Mitigating Environmental Pollution Using Eukaryotic Algae

By

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Kirk T. Semple

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I declare that **Mitigating Environmental Pollution Using Eukaryotic Algae** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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SIGNATURE DATE

I would like to express my gratitude and appreciation for the support offered by the following people and organisations:

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ABSTRACT

Aquatic systems are the main carrier of domestic and industrial wastes, including heavy metals (HM). HM are undegradable wastes that accumulate in the environment with time. The accumulation of HM has adverse human and environment health concerns. Applying chemical and physical treatment to immobilize or reduce the impact of HM is inefficient and costly. Biological treatment using eukaryotic algae has become an important subtract option for the treatment of polluted aquatic systems due to their low cost, efficiency, eco-friendly, sustainability and feasibility. Therefore, this thesis aimed to improve the resistance of eukaryotic algae to heavy metals through adaptation techniques to elevated concentrations of HM. The study shows the ability of algae to adapt to higher levels of heavy metals after a series of increases in metals concentration in the growth media. Also, the cell structure studies using Fourier Transform Infrared (FTIR) spectroscopy shows that algae have used the mechanism of cell structure alteration to cope with higher levels of metals. It is also found that the use of adapted microalgal phenotype significantly reduced the level of heavy metals used in the study from the solution. Therefore, the possibility of reusing the contaminated solution after treatment, has been studied through testing the ability of Daphnia magna to thrive in the algae-treated media solution. Results showed that daphnia was successfully able to live normally in the treated solution, suggesting that the treatment with adapted microalgal phenotype removed the metals and their toxic impact from the solution.

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directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.30. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)* , the control, and *Chlorella vulgaris* phenotype obtained from exposure to zinc (C.v-Zn). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.31. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris),* the control, and *Chlorella vulgaris* phenotype obtained from exposure to cobalt (C.v-Co). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.32. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris),* the control, and *Chlorella vulgaris* phenotype obtained from exposure to mercury (C.v-Hg). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.33. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris),* the control, and *Chlorella vulgaris* phenotype obtained from exposure to copper (C.v-Cu). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.34. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris),* the control, and *Chlorella vulgaris* phenotype obtained from exposure to heavy metals mix (C.v-Mix). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.35. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris),* the control, and *Chlorella vulgaris* phenotype obtained from exposure to nickel (C.v-Ni). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.36. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to cadmium (C.r-Cd). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.37. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to copper (C.r-Cu). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.38. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to mercury (C.r-Hg). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.39. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the

control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to heavy metals mix (C.r-Mix). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.40. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to nickel (C.r-Ni). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.41. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to zinc (C.r-Zn). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Figure 5.42. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to cobalt (C.r-Co). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Chapter six:

Figure 6.1. *Daphnia magna* survival in 0.01 mg/l zinc solution pre-treated with *Chlorella vulgaris*: the control, 0 mg/l of zinc (●); 0.01mg/l of zinc (■), and 0.01mg/l of zinc that has been treated with *C. vulgaris* phenotype (♦).

Figure 6.2. *Daphnia magna* survival in 0.001 mg/l copper solution pretreated with *Chlorella vulgaris;* the control, 0 mg/l of copper (●); 0.001mg/l of copper (■), and 0.001mg/l of copper that has been treated with *C. vulgaris* phenotype (♦).

Figure 6.3. *Daphnia magna* survival in 0.01 mg/l nickel solution pretreated with *Chlorella vulgaris; ;* the control, 0 mg/l of Ni (●); 0.01mg/l of Ni (■), and 0.01mg/l of Ni that has been treated with *C. vulgaris* phenotype (♦).

Figure 6.4. *Daphnia magna* survival in 0.01 mg/l mercury solution pretreated with *Chlorella vulgaris ;* the control, 0 mg/l of mercury (●); 0.01mg/l of mercury (■), and 0.01mg/l of mercury that has been treated with *C. vulgaris* phenotype (♦).

Figure 6.5. *Daphnia magna* survival in 0.01 mg/l cobalt solution pretreated with *Chlorella vulgaris; ;* the control, 0 mg/l of cobalt (●); 0.01mg/l of cobalt (■), and 0.01mg/l of cobalt that has been treated with *C. vulgaris* phenotype (♦).

Figure 6.6. *Daphnia magna* survival in 0.01 mg/l cadmium solution pretreated with *Chlorella vulgaris; ;* the control, 0 mg/l of cadmium (●); 0.01mg/l of cadmium (■), and 0.01mg/l of cadmium that has been treated with *C. vulgaris* phenotype (♦).

Figure 6.7. *Daphnia magna* survival in 0.001 mg/l mixture of metals solution pretreated with *Chlorella vulgaris;* the control, 0 mg/l of mixture of metals (●); 0.01mg/l of mixture of metals (■), and 0.01mg/l of mixture of metals that has been treated with *C. vulgaris* phenotype (♦).

Figure 6.8. *Daphnia magna* survival in 0.01 mg/l zinc solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of zinc (●); 0.01mg/l of zinc (■), and 0.01mg/l of zinc that has been treated with *C. reinhardtii* phenotype (♦).

Figure 6.9. *Daphnia magna* survival in 0.001 mg/l copper solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of copper (●); 0.001mg/l of copper (■), and 0.001mg/l of copper that has been treated with *C. reinhardtii* phenotype (♦).

Figure 6.10. *Daphnia magna* survival in 0.01 mg/l nickel solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of nickel (●); 0.01mg/l of nickel (■), and 0.01mg/l of nickel that has been treated with *C. reinhardtii* phenotype (♦).

Figure 6.11. *Daphnia magna* survival in 0.01 mg/l mercury solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of mercury (●); 0.01mg/l of mercury (■), and 0.01mg/l of mercury that has been treated with *C. reinhardtii* phenotype (♦).

Figure 6.12. *Daphnia magna* survival in 0.01 mg/l cobalt solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of cobalt (●); 0.01mg/l of cobalt (■), and 0.01mg/l of cobalt that has been treated with *C. reinhardtii* phenotype (♦).

Figure 6.13. *Daphnia magna* survival in 0.01 mg/l cadmium solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of cadmium (●); 0.01mg/l of cadmium (■), and 0.01mg/l of cadmium that has been treated with *C. reinhardtii* phenotype (♦).

Figure 6.14. *Daphnia magna* survival in 0.001 mg/l metals mixture solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of metals mixture (●); 0.001mg/l of metals mixture (■), and 0.001mg/l of metals mixture that has been treated with *C. reinhardtii* phenotype (♦).

List of Abbreviations

3NBBM- Bold Basal Medium with 3-fold nitrogen and vitamins

ABA- Phytohormone

AE - Adaptive evolution

Ag- Gold

ANOVA- Analysis of variance with one-way

As- Arsenic

CCAP – Culture collection of algae and protozoa

Cd- Cadmium

Co- Cobalt

CO_2-Carbon dioxide

COD- Chemical demand for oxygen

Cu- copper

DNA- Deoxyribonucleic acid

DO- Dissolved oxygen

DWB- Dry wight basis

EDTA- Ethylenediaminetetraacetic acid

Fe- Iron (Ferrer)

FTIR - Fourier-transform infrared spectroscopy

H_2- Hydrogen

H_2O-Water

HCO_3- Bicarbonate

Hg- Mercury

Hg°- mercury metallic form

HM – Heavy metals

HNO_3- Nitric Acid

ICP-OES - Coupled Plasma - Optical Emission Spectrometry

LC- Lethal concentration

Mn- Manganese

Mo- Molybdenum

NAS- National Academy of Sciences

NH_4- Ammonium

Ni- Nickel

NRCC- National Research of Council Canada

O_2- Oxygen

ºC- celosias

OD- Optical density

OECD - Organisation for Economic Co-operation and Development

Se- Selenium

Sr- Strontium

T- Temperature

V- Vanadium

Zn- zinc

CHAPTER ONE

INTRODUCTION

1.1 Mitigation of potentially toxic elements in the environment

An interesting definition for pollutants is that they are nothing more than a misplaced resource (Abbasi *et al.,* 1998). Metals like cobalt, iron, and zinc are essential for progression of vital process within a civilization; without metals, no development would be achieved (Abbasi *et al.,* 1998). Regardless of the importance of metals, they are also considered environmental pollutants of major concern. This is because they are not perishable or biodegradable like most organic pollutants (Azimi *et al.*, 2017). In addition, organic pollutants can be destroyed (disregarding the high cost of this process) by combustion and conversion to controllable forms such as carbon oxides, sulphur oxides, and nitrogen oxides. Combustion does not affect metals; it would at most change the metal's physical form or valence state unless an atomic reactor is used to break metals, leading to the production of an atomic bomb (Abbasi *et al.,* 1998). Metals exist almost everywhere: in the atmosphere, in waters, in soils and rocks, as well as in plant, animal and human tissues (Tchounwou *et al.*, 2012). In general, excessive amounts of metals released into the environment by anthropogenic activities including mining, burning fossil fuels, industrial effluent discharges; such actions induce metals at toxic levels leading to metal pollution (Alloway and Ayres, 1993). In addition, natural processes such as: volcanic eruptions, wildfires, weathering, and

acidic rains were also reported to remarkably contribute to heavy metals pollution (Fergusson, 1990).

Heavy metals are metallic elements and metalloids with high density, 5 g/cm^3 or above (Tchounwou *et al.,* 2012). Sometimes, terms like trace metals and toxic elements are incorrectly described as heavy metals (Abbasi *et al.,* 1998). However, each term has different specifications that make it uniquely different, as neither all toxic elements are heavy metals nor heavy metals are trace metals (Abbasi *et al.,* 1998). The mitigation of heavy metals is challenging due to the technical and economic complexations that face traditional approaches used for heavy metals treatment. In addition, most conventional methods of heavy metals removal are becoming outdated and publicly unacceptable (Gaur, *et al.* 2021). This is because of their high operational cost, generation of secondary pollutants, sludge production, and other method-related drawbacks. Therefore, biological remediation of heavy metals remains the most acceptable method for having more advantages than limitations.

1.1.1 Presence of heavy metals in the environment

Contamination of the environment due to the release of heavy metals is a major issue globally (Gaur *et al.* 2021). Heavy metals are persistent, acutely, chronically toxic, and can bio-accumulate in organisms and are potentially harmful to biota including humans (Abbasi *et al.,* 1998). Examples of selected heavy metals and their potential impacts are presented below.

Cobalt is a widely spread heavy metal that occurs in terrestrial and has been found in soils from 1 to 690 ppm (Adrian, 1980). In natural water bodies, it ranges from $4*10^{-6}$ to 1.7 ppm (Abbasi *et al.*,1998; Tchounwou *et al.,* 2012). In the atmosphere, cobalt's average natural background level is 0.001 ng/m^3 to 40 ng/m^3 in urban areas (Ernest, 1984). In plants, animals, and human tissues, cobalt levels range from 0.2 to 4.5 ppm on a dry weight basis (DWB) (Bockris, 1977). When the human body is exposed to cobalt the majority is concentrated in the kidney, lungs, skin, and urinary bladders (Abbasi *et al.*, 1998). Cobalt is typically used in alloy manufacturing, nuclear industries, and the bluing (steel) industry (Abbasi *et al.*,1998). Consequently, emissions and effluents from these industrial sites contribute to rising levels of cobalt in the environment.

Nutritionally, cobalt is an essential element for organisms, including humans e.g. vitamin B12. When reduced, may eventually cause pernicious anaemia, abnormal size red blood corpuscles, and neurologic abnormalities. Regardless of being an essential element at low concentrations that can be sourced from food, cobalt can be very toxic at levels higher than needed (Casey and Hambidge, 1980).Toxic effects of cobalt vary from causing hyperlipemia in rabbits and rats to lesions in muscles, myocardia toxicity, and tumours. It is also suggested that cobalt toxicity is more of a species function than bodyweight (Abbasi *et al.*,1998). In humans, excessive doses of cobalt were reported to cause severe cardiomyopathy, neurologic, hematologic, and endocrinal abnormalities (Berman, 1980). It also affects the haemoglobin, producing polycythaemia and hyperlipemia. Besides, cobalt may also cause goitres and other original specific symptoms like dermatitis to the skin and gastric mucosa irritation if ingested (Abbasi *et al.*, 1998).

Nickel is ranked 23rd in the order of abundance. In soil, Ni levels vary from 0 to 500 ppm (Adrian, 1980). In natural unpolluted water bodies, Ni levels are less than 0.01 ppm (Abbasi *et al.*, 1998; Tchounwou *et al.*, 2012). In the air, the average nickel level ranges from 3.7 ng/m^3 in a rural area to 74 ng/m^3 in air collected near industrial sites (Cawse, 1987). In benthic algae, Ni concentration is 52-74 ppm (DWB) (Abbasi *et al.*, 1998). In animal tissues, it reaches up to 45.9 ppm (DWB) (Abbasi *et al.*, 1998). In human tissues, nickel levels are up to 10 mg, but it varies between various tissues ranging from 0.002 ppm in muscles to 111 ppm in teeth and bones (Underwood, 1971; Sharma, 2013; Genchi, 2020). Nickel is involved in a vast array of industrial activities due to its various properties, such as the excellent looking finish, corrosion resistance, high standing to a wide range of temperature, and good alloy properties (Abbasi *et al.*, 1998). Nickel in the environment is sourced through two main ways, naturally via weathering of rocks and minerals and geothermal emissions, and anthropogenically through industrial and vehicular emissions. Nickel deficiency studies on animals showed pigmentation changes, thicker legs, swelling hocks, less friable liver dermatitis, and other symptoms in chickens (Nielsen and Ollerich, 1974). In rats, it has affected reproduction for several generations (Nilsson *et al.*, 1974). In humans, Ni deficiency is not clear due to the variety of ways that Ni enters humans' bodies through; food, drinking water, and air inhalation. Ni toxicity ranges from 0.03 ppm in freshwater to 11.2 ppm (Abbasi *et al.*, 1998). The most toxic form of nickel to humans is nickel carbonyl, an intermediate refining process liquid (NAS, 1975). Dermatitis was the most common symptom of industrial exposure to Ni (Falk, 1974; Horreth, 1976). Long term exposure causes lung cancer (Falk, 1974; Horreth, 1976). Females were found to be ten times more sensitive to nickel toxicity than males (Fisher and Shapiro, 1956; Sharma, 2013).

It is said that copper was the first metal used by humankind in ancient civilizations (Abbasi *et. al,*1998). It is ranked the 25th in the scale of availability, among other elements in the Earth's crust (Taylor, 1964). Its concentration in soils ranges between 2 to 100 ppm (DWB) (Bowen, 1966). Unpolluted waters have a range of 0.0006 to 0.4 ppm (Bowen, 1966). In the air, copper concentration found to be between 0.029 to 12 ng Cu/m^3 at remote locations, 30 to 200 ng/m^3 in the urban areas (Nriagu, 1979). Normal levels of Cu in plant and animal tissues are 1 to 50 ppm (DWB) (NAS. 1977). Humans have a range of 75 to 150 mg, with muscles, liver, and brain tissues as central depots (NAS, 1977). Copper in the environment introduced by windblown dust, volcanic emission, sea salts sprays, veg exudates, and anthropogenically from metal industries, it can reach up to 900 ppm in wastewater from processes such us Brossard and wire mills (Sittig, 1975).

For plants, animals, and humans, copper is an essential element and is a substrate of metalloenzymes and respiratory pigmies. However, the primary role of copper is the formation of blood and haemoglobin synthesis, oxidation processes through its presence in many oxidative enzymes, synthesis and cross-linking of elastin and collagen in the aorta and major blood vessels (Masironi, 1989; Perrin and Agarwal 1976; Horvath, 1976; O'Dell, 1976). Copper deficiency leads to anaemia, neutropenia, diarrhoea, demineralization of bone amyelination of the central nervous system (Horvath1979, NAS1977, Kirchgessner *et al.,* 1977). Toxic levels of copper range from 0.0005 ppm to 63.5 ppm for algae; however, the variation is very much dependent on metal species, algae species, pH, hardness, presence of other metals (Hodson, 1979; Taylor and Demayo, 1981). For other aquatic vertebrates, it ranges from 0.005 to 64.0 ppm (Abbasi *et al.*,1998). In human beings, copper poisoning effects are dependent on the phase of contact such as metals species powder or solution as well as the contact time. However, the main symptoms of copper toxicity are dermatitis,

greenish-black hair (Cooper and Goodman,1975), conjunction and oedema of eyelids, turbidity of ulceration of the cornea (Stokinger, 1963). It has been reported that copper at toxicity levels causes congestion of nasal mucous membrane and pharynx, metal fume fever, head stuffiness, and ulceration of nasal septum (Abbasi *et al.*,1998). If copper ingested, it causes; metallic taste, nausea, vomiting, diarrhoea, extensive and liver damage, coma, melena, hypotension, haemoglobinuria, and haematuria (Abbasi *et al.*,1998).

Zinc shares the 25th position with copper among metals abundance (Abbasi, 1989; Abbasi *et. al*,1998) in soils and rocks 100-300 ppm in natural freshwater 0.001 to 0.5ppm (Abbasi *et al.*,1998). Levels of zinc in aquatic plants and animals is widely varied in studies listed by Abbasi *et al.*, (1998), in general it was between 0.1 to 3500 ppm with an exceptionally high level of zinc found in *Mousealia sp.* from north of England with a concentration of 219000 ppm by Patterson and Whitton (1981). The highest concertation reported in human ashes was 5000 ppm (DWB) reported by Tipton and Cooke (1963), with a vast variation between different organs. Zinc is essential for its role in enzyme systems and in proteins and carbohydrates in animals (Abbasi, 1989). Zinc deficiency leads to retreated growth, anaemia, diarrhoea, immunity, hair loss, skin problems, dwarfism, impotence, bone disorders, low fertility in farm animals (Hart, 1982; Abdelhaleim *et al.*, 2019). Zinc toxicity to all forms of life depends upon biological factors, including: species, age, sex, weight, feeding habits, life stage, and adaptability to environmental stress (Abbasi *et al.*,1998). Also, physio-chemical factors have an impact on Zn toxicity, such as tested species, temperature, pH, hardness, presence of the other metals (Abbasi *et al.*,1998). Although it is common to have low toxicity for humans, zinc in levels beyond certain concentrations could be highly toxic (Sandstead 1975; Prasad and Overleas 1976).

Cadmium is one of the most notorious pollutants of the heavy metals; it is number 64 in the abundance order (Taylor, 1964). Its concentration in the soils and rocks ranges from 0.02 to 0.57 ppm (Forstner, 1980), in unpolluted water 0.01 to 0.4 ppb (Hart,1984; WHO, 2004) and in the air below 0.1 ng Cd/m^3 (Abbasi *et. al*,1998). The existence of Cd is always found to be associated with zinc at a ratio (Zn : Cd) of 100:5 (Abbasi *et. al*,1998). The main source of Cd is from anthropogenic origin, followed by weathering at a ratio of 20:5. A 20% of released Cd comes from zinc mining and smelting processes; 30% from Cd processing, use, manufacturing, and disposal; 50% from substances contaminated with Cd, such as phosphate fertilizers, sludge, and sewage effluents and driven from the combustion of fossil fuels (Maret & Moulis, 2013).

Cadmium is not recognized as an essential element to animals or humans. It is toxic to aquatic organisms even at very low levels (Maret & Moulis, 2013). Its toxicity is depending upon the type of tested organism, life stage, the period of exposure, and physical conditions like pH, temperature, dissolved oxygen (D.O), hardness, organic legends (Hart,1982). The effect of pH and hardness is on the metal biological activity. D.O. and temperature may impact the tolerant capacity of the tested species (Abbasi *et. al*,1998). Toxicity of Cd was found to range from 0.001 ppm in some aquatic species to 1.0 ppm in others like *Chlorella vulgaris* (Burnison *et al.*, 1973). Cadmium toxicity was found to be reduced in the presence of natural organic components in the water, such as humic water, probably because of the complexing part of the cadmium to lessen the concentration of toxic forms (Gessinf, 1981). Also, the presence of other materials like Pb, Ni, and Se was found to reduce Cd toxicity (Hotchinson, 1976; Hotchinson and Stokes, 1975; Dietilainin, 1976). Levels of 0.005 to 520 ppm were found to be the range of cadmium toxicity to invertebrates (Abbasi *et al.*, 1998). The proposed reasons for Cd toxicity in fish, is that gill damages occur leading to anoxia and

enabling oxygen exchange (Eisler,1971; Burton *et al.*, 1972). Benoit *et al.* (1976) explained that Cd inhibits the action of acetylcholinesterase causing death through respiratory muscle paralysis in humans. Chronic cadmium toxicity was found to cause kidney damage, lung disease, tubular proteinuria, which was found to be frequently reported among industrial workers (Lauways;1978; Dugdade 1978; WHO, 2004).

The most toxic among metal pollutants, mercury, is widely distributed in the environment but with lower levels than other common heavy metals. It is common in rocks, soils, and sediments, ranging from 0.03 to 1.7 ppm (Reader *et al.*, 1979; Abbasi *et al.*, 1998). The background levels of Hg in unpolluted air are up to 0.5 ng/m^3 (NRCC, 1979). In unpolluted natural water streams, mercury levels reported being below 0.1 ppb (WHO, 1978 and 2004). With an exceptional report for a river in Germany found to have 1.8 ppb (Schramel, 1973). The primary supply of Hg is from the natural degassing of Earth's crust (WHO, 1976 and 2004). The naturally sourced input of mercury to the ecosystem is about 80% (Lagerwerf, 1972). The other 20% is from human activities (Abbasi *et al.*,1998). However, the anthropogenic source of Hg can be more severe ecologically in a specific area of contamination. Mercury is widely used in industries of paints, electronics, equipment, pesticide, dental analogous, pulps, paper, batteries, thermometers, medicines, and mercury vapor lamps. The operation of these industries induces 25-50% of mercury to the ecosystem (NRCS, 1979). Contaminated discharges of effluents that content mercury will result in a significant build-up of the metal concentration in the aquatic environment.

In humans, methyl mercury high solubility in lipids which makes it more permeable to cross biomembranes easily, specifically into the brain, spinal cords, peripheral nerves, and across

the placenta (WHO,1976), leading to neurological disturbance (Abbasi *et al.*, 1998). Inorganic mercury is stored in the kidney, and other forms extend its significant effect there when exposed (Jaishankar *et al.*, 2014). In area where industrial activities take place, air may severely get contaminated. It has been reported that the metal levels range from 50-1000 ng/m^3 in such areas, which is 50-100 times higher than the background concentration (Abbasi *et al.*,1998). Methyl and ethyl mercury salts have been nominated as the most toxic forms of mercury. This nomination is due to the high solubility of Hg salts in lipids at 100 folds higher than solubility in water. The high solubility of these salts leads to high penetration to cells and inhibition effect on enzyme system (Jaishankar *et al.*, 2014).

1.1.2 Sources of polluted effluents

Dumping waste is the most commonly practiced method for the purpose of waste management. As human societies constantly move towards more sustainable waste management options, it is crucial that permanent ecological solutions are developed for the benefit of future generations. As human populations grow in both developed and developing nations the amount of wastes accumulating within landfills, also grow as a sequence (Manisalidis *et al.*, 2020).

More preferable over more primitive open dumping, landfills offer community health and environmental benefits, reducing contact between domestic wastes and the surrounding human and wildlife communities by confining environmental impacts (Edmundson, 2012). Despite these sanitary and ecological advantages of landfills, accumulated wastes unavoidably provoke both gaseous and aqueous contaminants. This process could last for

decades post-closure of landfills, and must be managed until subsided of the emissions of contaminants (Wojciechowska *et al.,* 2010).

1.1.3 The pollutant's journey from waste collection sites

All waste collection sites accumulate liquids and the emitted gases from biological and chemical degradation. However, outcomes from landfills as both gas and liquid wastes are concentrated for ease of management. Gaseous emissions include carbon dioxide, methane, and a variety of volatile organic compounds (Renou *et al.,* 2008, Edmundson, 2012). Effluents dissolve components within the waste as water from rainfall or biological degradation percolates through the solid refuse within a dump space. Effluents from landfills typically have high levels of chemical oxygen demand, ammonia-nitrogen, volatile organic compounds, suspended solids, xenobiotic organic compounds, and dissolved metals ions (Wasewar, *et al.,* 2020). Reduction or removal of these compounds needs to be done by treatment, due to their toxicity (Wiszniowski *et al.,* 2006).

Edmundson, (2012) frequently reported that extremely high concentrations of 2,000 mg/l, total ammonia-nitrogen in leachates worldwide, which can range from 0.2-13,000 mg/l. Ammonium is released from decomposing organic matter high in proteins and amino acids, such as food and yard waste. Elevated concentrations of ammonia nitrogen are toxic to biological systems (Edmundson, 2012). Ammonia has been regarded as the most troublesome constituent in polluted effluent over the long term as it may persist in the polluted effluents with time (Wasewar, *et al.,* 2020). Polluted effluents, without appropriate management, can cause severe adverse impacts on surface and groundwater quality (Manisalidis *et al.*, 2020).

The convenient management of these highly mobile environmental toxins is critically vital in the long-term protection of humans and wildlife communities.

1.1.4 Transportation of heavy metals into the environment

Pollutants added to the environment through natural phenomena and anthropogenic activity and transported into water bodies, atmosphere, and lithosphere, become a huge cause of environmental pollution (Walker *et al.,* 2005). Transportation of pollutants, including heavy metals, depends on movements and direction of surface water, temperature, airmass circulation, and the speed of the wind. In addition to factors related to pollutants like polarity, coefficient of partition, vapour pressure and stability (Walker *et al.,* 2005), the main sources of water pollution, including heavy metals, are urbanization and industry (Briffa *et al*., 2020). Runoffs carrying pollutants from these sources lead to the accumulation of the pollutants in the sediments of water bodies. Regardless of the amounts of pollutants transported to water bodies, they might still be toxic to humans and the environment. The harmful impact of heavy metals is related to factors like the metal species, the nature of metals presented, the biological role of the specific metal, the organism exposed and the stage of organism's life at the time of exposure. All elements in the food chain will be impacted if one organism is affected by metals toxicity (Walker *et al.,* 2005).

Movement of heavy metals begins from the release of domestic and industrial sources into sewage where they accumulate and are not degraded in the sewage treatment. Instead, they might be removed in the final effluent or the sludge (Briffa *et al.,* 2020). The remaining pollutants in the sewage that enters the water depend on the treatment applied before release.

Three stages of treatments are used for polluted effluents: primary, secondary and final (Walker *et al.,* 2005; Briffa *et al.,* 2020). In the first stage, polluted water will be filtered, producing sludge, which might further be processed. Secondary treatment may involve various technologies used to purify the polluted water, such as oxidation that can be done in three ways: oxidation ponds, biofiltration, and aeration (Enfrin *et al.,* 2019). The last stage of treatment usually uses activated carbon and sand to help remove the pollutants. To improve pollutants removal, new strategies must be adopted, and better technology developed to decrease the amounts of pollutants that are thrown into the water (Walker *et al.,* 2005; Enfrin *et al.,* 2019; Briffa *et al*., 2020).

Pollutants in water bodies can be transported over an extensive distance, where they can descend to the bottom or rise to the surface (Enfrin *et al.,* 2019). These distances depend on factors such as currants, stability, and physical state of the pollutant. When it reaches the oceans, wind and currents transport the pollutants further. The difference in density of water bodies also plays a factor in transportation as well as concentration of salt or temperature (Briffa *et al.,* 2020). Persistent pollutants such as heavy metals can then enter the food chain through marine life, which can then affect predators such as fish, birds, animals, and humans, which travel and transport the pollutant to different ecosystems (Walker *et al.,* 2005; Briffa *et al.,* 2020). The following diagram (1.1) illustrates the cycle of heavy metals in the environment.

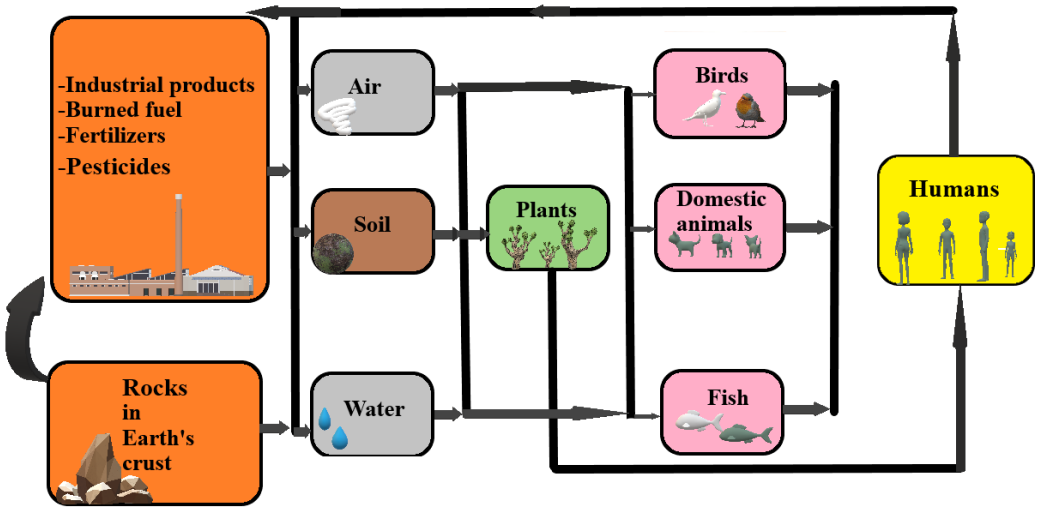

Figure 1.1: Sources of heavy metals and their cycling in the soil-water-air organism ecosystem. Content of metals in tissue generally builds up from left to right, indication the vulnerability of humans to heavy metal toxicity

1.2. Biological approaches to mitigating the impact of heavy metals in the environment

1.2.1 Bioremediation, biosorption, and bioaccumulation

Bioremediation means to heal, restore, amend or to fix the damage that has been done to ecosystems and the environment, by using biological processes (Vishwakarma, *et al.*, 2020). The concept of bioremediation is not new, for long time it has been practiced in many ways; the farmer by restoring farmland and the forester practicing it by forest replanting, also purification of water, waste management and sewage treatment (Chaudhry, 1994). It used to be believed, that nature will handle the chemicals, toxins and wastes we dispose of (Farhadian *et al.*, 2008). The issue about bioremediation is the recognition that sustaining

nature through biological processes is no longer applicable because of the overburdening practices that leads to the limits where natural processes cannot cope (Chaudhry, 1994).

Bioremediation is an environmental biotechnology that encompasses microbial activities, exclusively. Strategies of bioremediation have been found to be an attractive surrogate for waste management, due to their low cost and high efficiency (Chaudhry, 1994; Diep *et al.*, 2018). The evolution had achieved a progression in the physiology of microbial kingdom, rates that have never been seen before of discovered microorganisms possessing properties that never imagined in the past. For instance, thermophiles, which are very extreme, that they can grow and reproduce at over boiling temperatures. Another example is that formerly bacteria believed to be strictly heterotrophs, but they have been shown to grow autotrophically, using chemiosmotic mechanisms to generate energy under anaerobic circumstances, (Perelo, 2010). Furthermore, Microbes' cells also have mechanisms to deal with surroundings such as biosorption and bioaccumulation, when exposed to environments with overloads of pollutants (Chaudhry, 1994).

Biosorption is a physio-chemical process utilized to illustrate the solute passive uptake by non-living organisms based on many mechanisms including surface complexation, precipitation, absorption, ion exchange, and adsorption (Malik, 1999). It is a process independent of metabolism, unlike actively removed metallic ions which may be achieved by an organism's metabolic activities (Chaudhry, 1994). Biosorption takes place as a consequence of the interaction between solute and externally sited components constituting an organism's cellular structure (Chaudhry, 1994). In general, the key role in the sequestration of metals ions from the aqueous, is played by the cell wall since the sorption

conditions (pH, temperature ... etc will be presented in the factors impacting metals biosorption section) are distinct from the metabolic activities of the cell. Therefore, biosorption oversees binding and accumulating the metallic ions even in the case of dead cells (Malik, 1999).

It is important to distinguish biosorption from bioaccumulation for a better treatment process design (Figure 1.2). It could be a real issue to use living microbial cells in the environment with high toxic metal ion content, in terms of process optimization. It would be preferable that biomass growth and reproduction decoupled from its function as a biosorbent (Volesky, 2003).

Bioaccumulation: the interest in bioaccumulation began in the early1990s when environmental agencies increased the interest in bioremediation applications to reduce pollutant levels below regulatory limits (Diep *et al.*, 2018). *Bioaccumulation* is a metabolically active process that requires a living cell for the process to be accomplished (Malik, 2004; Mishra and Malik, 2013). The main advantage of bioaccumulation is that it is an irreversible process. When it occurs, the captured heavy metal will not realise until a physical or chemical disruption is applied to the cell (Diep *et al.*, 2018). Many studies reported the success of using eukaryotic algae in the bioaccumulation of heavy metals (Maeda *et al.*, 1990; Regaldo *et al.*, 2013; Anbalagan and Sivakami, 2018; Regaldo *et al.*,2021). Maeda *et al.(*1990) found that zinc was accumulated in the living cell of *Chlorella sp.;* and when it was attempted to desorb the metal through EDTA treatment, it did not reduce the metal concentration in the cell. Regaldo *et al.* (2013) reported the removal of 100% and

81.97% of Pb and Cu respectively from the tested aqueous solution when *Chlorella vulgaris* was used in their bioaccumulation studies.

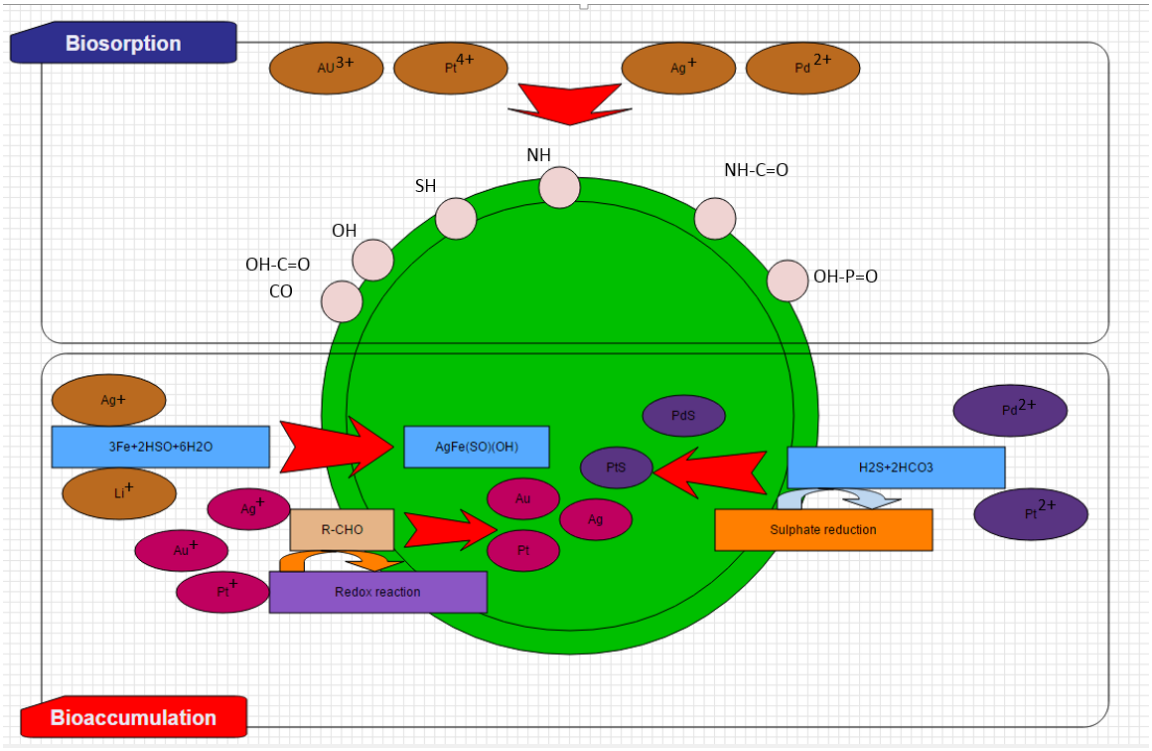

Figure 1.2. Biosorption and bioaccumulation on/ in the cell (Lo *et al.*, 2014)

1.2.2 Biological sorbents

Microalgae, bacteria, yeast, and fungi increasingly have received attention for their performance in metal ion removal and recovery (Wang and Chen, 2009). Especially since they are available in generous quantities with low cost (Wang and Chen, 2009). Biosorbents may include a variety of functional sites containing carboxyl, imidazole, amino, sulphydryl, sulphate, phosphate, thioether phenol, hydroxyl moieties and carbonyl amide (Romera *et al.*, 2006). For the metal removal, biosorbents are cheaper, more effective alternatives to

particularly heavy metals from aqueous solutions (Volesky, 1990). Due to their high sorption capacity with availability, both eukaryotic and prokaryotic microalgae are of special interest in research and biosorbent development. However, only a few publications on biosorption with algae have been published, compared to other microbial biomass applications (mainly bacteria or fungi)(Ismail and Mustafa, 2016); and even fewer for multi-metallic ion systems (Romera *et al.*, 2006).

It has been reported that algae were able to adsorb heavy metal ions (one or more) including Fe, Sr, Co, Cu, Mn, Ni, V, Zn, As, Cd, Mo, Pb, and Se with satisfying initial uptake capacity (Brinza *et al.*, 2007). Romera *et al.* (2006) provided a statistical analysis of biosorption capacity of non-living biomass of 37 different algae species (20 brown algae, 9 red, 8 green) from 214 publications. In this review, biomass-metal affinity (b) of the Langmuir equation for Cu^{+2}, Cd^{+2}, Pb^{+2}, Zn^{+2} and Cr^{+2} and the maximum sorption capacity (q_{max}) were all reported (Table 1). For heavy metals biosorption, brown algae stood out in a splendid way (Romera *et al.*, 2006). However, the details about multimetallic systems are limited.

Table 1.1. The sorption capacity of metals by untreated microalgal biomass in monometallic systems

Algae	Metal ions	Q_{max} (mg/g)	*Algae*	Metal ions	Q_{max} (mg/g)
Ascophyllum nodosum	Cd	126.5175	*Polysiphonia violacea (R)*	Pb	102.0046
Ascophyllum nodosum	Ni	107.4614	*Porphira columbina (R)*	Cd	45.50357
Ascophyllum nodosum	Pb	32.15744	*Sargassum baccularia (B)*	Cd	83.1834
Chaetomorha linum (G)	Cd	53.9568	*Sargassum fluitans (B)*	Ni	24.00421
Chlorella miniata (G)	Cu	23.25564	*Sargassum fluitans (B)*	Pb	330.2768
Chlorella miniata (G)	Ni	13.90953	*Sargassum hystrix (B)*	Pb	285.0036
Chlorella vulgaris (G)	Cd	33.723	*Sargassum natans (B)*	Cd	131.9693
Chlorella vulgaris (G)	Cr(VI)	102.9397	*Sargassum natans (B)*	Ni	24.00421
Chlorella vulgaris (G)	Cu	35.5824	*Sargassum natans (B)*	Pb	244.7032
Chlorella vulgaris (G)	Fe(III)	70.10391	*Sargassum siliquosum (M)*	Cd	82.0593
Chlorella vulgaris (G)	Ni	35.8009	*Sargassum sp. (B)*	Cd	157.374
Chlorella vulgaris (G)	Pb	97.384	*Sargassum sp. (B)*	Cr(VI)	130.9869
Chlorella vulgaris (G)	Pb	73.556	*Sargassum sp. (B)*	Cu	68.6232
Chlorella vulgaris (G)	Zn	24.1906	*Sargassum vulgare (M)*	Ni	4.98865
Chondrus crispus (R)	Cd	9.296307	*Sargassum vulgare (M)*	Pb	227.92
Chondrus crispus (R)	Pb	194.9752	*Scenedesmus obliquus (G)*	Cr(VI)	113.0887
Cladophora glomerata (G)	Ni	25.99967	*Scenedesmus obliquus (G)*	Cu	33.29496
Codium fragile (G)	Ni	5.81031	*Scenedesmus obliquus (G)*	Ni	30.19601
Codium taylori (G)	Cd	29.69872	*Ulva lactuca (G)*	Pb	126.392
Codium taylori (G)	Pb	376.068	*Undaria pinnatifida (B)*	Pb	403.004
Corallina officinalis (R)	Cd	72.95409			
Fucus vesiculosus (B)	Ni	23.00648			
Fucus vesiculosus (B)	Ni	10.97503			
Fucus vesiculosus (B)	Pb	310800			
Galaxaura marginata (R)	Pb	25.0712			
Galaxaura marginata (R)	Pb	47.656			
Gracilaria edulis (R)	Cd	26.9784			
Gracilaria Salicornia (R)	Cd	17.9856			
Padina gymnospora (B)	Ni	9.9773			
Padina gymnospora (B)	Pb	65.0608			
Padina sp.(B)	Cd	59.5773			
Padina tetrastomatica (B)	Cd	59.5773			
Padina tetrastomatica (B)	Pb	217.3528			

Source: (Romera *et al.*, 2006) B: brown algae; G:green algae; R: red algae

1.3. Factors influencing heavy metal biosorption

Biosorption is a physio-chemical process that is impacted by various influences related to the physical and chemical environments of the process. In addition, the factors that impact the biosorbent, which is a prominent part of the process, will also affect the biosorption process. Factors such as pH, temperature, biosorbent dosage, initial metal concentration in solution, agitation rate and contact period influence the biosorption process rate and contact period influence the biosorption process significantly (Figure 1.3).

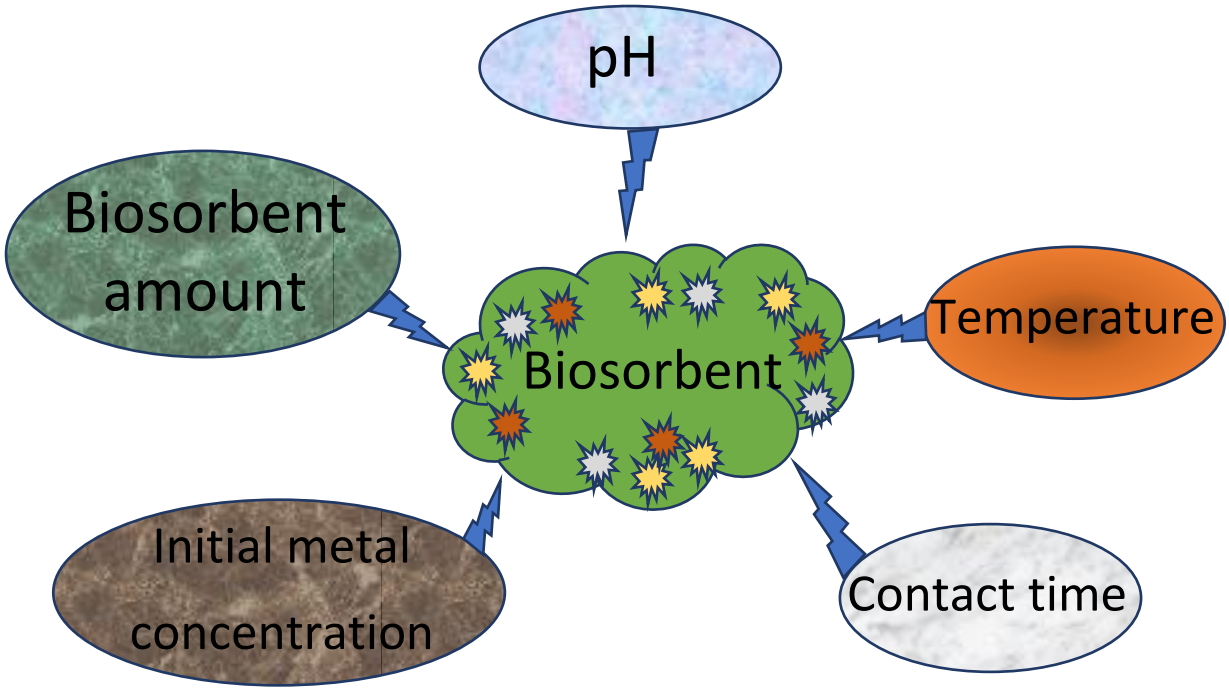

Figure 1.3. The factors that affect heavy metals biosorption

1-3-1 Impact of pH on metal biosorption

One of the most important parameters that affects biosorption process is pH. At low pH, the concentration of protons is high, and the ion exchange sites become strongly protonated (Volesky, 2007). Solution chemistry of the metals, metallic ions competition and functional groups activity are all influenced by pH (Volesky, 2007). Studies have shown that the pH solution has a significant effect on the biosorption of metal and as the solution pH increases, there is an increase in cation biosorption (Peng *et al.*, 2009). For example, Cu ions biosorption on biomass is mostly due to ionic attraction and at lower pH values the ionic metals are in competition with the H^+ in the solution (Gulnaz *et al.*, 2005). Studies on the effect of pH on the biosorption of copper have been carried out using different types of biomass. For example, a study on the biosorption of copper using *Spirogyra* species reveals that an increase in pH from 2.5 to 5, the biosorption capacity rises at all the selected Cu ions levels (Peng *et al.*, 2009).

A study on the effect of pH on the adsorption of metal ions by *C. vulgaris* showed that the optimal pH range for absorption of cadmium, zinc, chromium, copper, nickel, and cobalt was pH 6-7. The study also showed that reducing the pH, leads to desorption of metal ions and could adversely affect the absorption of metals (Greene, 1990). Another study on the effect of pH on the biosorption of copper by *C. vulgaris* concluded that the biosorption of Cu was enhanced when pH increased above pH 3 (Al-Rub *et al.*, 2006).

1-3-2 Impact of water temperature on metal biosorption

Temperature has a substantial influence on biosorption, which increases within a temperature range of about 20-30 °C but decreases when temperature goes above a critical value. An increase of temperature might increase the metal ions kinetic energy that makes it easier for the metal ions to be attached to the biosorbent surface (Abdel-Ghani and El-Chaghaby, 2014). Another piece of research has shown that the binding of metal ions to the surface of the biosorbent at a low temperature is more rapidly, easily, and reversible because of the low requirement of energy (Rathnakumar *et al.* 2009). Studies by Rathnakumar *et al.* (2009) on biosorption of Cu ions at different temperatures, showed that biosorption decreases with an increase in temperature between 20^{o}C to 40^{o}C, and also the capacity of the sorption decreases thereby making biosorption affinity decrease at high temperature. Another study by Gulnaz *et al*. (2005) reported that biosorption capacities decreased as the temperature increased from 20 to 50 °C, 20 °C was found to be the optimum between the studied temperature ranges. Batch biosorption experiments carried out by Pamukoglu and Kargi (2007), at a temperature range of 30 °C to 50 °C showed the variation of first and second order rate constants with temperature. Both rates were increased as the temperature increases from 30 – 50 °C due to the high frequency of contact between the Cu ions and adsorbent particles. In a study on the impact of temperature on metals adsorption by *C. vulgaris*, Aksu (2002) observed that increased temperature led to an increase in the adsorption of zinc ions by *C. vulgaris*. However, she reported a decrease in Cd adsorption from 83.3 mg/g (in 20°C) to 51.2 mg/g (in 50°C). Yang *et al.* (2014) investigated the effect of temperature on Cu, Cd, Zn and Mn heavy metals removal using *Chlorella sp.* The incubation period in this study was seven days, pH = 6 and initial concentration was 230 mg/l, and the temperature was set to 3 different levels, 10, 28 and 37°C. The best temperature, representing the highest heavy metal removal, is 28°C.

Except for cadmium, where removal amounts are almost equal in different temperatures. Three other metals show a significant decrease in removal in low temperatures and raising temperature excessively (higher than 28°C) will decrease the efficiency of Mn, Zn, and Cu removal.

1.3.3 The impact of biomass concentration on metal biosorption

Biomass concentration is one of the factors affecting biosorption. Studies have shown that as biomass dosage increases, the metal uptake decreases. Due to the increase in the surface area of the adsorbent that in turn increases the number of sites available for binding (Esposito *et al.,* 2001). In a study of Cu removal, the biosorption efficiency was found to increase when the biosorbent concentration was increased (Rathnakumar *et al.,* 2009). Because of the decrease in surface area per unit of adsorbent, Olguín *et al.* (2003) found that the equilibrium concentration of copper rises when adsorbent concentration increases. Due to the increase of pretreated powder waste biosorbent concentration, the first order rate and second order rate constants also increased, this is due to the increased of binding sites of the biosorbent available (Pamukoglu and Kargi, 2007).

In their study, Abdel-Atty *et al.* (2014) evaluated the effect of different biomass dosage of the water algae *Anabaena sphaerica* on the biosorption of Cd and Pb ions. The data revealed that the biosorption of Cd and Pb ions increased with subsequent increases in the biosorbent amount and almost became constant at higher biomass dosage. This behaviour could be explained by the decreases of effective surface area for biosorption as a result of the formation of aggregates of the biomass at higher doses. The effect of variation of *C.*

vulgaris algal cells dosage on copper uptake and removal was reported by Al-Rub *et al.* (2006). They concluded that while the percentage removal of copper increases with the increase in algal cells dosage, copper uptake decreases with increased algal cells dose. Many reasons explain this behaviour, such as solute availability, electrostatic interactions, interference between binding sites, and reduced mixing at high biomass densities (Meikle *et al.,* 1990). The biosorbent mass may also affect solution pH because of the release of some organic compounds due to biomass degradation. Therefore, experiments on the interaction effect of biomass dose and pH were conducted on blank solutions with initial pH of 5.0. These experiments showed that solution pH increased slightly with increased biomass dose, where a maximum increase in solution pH of 0.4 was obtained with a biosorbent mass of 0.20 g (Al-Rub *et al.,* 2006).

1.3.4 Metal concentration effect on metal biosorption

Metal concentration is another factor that affects biosorption process. Studies have verified that at higher metal ions levels the amount of adsorbed ions is greater than the value at lower concentration since there are more binding sites for interaction (Mukhopadhyay *et al.,* 2008; King *et al.,* 2008). There is a greater driving force at a higher level between the solid and liquid interface thus enabling mass transfer. The initial metal concentration provides a driving force that overcomes mass transfer resistance of metal ions between the adsorbent and the biosorbent solution (King *et al.,* 2008). The biosorption percentage decrease may be due to lack of enough surface area to absorb the available metal in the solution (King *et al.,* 2008).

The biosorption of Cu ion by the wheat shell was studied at several different initial copper concentrations ranging from 10 to 250 mg/l. Biosorption efficiencies decreased with the increasing of initial metal concentrations (Basci *et al.,* 2004). Chatterjee *et al.* (2010), studied the effect of different initial ions concentrations on the biosorption of Cr, Cu, Cd, and Pb by the dead biomass of *G. thermodenitrificans*. He found that the increase of initial metal levels increased the capacity of metal adsorption from 25 to 175mg/l with a maximum adsorption at 175mg/l for all the metals used in the study. Yang *et al.* (2015) investigated the effect of initial metal concentration on Cu, Cd, Zn and Mn heavy metals removal using *Chlorella sp*. The incubation period in this study was seven days, pH = 6 and the temperature was 28 °C. They investigated the effect of initial concentration on the amount of metal ions removed, applying three different initial concentrations for each metal ion. The results showed that increasing the concentrations significantly reduces removal efficiency, so that the maximum removal efficiency of the considered metals with an initial concentration of 92 mg/l was 62.05 and 83.68 respectively for Zn and Mn, and 83.6% and 74.34% for Cu and Cd, with initial concentrations of 9.2 mg/l. In another study, Malakootian *et al.* (2016) investigated the effect of initial concentration of Zn on the amount of its removal using *C. vulgaris* dry biomass. In this study, microalgae concentration was 2 g/l, and temperature and pH were set to 25°C and 7, respectively. Results showed that with an increasing initial metal ion concentration from 50 to 250 mg/l, its removal decreases by 44.9%.

1.3.5 Particle Size impact on metal biosorption

Particle size of adsorbent is another vital factor that needs consideration in biosorption research. The surface of contact with the biosorbent and metal ions in solution plays a

significant role in biosorption processes. Biosorbent particles size effects both surface area and the equilibrium time, the smaller the particles size the greater the surface area and the shorter equilibration time. In a study using eggshell, Vijayaragharan and Joshi (2013) found that reducing the particle size from 750 to 100 μm, increased the lead removal efficiency from 30.7% to 99.6%, equilibration time was 90% faster. One important factor to consider when reducing particle size, is to make sure that they are resilient to withstand extreme conditions at regenerated cycles (Volesky,2001). Vijayaragharan and Yun (2007) found that using fine powdered biosorbent which formed dense slurry making the solid-liquid separation tedious.

1.3.6 Contact time

The period of contact time between the adsorbent and adsorbate is one of the critical factors that impact the biosorption of metal ions in the aqueous solution (Safa and Bhatti, 2010). The time range of optimum biosorption of metals was frequently reported in minutes, most of the ion's sorption occurred in the initial minutes of contact between the biomass and the ions in the solution (Roy and Mondal, 2017). Rapid sorption during the initial contact period may be due to the presence of many vacant active sites. Initially, the metal ion's sorption rate is higher, and then this rate decreases with time. Under the same biosorption condition, the optimum contact time differs between metals (Roy and Mondal, 2017). Malakootian *et al.* (2016) investigated the effect of contact time of biomass and pollutants in removing zinc using *C. vulgaris* biomass. This study used biomass with 2 g/l concentration, T = 25°C, pH = 7 and initial concentration of 50 mg/l of Zn. The time required to reach the maximum removal percentage of zinc was 60 minutes.

1.4. Organisms used in the study

1.4.1 Green microalgae

The term algae were used by phycologists formerly to refer to any eukaryotic organism with chlorophyll *a* and lack of roots, stems and leaves. It also included cyanobacteria, which are nowadays regarded as prokaryotes (Engström, 2012). Microalgae can transform simple inputs like CO_2 and H_2O, with some mineral salts, into organic carbon through photosynthesis. Almost everywhere, microalgae could be found in marine, brackish, and freshwater. Scientists have estimated the total number of algal species to be over 50,000 globally, while only 30,000 species are identified and tested (Frac *et al.,* 2010). However, fewer species have been evaluated for their biotechnological application.

Biomass from microalgae mainly consists of carbohydrates, proteins, and oils (Frac *et al.,* 2010). The microalgal cell wall builds up from a microfibrillar layer of cellulose, which can sometimes be enclosed with an amorphous layer, a protective layer, and both comprise the cell wall. The cell wall may be protected with plates and scales (Engström, 2012). Sometimes there may be layered polysaccharides outside of the external amorphous coating. The plasma membrane is very slim and surrounds the cytoplasm that embraces organelles (Engström, 2012). Microalgae have different cell chambers where Golgi, mitochondria, endoplasmic reticulum, plastids, chloroplasts, ribosomes, lipid droplets, centrioles, and vacuoles are common (Sheehan *et al.,* 1998). Flagella and microtubules may also occur (Sheehan *et al.,* 1998). A double nuclear membrane encloses the nucleus and contains nucleolus, genetic information as DNA molecules in chromosomes. Most microalgae are uninucleate, but multinucleate species exist (Engström, 2012). A crucial organ in algae is the chloroplast, a

levelled vesicle, plus chlorophyll and other pigments, which is the site where the photosynthesis progress. From the main three groups of algae, Green (chlorophytes), red (Rhodophytes), and brown algae (Phaeophytes), the two species enclosed in this study belonged to the green division.

1.4.1.1 *Chlorella vulgaris*

Chlorella is a genus of about thirteen species of single-celled green eukaryotic algae belonging to the division Chlorophyta. The cells are spherical in shape, about 2 to 10 μm in diameter, and are without flagella. *Chlorella vulgaris* is predicted to have been on Earth for more than two billion years (Safi, 2014). It has been evolving for survival during that time, resulting in many of the valuable features that we know today (Safi, 2014). The shape of *Chlorella* cells is close to spherical with a width of 1.5-10 μm. The pyrenoid is ellipsoidal or spherical and has 2-4 starch grains surrounding it. The autospores are spherical, and each sporangium contains 2, 4, 8, or even 16 cells (Engström, 2012). *C. vulgaris* has a high carbon fixation capacity. Some strains of *C. vulgaris* have shown the capability to produce a 37 % dry weight starch content. Also, this species has the potential to grow in a heterotrophic way with an organic carbon source (Lee, 2007).

Important features found in *C. vulgaris* are due to its ability to multiply rapidly. Standard practice involves typically growing populations in photobioreactors or growth chambers (Sacasa, 2013). These chambers are consistently shaken and used to control firm aspects of metabolism in microalgae *C. vulgaris*. Variables such as growth media, carbonation, and light

have been researched deeply to understand the best means of optimal growth (Yuvraj *et al.*, 2016).

1.4.1.2 *Chlamydomonas reinhardtii*

Chlamydomonas is a genus of green algae consisting of about 150 species all unicellular flagellates, found in stagnant water and on damp soil, in freshwater, seawater, and even in snow as "snow algae". It is an eukaryotic algae that belongs to the Chlorophyceae family. *Chlamydomonas reinhardtii* is a unicellular green microalga (Bilanovic *et al.*, 2009). Cells are rounded, or close to rounded, with a width of 8-22 µm and a length of 10-22 µm. It has a distinct cell wall and a single chloroplast in close proximity to the nucleus. The nucleus is typically located in the centre and with a distinct nucleolus (Harris, 2009; Engström, 2012). A sizeable elementary pyrenoid exists within the cell. It also has two anterior flagella that are used for sensing and swimming. *Chlamydomonas* are distributed throughout the world in a variety of regions. However, *C. reinhardtii* is found explicitly in fresh-water ponds and lakes, in garden and farm soils (Harris, 2009). It is a commonly used model organism and is one of three algae where the genome has been resolved (Malcata, 2011). Malcata continues by pronouncing that refined metabolic engineering only has been possible with *C. reinhardtii*. This species has the potential to grow in a heterotrophic way with an organic carbon source such as acetate and even mixotrophically when utilizing acetate as the carbon source. At the same time, CO_2 is assimilated during photosynthesis (Harris, 2009). *C. reinhardtii* is also attractive due to its ability to switch from producing O_2 to H_2 (Melis and Happe, 2001). *C. reinhardtii* is known for its ability to photo produce H_2 when photosystem II is blocked. Yet, the reason for this is unknown (Melis and Happe, 2001).

1.4.2 Water flea (*Daphnia magna)*

Daphnia magna spices are found in various habitats, including freshwater and brackish of up to 8 ppt salinity, habitats like rivers, lakes, and temporary pools such as rock pools (Ebert, 2005). The optimum temperature for *D. magna* is between 18-22c; however, they can tolerate a much more comprehensive range (Ebert, 2005; Haney, 2010; Vanoverbeke *et al.*, 2007). The plankton *D. magna* are part of the diverse crustaceans that belong to the Phyllopoda; they are known for their flattened fins-like legs that are used to produce a water current for the filtering apparatus. Within the branchiopods, *Daphnia* belongs to the Cladocera. *Daphnia* bodies are surrounded by an uncalcified shell, known as the carapace. It has two parallel walls, between which haemolymph flows and forms part of the body cavity. In addition, *Daphnia* has six limbs on the trunk (Freyer, 1991). The limbs function as an apparatus for feeding and respiration; the body length ranges from less than 0.5 mm to more than 6 mm. Males are smaller in size, have larger antennules than females, have modified post-abdomen, and have different first legs armed with a hook that helps in clasping (Kästner. 1993; Freyer, 1991; Ebert, 2005).

The life cycle of *Daphnia* during the growth season is described by its asexual mode of reproduction, generating offspring that are identical to the mother daphnia. A female produces a clutch of parthenogenetic eggs after every adult moult if feeding conditions are optimum. At 20ºC, an adult female may have a clutch of eggs every three to four days until passing away. Females may live up to two months in the laboratory, with a higher age recorded under poor feeding conditions (Ebert, 2005). Clutch sizes vary among daphnia species and are up to 100 in *Daphnia magna* (Ebert, 2005).

1.5. Adaptation of living organisms to environmental stress

Adaptation is an adjustment of microorganisms, or any of their organelles, to a frequent or sustained stimulus in the surrounding conditions either for a period or the entire organism's lifetime, leading to natural selection and making the organisms better fitted to the new conditions. This change might be physiological or, over generations, evolutionary (Forbes and Krimmel , 2010).

Acclamation is the process in which an individual organism adjusts to a change in its environment, such as temperature, humidity, altitude alteration, photoperiod flocculation, or pH, allowing it to uphold functioning through a variety of environments. Precisely, acclamation can be defined as a behaviour that an organism follow to cope with an unideal event that strikes for hours to weeks, and within the organism's lifetime (short period); in contrast to adaptation, which is a development that takes place over many generations (Gatten *et al.*, 1988).

The acclamation may be a discrete phenomenon for example, human bodies acclimate to new temperatures with remarkable speed or may instead represent part of a periodic cycle, such as a mammal shedding heavy winter fur in favour of a lighter summer coat. Organisms can adjust their physical, behavioural, morphological, and/or biochemical traits in response to changes in their environment (Gaur & Rai 2001). Although a lot of work on numerous species have been reported about their ability to adapt to new environments, there remains a lot of unanswered questions about why and how organisms act so.

Adaptive evolution (AE) is a widely applied strategy for the economically valuable traits' selection and engineering. The natural process typically starts with an environmental change and results in genetically inheritable conversion of a living organism. Unlike shock (an initial cellular response, which usually includes a transient reprogramming of cellular activities) adaptation is the subsequent cellular state that involves inheritable traits resulted from long-term exposure and selection (Velmurugan *et al.,* 2013). For an AE, new conditions must be defined for prolonged sets of time during cultivation of a microorganism. The period of time is in the range of weeks to years depending on the species under study, which allow the selection of strongest phenotypes for the AE process to begin. Important advantages will be obtained when microbial cells are used in AE studies as they i) require minimum nutrient ii) are easily cultivated and iii) grow rapidly, which allow the microbial cells to be cultivated for considerable number of generations within weeks or months (Dragosits *et al.,* 2013).

Algae has been used frequently in AE studies, for carbon limitation, light stress, pH stress, salinity stress, nitrogen starvation, phosphorus starvation and heavy metals tolerance experiments (Fu *et al.,* 2012; Fu *et al.,* 2013; Yu *et al.,* 2013; Yokota and Canvin, 1986; Yun *et al.,* 1996; Umino *et al.,* 1991; Chiu *et al.,* 2008). When exposed to nitrogen starvation stress, green microalgae *Chlamydomonas reinhardtii* were enhanced by AE producing higher growth rates, biomass and total lipid content from 32% in the original strain to 36.67% in the obtained phenotype (Yu *et al.,* 2013). *Chlorella vulgaris* and *Dunaliella salina* growth yield was increased by combined AE and LED-based photobioreactors PBRs (Fu *et al.,* 2012). Besides, AE improved the carotenoids yields in *D. salina* when redesigned LED- based PBRs were used (Fu *et al.,* 2013).

Many studies reported the approaches to adapt *Chlorella* to different light conditions (Blanc *et al.,* 2010; Nielsen *et al.,* 1962; Hutchinson, 1969; Wilhelm and Wild, 1984; Senge and Senger, 1990 and Senge and Senger, 1991), temperature (Maxwell *et al.,* 1994; Hosono *et al.,* 1994; Sayed and El-Shahed, 2000), and CO_2 levels (Yokota and Canvin, 1986; Yun *et al.,* 1996; Umino *et al.,* 1991; Chiu *et al.,* 2008).

For remediation purposes, *Chlorella sp.* were adapted to municipal wastewater treatment. *Chlorella sp.* could successfully remove ammonia, total nitrogen, total phosphorus, and COD by 93%, 99.1%, 80.9% and 90.8% respectively from wastewater (Li *et al.,* 2011). Selenium resistance green microalgae, *C. vulgaris,* were developed by AE (Shrift *et al.,* 1961). Also, resistance to selenium remained after *C. vulgaris* was sub-cultured in selenium free medium indicating that AE has occurred (Shrift, 1954). AE was performed for phenolic wastewater treatment by *Chlorella sp.* after 31 rounds, the obtained strain was able to grow under 500 mg/l and 700 mg/l phenol without significant inhibition. The 500 mg/l phenol was totally removed by the strain obtained within seven days when initial cell density of $0.6*10^9$ was used (Wang *et al.,* 2016). *Chlorella sp.* was successfully able to grow in diluted landfill leachate after short period of adaptation with a removal performance of COD 60% and NH_4 100% in the diluted leachate (El Quaer *et al.,* 2016).

All living organisms have the characteristic feature of adaptation. It is a beneficial structural, functional and/or behavioural alteration developed in living organism over time as a response to changes in the surrounding habitat. On evolutionary basis, adaptation could be classified into two categories: short-term adaptation (temporary) and long-term adaptation (permanent). Changes in the short-term adaptation are temporary, not inherited, has no DNA alteration,

and has no role in evolution. On the other hand, changes in the long-term adaptation are permanent, inherited, influences DNA and play an important role in evolution (Fisher, 1930; Pianka, 2000).

1.5.1 Algal adaptation to heavy metal toxicity

As microalgae have been found in potentially polluted environments, studies reported in the literature indicate that microalgae may be able to adapt to heavy metal toxicity (Pandey, 2017; Gaur & Rai, 2001). These environments occur as a result of the disposal of domestic and industrial wastes into the water bodies (Stokes & Krauter, 1973). In the laboratory, strains of microalgae were successfully grown in elevated levels of heavy metals (Shehata and Whitton, 1982; Gaur & Rai, 2001). Toleration of algae to metals could be genetic or physiological and toleration to heavy metals varies between different algae groups (Mehta & Bisen, 1992). It is presumed that the first step of adaptation is selection, if it is mixed habitat, then the most tolerant group or species will last longer and less resilient group will be eliminated sooner (Gaur & Rai, 2001).

Gater *et al.* (1987) found a distinct variation in the combination of an algal consortium with increase of Zn levels in the following order diatoms>filamentous green algae> unicellular green algae suggesting the later to be the most tolerant to Zn than other groups. In the same group of algae there is a natural diversity in the susceptibility of heavy metals amongst species, Kesslar, (1985) demonstrate that one of *Chlorella saccarophita* strains was a hundred folds more sensitive than the other fourteen strains belonging to five species of *Chlorella*, he used in his study.

1.5.2 Adaptation to pH stress

Another crucial factor for the photosynthesis process is pH, and this is because pH impacts almost any biological and chemical process, especially in aquatic organisms like algae. Different algae cultures may have different pH ranges, and optimal pH environment can help algae cultures, not only survive, but also thrive (Pick, 1992). For instance, marine algae strains favour pH at around 8.2, while freshwater strains need pH of approximately 7.0 (Abu-Rezq *et al.*,1999).

Another form of pH effect on algae, is that algae require carbon dioxide for growth and pH can affect how much CO_2 is available. Depending on pH, dissolved carbon dioxide in the water can be found in three species. These species are: carbon dioxide (CO_3^{-2}) which found at low pH, bi-carbonate (HCO_3^-) is dominate at neutral pH, and carbonate (CO_3^{-2}) at high pH. Algae can only make use of carbon dioxide and bicarbonate, and cannot use carbonate, which suggests that high pH range will prevent algae from practicing photosynthesis (Fondriest Environmental, Inc.,2016). Naturally acidophilic algae can constantly keep their internal pH at values around neutral, they can maintain an internal pH of 7 even at external pH of 1 (Gaur & Rai, 2001). Neutralophilic unicellular algae also behave similarly. However, acidophilic algae have approximately double the buffer capacity than neutralophilic algae (Gimler and Weis, 1992). It is suggested that lipids play important role in the ability of acidophilic algae to tolerate pH variation (Pick, 1992).

1.5.3 The relationship between pH and heavy metal tolerance

In most cases, studying pH tolerance is coupled with resistance to heavy metal toxicity. This is because most heavy metals, with the exception of lead, are more soluble in acidic environments than at neutral or alkaline pHs. It is believed that acid tolerant algae are also resistant to heavy metals because they use the same mechanisms to overcome both stresses (Klimmek *et al.,* 2001). When cationic metals come into contact with the microalgal cell wall, metals then transfer to the cell plasma membrane, and then enter the cell thereby affecting cellular metabolism (Kumar and Nanda, 2018). This exchange capacity is metal species specific and pH dependent (Rai *et al.,* 1993). With a decrease of pH, adsorption to cell wall decreases. Acidic environments, encouraging cations to approach the cell plasma membrane. Between pH 3 to 5, considerable quantities of metal ions will be captured by the cell wall. pH increase will raise the number of metal ions binding to cell wall substances in acidophilic algae (Hirooka *et al.,* 2017). This implies that resistance to acid environments induces resistance to heavy metals. It is supported by a study on the acidophilic strain of *C. vulgaris* when performed better metabolic activities, then wild type at lower pH (Rai *et al.,* 1993).

1.5.4 Mechanisms of tolerance to heavy metals in microalgae

The means of heavy metals resistance, when it comes to the susceptibility to more than one metal, could be the result of developing more than one mechanism for each metal or it could be that the development of one metal tolerance automatically confers resistant to other heavy metals (Rai *et al.,* 1981a; Rai *et al.,* 1981b) (Figure 1.4). The prior opinion has been

supported by Hall's research (1980) in which the co-tolerance of Cu- resistant culture of *Ectocarpus siliculosus* to zinc and cobalt was demonstrated. Similarly, Fakamura *et al.* (1989) confirmed cadmium and zinc co-tolerant in the same freshwater algae. Further, Bariaud *et al*. (1998) reported that one of *Englena gracilis* strains that is Cd tolerant was also co-tolerant to zinc and cobalt. Foster (1982) argued that under some conditions, it is preferred that algae that has an unspecific metal tolerance because it is easier to induce comber to the metal specific tolerance.

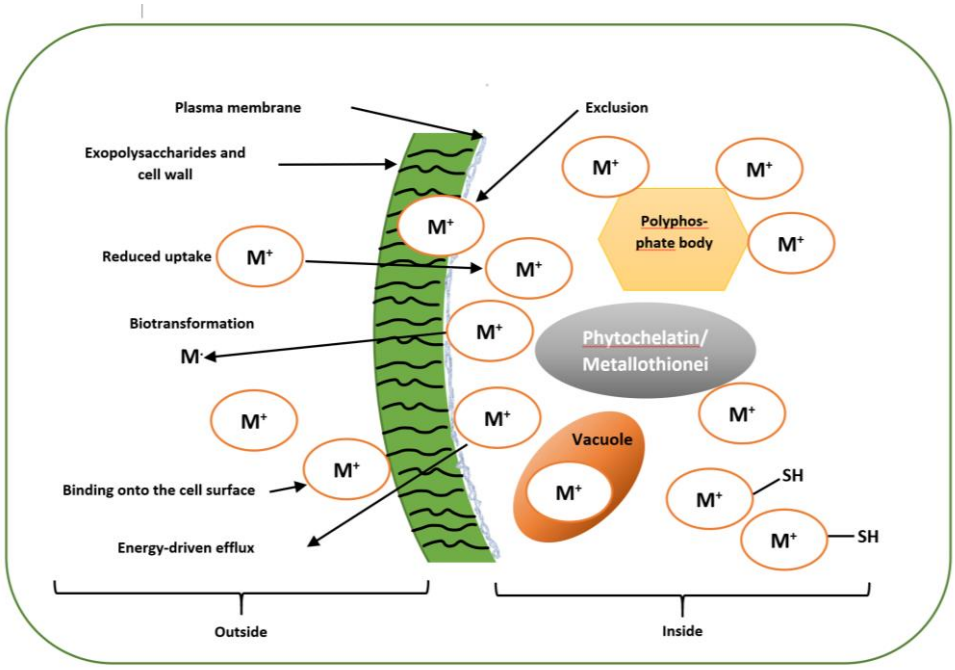

Figure 1.4. Common mechanisms for metal tolerance in algae

Mechanisms involved in the tolerance to high concentrations of heavy metals generally involve the retention of metals on the external component of the cell and thereby reducing metal bioavailability and diffusion into the cell. In addition, they are responsible for the increase of the efflux of metals from the cell. Furthermore, they are the intercellular group of detoxification mechanisms. The latter includes sequestration in polyphosphate bodies,

metallothionein, phytochelatins and form strong ponds with chelators. Detoxification by oxidation and reduction of metals to produce a form of metals that are less soluble or less toxic. The synthesis of enzymes or meta that alters oxidation stress caused from exposure to heavy metals is also a way of detoxification that might enhance metal resistance. The development of toleration to a single metal might involve the combination of more than one strategy (Tchounwou *et. al*, 2012).

1.5.4.1 Extracellular mechanisms

There are a number of extracellular mechanisms for tolerating high concentrations of heavy metals by microalgae and other micro and macro-organisms such as higher plants. Below a list of the most frequently mechanisms that have been reported by researchers who studied the mechanisms of heavy metals tolerance in eukaryotic and prokaryotic microalgae.

Binding to cell surface: This strategy has been frequently reported as an important mean of metal binding in algae. The surface polymeric components in the cell wall works as a good medium for metal ions. About 85% of metal ions accumulation was found to be in the cell surface in some cases (Reed and Gadd, 1990). The presence of carboxyl and other groups in the cell wall gives it the negative charge that plays an important role in attracting metal ions. This is supported by the evidence derived from research that has been made on *C. reinhardtii* which has a cell wall mutant, it was more sensitive to cobalt, cadmium, and nickel, than the wildtype (Gentr, 1996). Another piece of evidence is provided by Rijstenbil *et al.* (1998), in which the authors reported that algae species with thicker cell walls (1-9 μm) were more tolerant to copper than the species with thinner cell walls (0.5-5μm). On the other hand, many

researchers believed that adsorption to cell surface is only a transitional stage of ion uptake inside algal cell (Kraver *et al.,* 1997). Whether adsorption to the cell surface component is perpetual or just transitional, the most valuable feature is if this step is actually reducing the bioavailability of heavy metals (Volskey, 1990).

Production of metal-affinity ligands: Aquatic microorganisms' responses to the toxicity of available metal ions not the total metal concentration. The ionic form of metals is the effective form when it comes to toxicological effect. Natural water has variety of ligands that metal ions tend to adsorb, thus reducing their availability to living organisms. Algae participate in releasing such substances. This demonstrates that *Anabaena cylindrica* produces an extracellular material that has the trait of complexation and detoxification of copper ions (Fogg and Westlake 1955). Similarly, eukaryotic algae and some cyanobacteria species can produce organic acids as copper complexing agents (Mokght and Morel, 1979). Similarly, Mokght and Morel (1979) also stated that four species of cyanobacteria were able to produce hydroxamates that are a strong complexation agent.

In some cases when the produced complex of cupric schizorium is recognised by the ferric schizokinen uptake systems, the toxicity of copper will be emphasized as occurred in species *Bacillus megaterium* (Arcereak *et al.,* 1984). Clark *et al.* (1987) stated that *Anabaena* sp. developed a siderophore system to tackle ferric starvation, but also fortuitously displayed improved copper tolerance. It was also reported that eukaryotes are able to produce ligands with weak and strong complexation abilities (Xue and Sigg, 1990) disputed whether these substances were already existing in the surroundings and complexed with copper ion or had been released to the external environment after exposure to Cu Ions (Gledhill *et al.,* 1999).

The first opinion is favoured in case of copper detox by *Fucus vesiculosus*. Evidence points to algae being able to extract both weak and strong complexation agents. A particular kind of ligand is normally produced by a specific organism. However, organisms sometimes produce more than one type of ligand (Morelli *et al.,* 1989). Initially strong ligands get saturated with each metal and then weaker complexing agents start to form bonds with metal ions. They could play a significant role especially if they are present in a high concentration. Weak and strong complexing ligands are important in protecting algae from metals toxicity (Stordub *et al.,* 1987).

1.5.4.2 Intracellular mechanisms (sequestration by protein and peptides)

There are several intracellular mechanisms for tolerating high concentrations of heavy metals by microalgae that is shared with other organisms such as yeast, higher plants, and even animals and humans. *Efflux of Ions*: Exclusion of metals in this way is one of the mechanisms used by algae for metal tolerance and is rare to be seen in higher plants (woodhouse, 1983). Strains that use these mechanisms are found to accumulate less metals intercellularly in comber to nontolerant ones (Foster, 1977; Hall *et al.,* 1979; Bariaud *et al.,* 1985; Rai *et al.,* 1991). Under this concept, metal tolerant organisms can control metal influx by; cell wall modification or membrane functional alteration to reduce permeability of metal ions (Silver and Phung, 1996; Twiss and Nalewajko, 1992). A copper tolerant strain of *Anabaena doliolum* was isolated and its morphology and biochemical structure, were studied by Rai *et al.,* (1991), they found less copper transport, more lipid content and better membrane integrity, in comparison to copper sensitive strain of some species of that *Anabaena doliolum.* Change in membrane permeability were also reported by Asthana *et al.* (1993) which explains the Ni tolerance in *Nostoc muscorum*. Algae with a strong negative charge on the cell wall could attract more Al ions that are positively charged, which increases algae

sensitivity to this ion (Wagatsuma and Akiba 1989). Metal efflux is an active process that requires energy to accomplish, in their research using *Nostoc calcicole*, Verma and Singh (1991) demonstrated an energy dependent copper efflux system. Yoshimura *et al.* (1999) also supported this fact with his research on *Cyanidium caldarium* which is an Al tolerant species. *Chlorella* sp. has an enzymatic system that oxidise mercury chloride to metallic form (Hg°), that is volatilised easily from algal cells (Tchounwou *et al.,* 2012).

Rai (1990) reported that a family of metal-agent proteins named metallothionein work as regulators of exceeded levels of metal ions in many organisms including plants and algae. They have found metallothionein to be able to sequester metal ions, particularly Zn and Cu with high affinity to these two ions. In algae it is reported that *Anacystis nidulans* and *Synechococcus sp.* contain metallothionein (Gaur & Rai, 2001). Eukaryotes endowed with metals tolerance may have several metallothionein genes copies and therefore increase in the metallothionein mRNA and protein (Palmiter, 1987). The metal induces of metallothionein production differ between different microorganisms. For example, copper is the preferred reported inducer of metallothionein in yeast (Volskey, 1990). A similar role has been described for phytochelatins a peptide that is synthesised enzymatically (Steffens, 1990) (Figure 1.5). Phytochelatins have a foundational feature of binding to metals ions. They have been isolated from contingent of organisms (Robinson *et al.,* 1993). When biochemical properties of phytochelatins were investigated, cadmium and copper were found to be participating in formation of peptide-metal complex in high plants and algae (Grill *et al.,* 1987; Robinson 1989) and weak bonds with zinc (Robinson, 1989). It has been reported that phytochelatins biosynthesis only occurs when cells exposed to high concentrations of heavy metals (Robinson 1989). In algae production of phytochelatins could be induced by variety of metals including Cd, Ag, Pb, Zn, Cu and Hg (Robinson 1989). It is reported that

Thalassiosira weissflogii found to synthesize phytochelatins when exposed to Pb, Ni, Cu, Zn, Co, Ag and Hg (Ahner and Morel, 1995a.b). Complexation of phytochelatins with metal ions can be quite significant; for example, Howe and Merchant (1992) reported that 70% of cytosolic cadmium has been sequestrated by the polypeptide aggregates in *C. reinhardtii.*

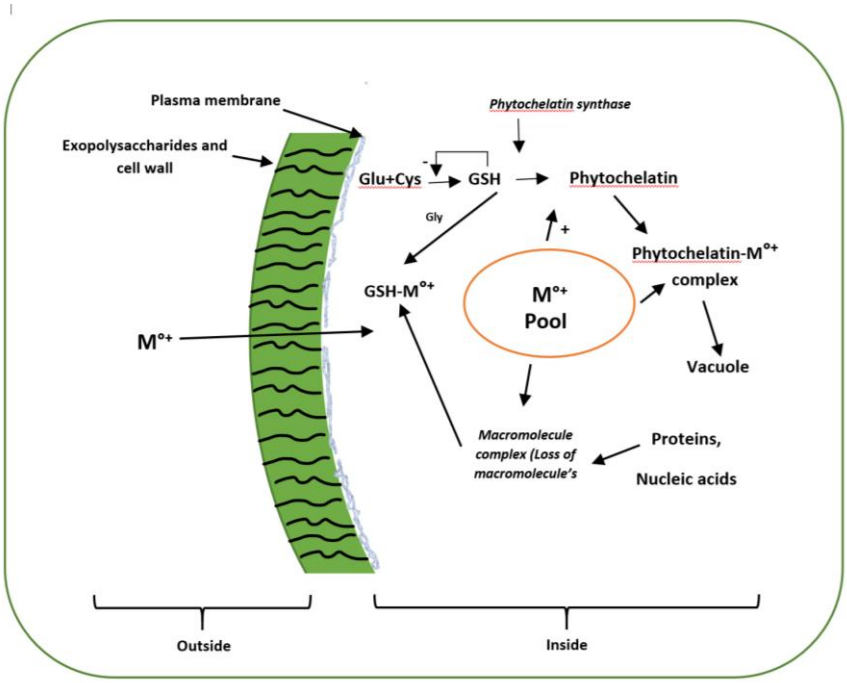

Figure 1.5. Phytochelatins synthesis model and metal detox. The accumulation of metals inside cells will form a metal pool (M°+). (GSH) is the Glutathione that exists in the cell and used for phytochelatins biosynthesis. GSH in the cell, when overproduced, can bind directly to metals like Hg and Ag in an efficient way and less efficiently with Cd. Cytosolic metal catalyses the formation of phytochelatins; in turn, they bind metals to form cytosol and detoxifies it, or safely transfers into the vacuole. Metals can also be compartmentalized by proteins, nucleic acids, and enzymes. Apoenzymes and proteins may use metals from the pool for metabolic processes.

Phyto-detoxification enzymes are triggered when metal ions accumulate in the cell. The presence of metals is followed by the synthesis of phyto-detox enzymes. The produced phyto-

detox enzymes twitched forming ponds with metal ions. which reduces the in-cell concentration of heavy metals. As a result, toxicity is reduced due to the low concentration of unbound metal ions (Gaur & Rai, 2001). Capturing metals in this way protects living cells from ions activity that disturbs their biological process. Phytochelatins work as metal transporting agents from cytoplasm to a specific vacuole (Vogeli-Lange and Wagner, 1990).

On the other hand, the role of metallothionein include zinc ion homeostasis and distribution, gene expression regulation, programming expression during development, monitoring bioindicators of low levels of metals in the surroundings (Erb *et al.,* 1996). It also plays a role in optimising metal levels inside living cells (Gaur & Rai, 2001).

1.6 The aim and objectives of the thesis

This thesis investigates the possibility of using green microalgae to mitigate heavy metal mobility and impact in aquatic systems through adaptation. The hypothesis is that applying selection and adaptation on nominated green microalgae will improve their resistance to heavy metal toxicity for better bioremediation. This thesis incorporates relevant works and methodological development into experimental findings to achieve the following objectives:

- Review literature about bioremediation, mechanisms, factors, and challenges, in particular, the work related to adaptation strategy and its implements in microbiology. Moreover, research related to existence, background values, pollution, and impact of heavy metals on humans and the environment.
- Evaluate the impact of selected heavy metals on green microalgae species, C*hlorella vulgaris* and *Chlamydomonas reinhardtii,* used in the research and *Daphnia magna* to find $LC_{50,}$ which was used as a reference value to be castoff in the first stage of the adaptation approach.
- Investigate the adaptation capacity of the studied green microalgae *C. vulgaris* and *C. reinhardtii* over an extended period of exposure to heavy metals in different and gradual concentrations.
- Investigate the changes that might occur in the cell structure due to exposure to heavy metals during the adaptation process. This was achieved by using Fourier-transform infrared spectroscopy (FTIR).
- Use *Daphnia magna* as an indicator of the efficiency of the produced microalgal phenotype in removing heavy metals from the solution.

CHAPTER TWO

MATERIALS AND METHODOLOGIES

2.1 Preliminary microalgal sampling, growing, and monitoring

2.1.1 Green microalgae growth

2.1.1.1 Growth media

Bold Basal Medium with 3-fold nitrogen and vitamins (3N-BBM+V) was used to grow both *Chlorella vulgaris* CCAP 211/111 and *Chlamydomonas reinhardtii* CCAP 11/45. This medium was a modification of Bristol's solution (Bold, 1949). Stock solutions of each of the following salts were prepared: $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $K_2HPO_4.3H_2O$, KH_2PO4, NaCl, $NaNO_3$, trace element solution (Na_2EDTA, $FeCl_3.6H_2O$, $MnCl_2.4H_2O$, $ZnCl_2$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, vitamin B1 (Thiaminhydrochloride), vitamin B12 (Cyanocobalamin). Stocks were prepared according to the CCAP recipe, kept in the fridge, and used during the course of the study (Appendix 1).

For *Chlorella vulgaris* 211/21A, f/2 Medium was used. Stock solutions of each of the following salts were prepared: NaNO3, NaH2PO4.2H2O, trace element solution (Na2EDTA, FeCl3.6H2O, CoCl2.6H2O, MnCl2.4H2O CuSO4.5H2O, ZnSO4.7H2O, MnCl2.4H2O, Na2MoO4.2H2O), and vitamin mix (Cyanocobalamin "vitamin B12", Thiamine HCl "vitamin B1", and Biotin). Stocks were prepared according to the recipe of the culture

collection of algae and protozoa (CCAP), kept in the fridge, and used in the experiments (Appendix 2).

2.1.3.2 Growth conditions

Algae cultures were obtained from CCAP and grown in 250 ml conical flasks. Glass flasks were filled with 100 ml of the 3-N-BBM, or f/2 medium then autoclaved at a temperature of 121˚C for 45 minutes. The media were inoculated with 5% v/v from stock cultures that were approximately in the mid-exponential phase. Then cultures were incubated at 25˚C under 12h:12h photoperiod with light intensity varying between 200-250 μE. Continuously, cultures were shacked at 110 rpm (IKA orbital rotary shaker KS 501 d). Optical density (OD) was measured every 24h at 700 nm.

Optical density (OD) analysis OD700 was used to follow growth in a spectrophotometer, Ultrospec 2100 Pro UV/Vis. The time intervals from sampling to OD 700 nm measurements were approximately the same at all samplings every day. The samples were diluted with the medium appropriately to result in OD 700 nm of 0.2-0.5, if required. The spectrophotometer counts an average of three readings on each sample. Before running the OD 700 nm measurements with cells, the spectrophotometer was zeroed with medium only, which was exchanged every day to avoid errors from probable contamination.

Cell count analysis as a complement to OD 700 nm measurements total cell counts was performed on samples in a counting chamber, Bürker with 0.1 mm depth, on ZEISS Axiostar microscope, with 40 times magnification. The chamber was mopped using tissue with ethanol

before each counting. A cover glass was put on, and 100 µl samples were dragged in. The same dilution as OD 700 nm was used to get an adequate number to count, 20-100 cells per square. Five squares were calculated for all samples. Cells on the left line and the top line were also counted. Limitations associated with the procedure of cell counting were the difficulty of separating dead or empty cells from the live ones, except when a stain is used, a cluster of small cells were difficult to count with absolute precision. The process of counting the cells is undoubtedly time consuming and monotonous.

Snijder Chambers with controlled light, temperature, CO_2, and humidity were used to incubate the starter cultures obtained from CCAP. The chamber conditions simulate the global climate in a reproducible manner by controlling day and night cycles in addition to the previously mentioned conditions. These growth chambers have been used in the first six months of the study.

For the rest of the study period, a growth chamber was built to accommodate experimental units under study (Figure 2.3). The conditions were controlled using a commercial temperature controller and timer switch to control the light. Temperature was monitored using data logger (Figure 2.4 a, b and c).

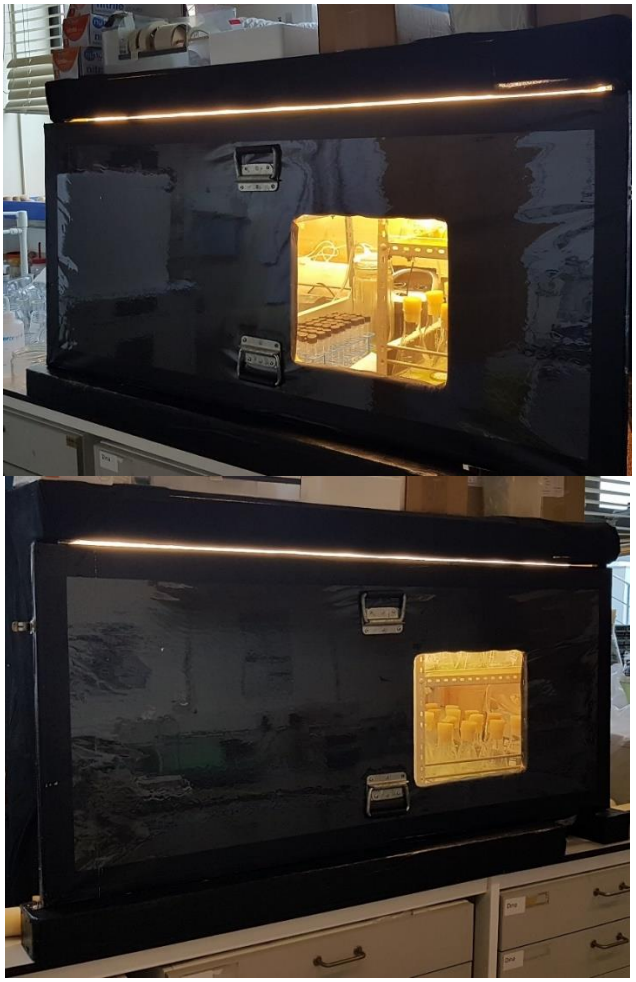

Figure 2.3. Built growth chamber

Figure 2.4. (a) Data logger, (b) temperature controller, (c) timer switch

2-2 The impact of heavy metals on microalgae and daphnia

2.2.1 Chemicals

The chemicals used to prepare the toxicity test solutions are $Fe(NO_3)_3 \cdot 9H_2O$, $Pb(NO_3)_2$, $Mn(NO_3)_2 \cdot 4H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Cu(NO_3)_2 \cdot 3H_2O$, $Co(NO_3)_2.6H_2O$, $Cd(NO_3)2 \cdot 4H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, $As_{21}8O_3$, and $HgCl_2$.

2.2.2 Procedure

To determine the susceptibility of microalgae to heavy metals, different concentrations of selected heavy metals were added together and separately to the 3NBBM and f/2 medium. To establish the concentration of heavy metal/s that causes a fifty per cent reduction of algae population (LC_{50}), following the protocol of the Organisation for Economic Co-operation and Development (OECD) 201 (OECD, 2011). Five concentrations of each of the metals were used: 0 (control), 0.1, 1, 10, and 100 mg/l. Cell density measurement used for growth monitoring on algae for up to 5 days (the beginning of log-phase). The matrix used to prepare metals solutions is 3-NBBM and f/2 medium. Metals solutions of the five concentrations were distributed to 250 ml Erlenmeyer flasks at 100 ml each. Thus, each metal (Cu, Zn, Ni, Hg, Co, Al, Mg, As, and Cd) concentration/ treatment has three replicates.

The inoculum was prepared no more than 2 to 3 hours prior to the beginning of the test, using *the microalgal cultures* harvested from a four to seven-day stock culture. Each millilitre of inoculum contained enough cells to provide an initial cell density of approximately 1 x 10^4 cells/ml (± 10%) in the test flasks. Inocula of sufficient numbers of log-phase-growth

organisms then were transferred to each flask. The experiment units were incubated under 12:12 light: dark cycle illumination at 86 ± 8.6 μE/m^2/s (400 ± 40 ft-c), at 25 ± 1ºC, and were shaken continuously at 100 rpm on a mechanical shaker. Flask positions in the incubator have been randomly rotated each day to minimize possible spatial differences in illumination and temperature on growth rate. pH (Fisher brand pH meter was used) and temperature were monitored at the beginning and end of the test (Appendix 4).

2.2.3 LC_{50} calculation

At the end of the test, the LC_{50} was measured for each organism and calculated using Probit's analysis. In this method, the survival percentage at each concentration of the toxin is compared with the growth percentage of the control culture of the strain under study. In excel, the toxin concentrations have been listed and, log10 of these concentrations have been calculated. Then the death percentage is also calculated and listed. The dead percentage then transformed to Probit using the Probit transformation table (Appendix5). The regression analysis has been performed after that, and from the analysis table, the values of x and intercept have been used in the following equation:

$$Y=ax+b$$

Where a=x variable

b= intercept

To calculate the LC_{50}, the value is transformed to Probit using the transformation table. So, the Probit of 50 is 5 in the transformation table and that will substitute Y in the equation Y= ax+b. By solving the previous equation, x value is founded and LC_{50} is the antilog of x, which represents the concentration of toxin that causes 50% of population death.

2.2.4 The impact of heavy metals on *Daphnia magna*

OECD protocol 202 (1996, 1998, 2000) was used to test heavy metals impact on daphnia. The same general aspects that were adopted in the metal toxicity test on algae have been used with the following principles: young daphnids of age less than 24 hours have been used, exposed to a range of metal ions (Cu, Zn, Ni, Hg, Co, Cd and their mixture) at concentration: 0.001 mg/l, 0.01 mg/l, 0.1 mg/l, 1, 10 mg/l and the control, for a period of 48 hours. The mixture was prepared by adding equal amounts of each metal in the mixture to prepare the required concentration. For example, to prepare 10 mg/l of the metal mixture, an amount of 1.67 mg/l of each metal (Cu, Zn, Ni, Hg, Co, and Cd) were mixed in one litre of the growth medium to reach a final concentration of 10 mg/l of the metal mixture. In addition, counts of living daphnids were taken every two hours and compared to the control.

Matrix solution used to prepare the metals stock solutions and for the control, prepared from melting the nutrition ballet provided by the daphnia starter culture supplier (Aquaria). In addition to the regular count of surviving daphnia, pH, temperature, and dissolved oxygen have been monitored at the beginning and end of the test (Appendix 6).

2.3 Method development for acclimatization of green microalgae to heavy metals

During the adaptation of microalgal cultures to increasing concentrations of metals, a defined set of conditions for prolonged time sets were used to cultivate a microorganism. After

determining the exponential phase of our strain from the initial growth curve, repeated generations of 7 days were applied. Considering maintaining cell density at the same level during all adaptation stages as much as possible. During generations of the same stage (or two stages), metals' concentrations remain the same. Thereafter, the concentration of the aqueous solution of heavy metal/s was gradually increased, starting after the tenth generation of each stage. Five stages of adaptation were conducted and lasted for 315 days. The initial level of heavy metals used in stage 1 is derived from the inhibitory experiment, as shown in chapter four (Table 4.1). Metals' concentrations used in the subsequentially stages were increased by approximates of 10%.

Samples solutions from the last generation of the last stage of adaptation were analysed chemically using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES 7200Thermo Fisher Scientific). The ICP-OES is a system in which samples that contain a configuration of elements, mainly water-soluble, can be determined using plasma and spectrometer. It is a preferred technique because of the multi-element options and high throughput.

2.3 Cell structure analysis by Fourier-transform infrared spectroscopy (FTIR)

For cell structure analysis, Fourier-transform infrared spectroscopy (FTIR) is used. This system is used to attain an infrared spectrum of absorption or emission of a solid, liquid, or gas. A high-spectral-resolution data over a broad spectral range is simultaneously collected

via an FTIR spectrometer; this deliberates a significant advantage over a dispersive spectrometer, which measures intensity over a narrow range of wavelengths at a time. The name FTIR is since the signal first needs to be Fourier-transformed to generate the more familiar IR plot of intensity versus wavenumber. Alpha Bruker FTIR spectroscopy was used in the study.

Sample preparation is the most important step in the process of spectra collection. After centrifuging the sample and decanting the solution, samples then need to be washed. It is important to carefully remove any contaminants by washing a sample several times with distilled water to remove any other component/residue from the experimental solution. In addition, a sufficient amount of the sample needs to be taken for analysis; after washing the samples, they have to be snap frozen with liquid nitrogen; and then lyophilize (freeze dry) to be ready for scanning with FTIR or to be kept in the freezer for a later process at -80^{o}C.

2.5 The survivability of *Daphnia magna* in heavy metal solution after microalgal bioactivity during incubation time

Metal solutions were prepared for this experiment were 0.01 for Zn, Ni, Hg, Co, and Cd and 0.001 for Cu and heavy metals mixture. Solutions were added to the 250 ml Erlenmeyer flasks, 100 ml each. The matrix used to prepare the solutions was distilled water that contains nutrition pills provided with *Daphnia magna* from the supplier, one pill per litre. The flasks were divided into two sets, one to be treated with *Chlorella vulgaris* phenotypes and the

second with *Chlamydomonas reinhardtii* phenotypes. Each metal solution was treated with the microalgae adapted to that metal.

Microalgal cultures during exponential phase were washed three times with distilled water after harvesting from the original culture and then resuspended in the matrix. An inoculum of 5% v/v of microalgae was added to each flask. After three days of microalgae bioactivity in the solutions, both sets were filtered, and microalgae were removed. The obtained solutions were then used to test *Daphnia magna* survival in the algae-treated solutions.

The algae-treated solutions were distributed in test tubes, 10 ml of solution in each tube, three replicates of each treatment. Moreover, ten young daphnia were added to each tube, monitored, and counted every two hours for a total of 10 hours. Additionally, two controls were used in this experiment: the first (control+) was the untreated metal solution and the second (control) was the matrix without metals addition.

2.6 Statistical analysis

Excel 365 version 2102 was used for analysis of variance with one-way (ANOVA) to find the significance of difference from the control (wherever needed), LC_{50} calculations, and graphs presentation. In addition, SPSS version 26 was used for Mann-Whitney test to compare nonparametric values of FTIR spectra in chapter five and Cohen's d number to find the size of effect in the fate experiment in chapter six.

CHAPTER THREE

PRELIMINARY EXPERIMENTS

This section examines and describes a pre-study investigating the growth of *Chlorella vulgaris* 211/111, *Chlorella vulgaris* 211/21A, and *Chlamydomonas reinhardtii* 11/45 using two methods: optical density (OD) and cell count. The relationship between OD and cell count was also examined. The calibration curve that can be used to find unknown cell numbers from absorbance data, was also included. In addition, results from preliminary FTIR scans of the three strains, are shown in this chapter.

3.1 Microalgal growth curves

3.1.1 Growth curves by optical density

The growth curves for *C. vulgaris* 211/111, *C. vulgaris* 211/21A, and *C. reinhardtii* 11/45 were measured using optical density (Figure 3.1). *C. vulgaris* 211/111 did not clearly show a lag phase when OD was used to monitor growth (Figure 3.1a, Table 3.1). Cell density increased as a function of time from day 1 to day 10 in the exponential phase. The growth rate was 0.57 day^{-1}, with a maximum growth with an absorbance of 1.688 on day10. *C. vulgaris* did not show a clear stationary phase as the growth started to decline after day 10, where the death phase started to occur mainly because of the reduction of nutrients.

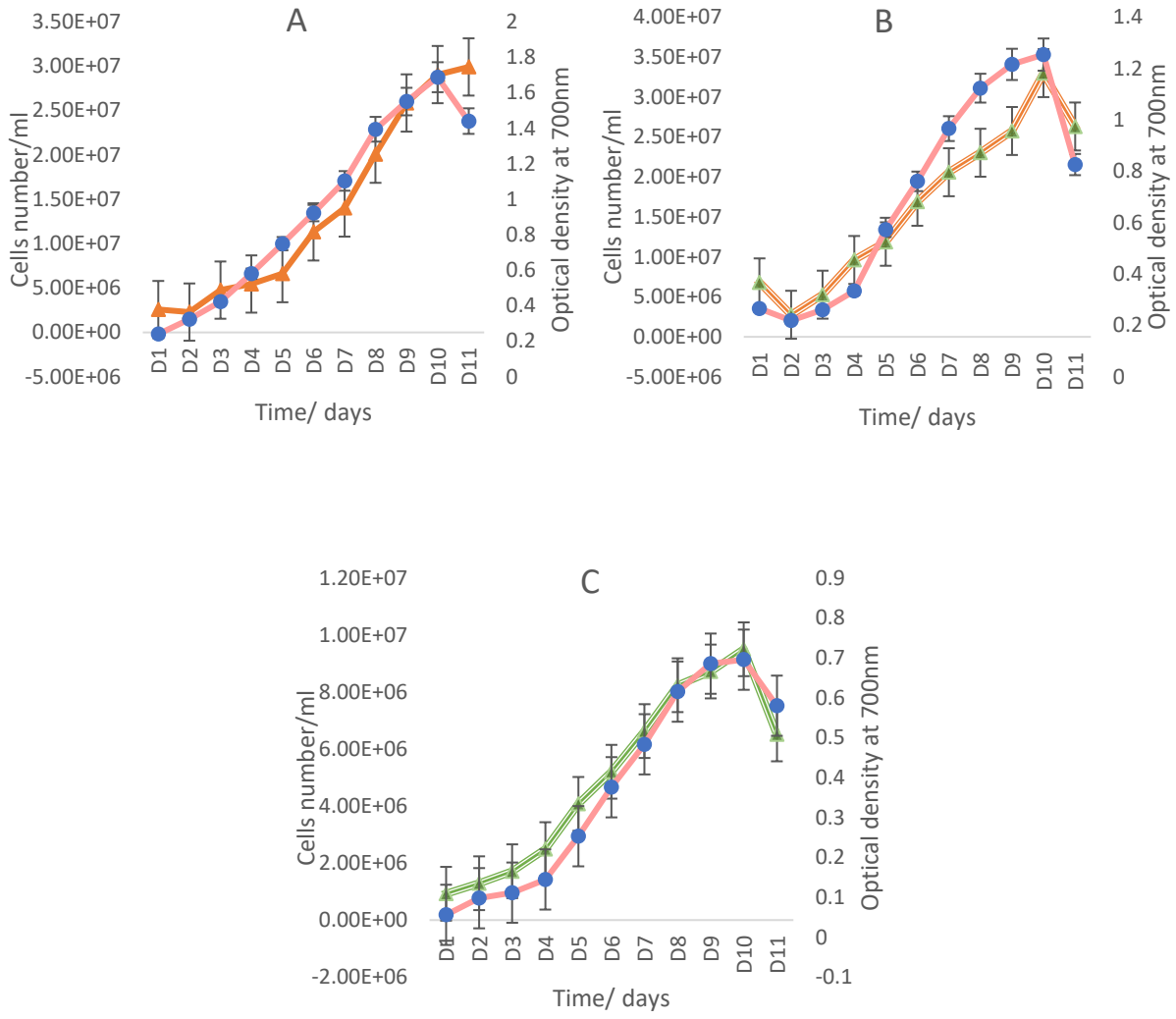

Figure 3.1 Comparison between cells number (▲) and optical density (●) readings for (a) *C. vulgaris* 211/111. (b) *C. vulgaris* 211/21A and (c) *C. reinhardtii* 11/45.

Table 3.1. Growth parameters for *C. vulgaris* 211/111, *C. vulgaris* 211/21A, and *C. reinhardtii* 11/45 by optical density

Optical density	*C. vulgaris*	*C. vulgaris*	*C. reinhardtii*
Lag phase	No	3 days	4 days
Log phase	1-10	3-9	4-9
Stationary phase	10	9-10	9-10
Maximum growth	1.688	1254	0.695
Death phase	After day10	After day 10	After day 10
Fastest growth rate	0.57 /day	0.44 /day	0.54 /day

On day 2, the growth of *C. vulgaris* 211/21A displayed a reduction in the cell density. However, the growth curve retrieved exponential tendency from day 3. This difference in the behaviour of strains in the medium could be due to a nutritional shock that some organisms display when first sub-cultured. *C. vulgaris* 211/21A showed a lag phase between days 1 and 3 with a meagre growth increase (Figure 3.1b, Table 3.1). From day 3 to day 9, the cell density increased as a function of time in the exponential phase. The growth rate was 0.44 day^{-1}, with a maximum growth of 1.254 on day 9. A stationary phase was observed between days 9 and 10. After day 10, the growth started to decline, where the death phase started due to nutrients exhaustion.

C. reinhardtii 11/45 showed a lag phase between days 1 and 4 with a low growth increase (Figure 3.1c, Table 3.1). From day 4 to day 9, the cell density increased in the exponential phase. The growth rate was 0.54 day^{-1}, with a maximum growth of 0.695 on day 10. A stationary phase was observed between days 9 and 10 where low to no growth increase was recorded. After day 10, the growth started to deteriorate in the death phase.

3.1.2 Growth curves using cell counts

C. vulgaris 211/111 displayed a lag phase between day 1 and 4 when cell count was used to monitor growth (Figure 3.1a, Table 3.2). There was a negligible growth during lag phase. However, cell number increased as a function of time from day 4 to day 10 in the exponential phase. The growth rate was 0.57 day^{-1} during exponential phase, with a maximum growth of 2.91E+7 cells/ml on day10. A stationary phase was observed between days 10 and 11. The death phase started to occur after day 11 when nutrients started to deplete.

C. vulgaris 211/21A showed a lag phase between days 1 and 3 with a meagre growth increase (Figure 3.1b, Table 3.2). From day 3 to day 10, the cell density increased as a function of time in the exponential phase. The growth rate was 0.46 day^{-1}, with a maximum growth of 3.31E+7 cells/ml on day 10. No stationary phase was observed as the growth suddenly shifted after day 10; the growth started to decline, where the death phase has started.

Between days 1 and 3, *C. reinhardtii* 11/45 displayed a lag phase with a low growth increase (Figure 3.1c, Table 3.2). From day 3 to day 10, the cell density increased in the exponential phase. The growth rate was 0.51 day^{-1}, with a maximum growth of 1.00E+7 cells/ml on day 10. *C. reinhardtii* 11/45 did not show a clear stationary phase as the growth declined after day 10, in the death phase.

Table 3.2. Growth parameters for *Chlorella vulgaris* 211/111, *Chlorella vulgaris* 211/21A, and *C. reinhardtii* 11/45 by cell count

Cell count	Chlorella Vulgaris	Chlorella Vulgaris	C. reinhardtii
Lag phase	4 days	3 days	3 days
Log phase	4-10	3-10	4-10
Stationary phase	10-11	10	10
Maximum growth	2.91E+7 cells/ml	3.31E+7 cells/ml	1.00E+7 cells/ml
Death phase	After day11	After day 10	After day 10
fastest growth rate	0.57 /day	0.46 /day	0.51 /day

3.2 Comparison of growth curves in optical density and cell count

To determine how microalgal growth patterns fit together using the two methods, a comparison between OD and cell count has been made for the three strains (Figure 3.1). *C. vulgaris* 211/111 growth shown in Figure 3.1a compares data collected in two ways, cell count and OD. The growth tendencies in both methods are similar except for the appearance of lag phase in the cell count curve, and the last reading on day 11; where a stationary phase is shown in the cell count method but a sudden decline in the growth is shown when OD is used. Also, OD looks more constant, and errors between the three replicates are reduced than the cell count method.

The growth of strain *C. vulgaris* 211/21A (Figure 3.1b), shows that both ways of growth measurement are representative and showed a similar pattern. However, the errors between replicates measured by OD are smaller than errors that occurred when cell count was conducted. *C. reinhardtii 11/45* is also well represented by both OD and cell count. In

addition, similarity of growth curves for both OD and cell count for *C. reinhardtii* 11/45, is higher than *C. vulgaris* 211/111 and *C. vulgaris* 211/21A (Fig. 3.1c). this suggest that the estimated number of cells per millilitre from absorption data, is more accurate.

3.3 Calibration curves of optical density and cell count for microalgae

The relationship between cell number and optical density was found for each strain (Figure 3.2). The correlation between OD and cell number was demonstrated for *C. vulgaris* 211/111, to find an unknown cell concentration from a known OD the following equation can be used; y = 5E-08x + 0.3439 (Figure 3.2.). For *C. vulgaris* 211/21A, the correlation between optical density and cell number was also found. This relation is better demonstrated by the equation: y = 4E-08x + 0.0565 (Figure 3.2b). Optical density when plotted against cell number demonstrates a linear relationship for *C. reinhardtii 11/45* (Figure 3.2c). The calibration curve can then be used to determine cell numbers based on the absorbance reading using the equation y=8E-08x - 0.0473.

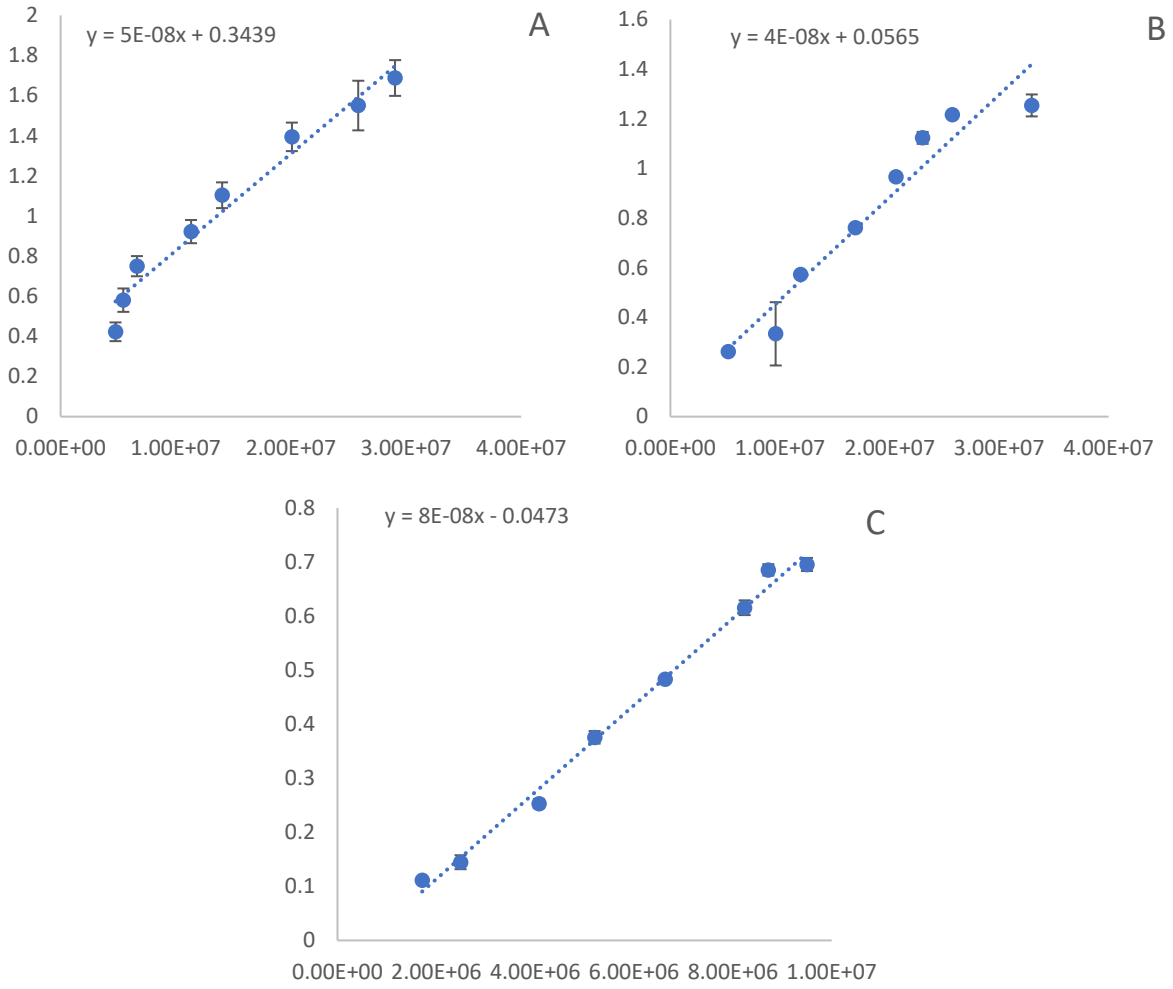

Figure 3.2 Calibration curve for optical density and cell number for (a) *C. vulgaris* 211/111 (b) *C. vulgaris* 211/21A and (c) *C. reinhardtii 11/45*.

3.3 Fourier transform infrared (FTIR) spectroscopy of microalgal strains

Preliminary analysis of microalgal strains under the study have been conducted to find the biological print of each strain through FTIR. Figure 3.5 shows the cell molecular groups of *C. vulgaris* 112/111. General identification of absorption bands was obtained from published information (Benning *et al.* 2004; Giordano *et al.* 2001; Keller,1986; Naumann *et al.* 1996; Stuart, 1997; Wong *et al.* 1991; Dean *et al.* 2007; Sigee *et al.* 2002; Maquelin and Kirchner, 2002). Peaks in the curve obtained from FTIR was assigned a functional group for each.

Protein spectra were characterized by a peak at 3270 cm^{-1} (amide A); lipid spectra were categorized by three sets of strong vibrations, the C-H at 2974 cm^{-1}, 2907 cm^{-1}, and 2897cm^{-1}; protein also featured at 1645 cm^{-1} (amide I) and 1552 (amide II). Position 1380 cm^{-1} represent protein and lipids bending of methyl. Band of position 1242 cm^{-1} stands for nucleic acids. Carbohydrate absorption bands due to C-O-C of polysaccharides found at 1148 cm^{-1}, 1045 cm^{-1} up to 883 cm^{-1}.

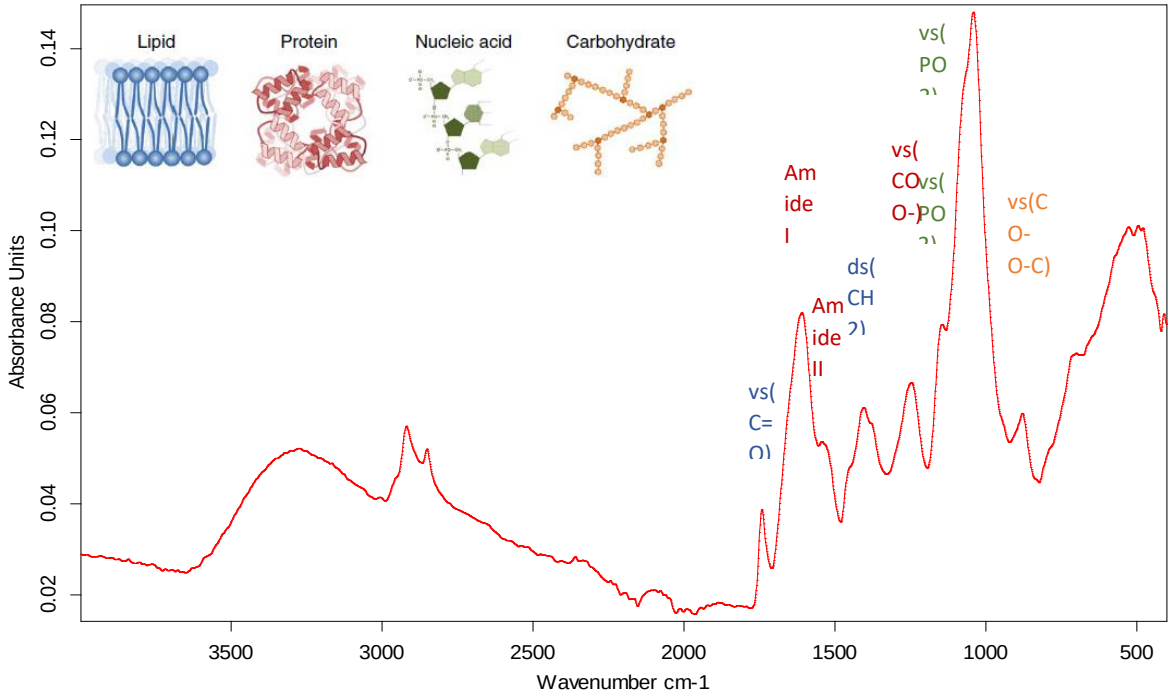

Figure 3.5. FTIR spectra of *C. vulgaris* showing biomolecular peak assignments from 3,500–500 cm^{-1}. ν = stretching vibrations, δ = bending vibrations, s = symmetric vibrations and as = asymmetric vibrations. The spectrum is a transmission type micro spectrum from algae (*C. vulgaris*). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units

Analysis of *C. reinhardtii* cell structure was made using FTIR (Figure 3.6). Published information obtained from Coates (2006), Bednarz *et al.* (2004), Andrew *et al.* (2010), Benning *et al.* (2004), Giordano *et al.* (2001) Keller (1986), Naumann *et al.* (1996), Stuart

(1997), Wong *et al.* (1991), Dean *et al.* (2007), Sigee *et al.* (2002), Maquelin and Kirchner (2002) was used for general identification of spectral absorption bands of *C. reinhardtii*. Peaks in the scans were assigned a functional group per each. Protein spectra were categorised by a clear feature at 3280 cm-1 (amide A). Three sets of strong vibrations, 2975 cm^{-1}, 2927 cm^{-1}, and 2900 cm^{-1} were for lipids spectra. Protein also presented at 1641 cm^{-1} (amide I) and 1546 (amide II). Position 1380 cm^{-1} represent protein. Band of position 1271 cm^{-1} stands for nucleic acids. Due to the C-O-C of polysaccharides, carbohydrate absorption bands were found at 1148 cm^{-1}, 1045 cm^{-1} up to 878 cm^{-1}.

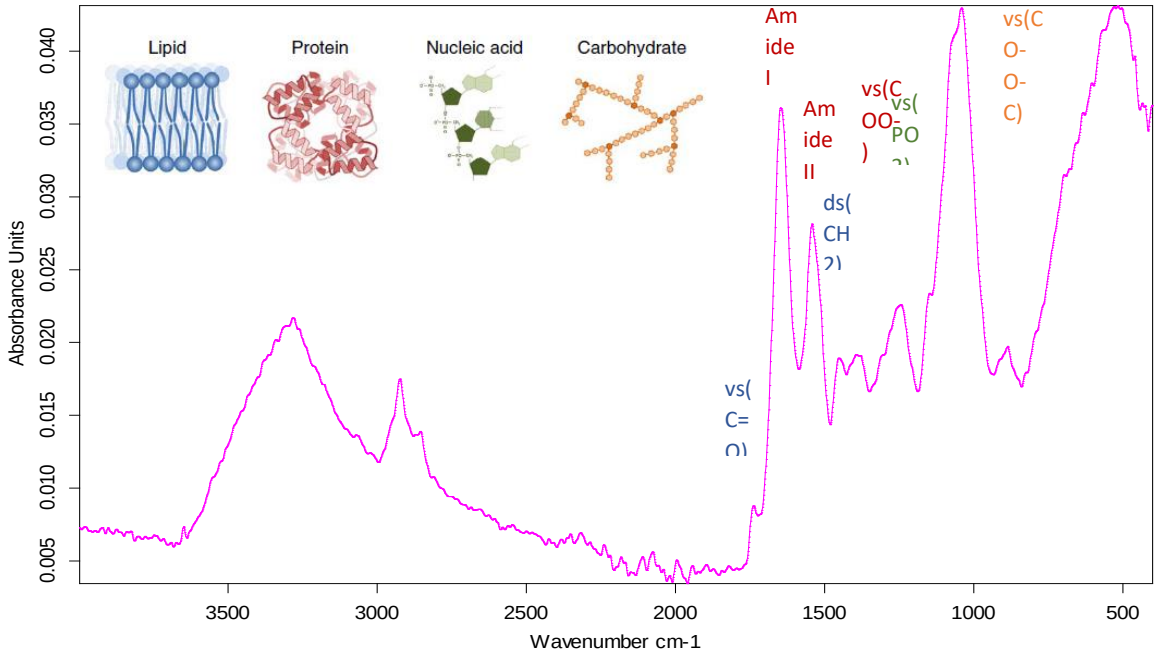

Figure 3.6. FTIR spectra of *C. reinhardtii* showing biomolecular peak assignments from 3,500–500 cm^{-1}.ν = stretching vibrations, δ = bending vibrations, s = symmetric vibrations and as = asymmetric vibrations. The spectrum is a transmission-type micro-spectrum from algae (*C. reinhardtii)*. The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units

C. vulgaris 211/21A cell structure was demonstrated using FTIR and is shown in Figure 3.7. Each peak in the scans represent a functional group. In general, *C. vulgaris* 211/21A shows the same peaks positions as in *C. vulgaris* 211/111 but differ quantitively.

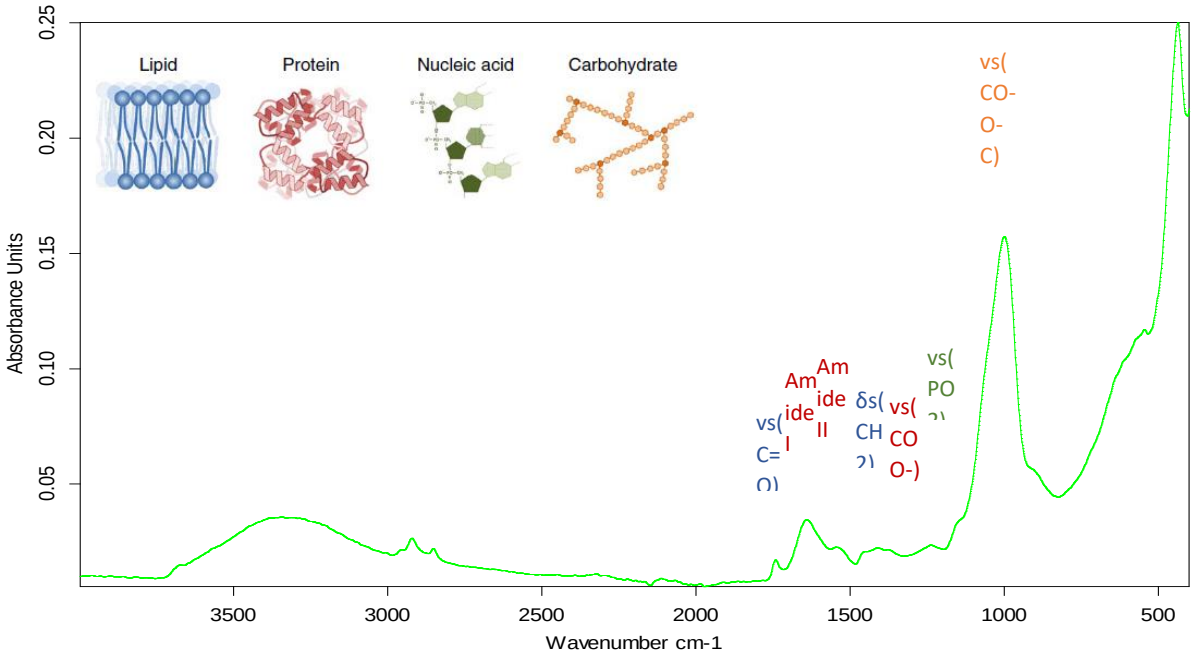

Figure 3.7. FTIR spectra of *C. vulgaris 211/21A* showing biomolecular peak assignments from 3,500–500 cm $^{-1}$.ν = stretching vibrations, δ = bending vibrations, s = symmetric vibrations and as = asymmetric vibrations. The spectrum is a transmission-type micro-spectrum from algae (*C. vulgaris*). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units

3.4 Results discussion

The growth curves of the green microalgae (*Chlorella vulgaris* 211/111, *Chlorella vulgaris* 211/21A, and *Chlamydomonas reinhardtii* 11/45) were measured in two ways (optical density (OD) and cell counts). For up to 11 days, sustainable microalgal growth was obtained when the Bold Basal medium with 3-fold nitrogen and vitamins (3NBBM) medium was used due to the high content of nutrients in this medium; this was supported by Wong *et*

al. findings (2017) when they used 3NBBM they obtained a longer log phase compare to other media. In addition, this high content of nutrients led to the absence of lag phase, high growth rates and long exponential phase in the growth curve of *C. vulgaris* 211/111. *C. reinhardtii* 11/45 and *C. vulgaris* 211/21A showed a lag phase of 3 to 4 days for OD and cell counts, respectively. This difference in the behaviour of microalgae in the medium is due to the difference in the reaction that microorganisms adopt when first introduced to new conditions, which is so-called: nutrient shock, as described by Azevedo *et al.* (2012). Azevedo *et al.* (2012) notice that the use of medium with high content of nutrients led to growth reduction at the beginning in some of the strains used in their study, this was explained as a shock caused by the high volume of nutrient compare to the relatively law number of cells in the inoculum. The correlation between OD and cell count showed a good fit in the calibration curve of both measurements for the examined microalgae (Stevenson *et al*., 2016). This led to more accuracy in estimating cell numbers from absorbance data.

Data collected through FTIR for the observation of functional groups on microalgal cells for the strains under investigation; and to obtain general identification of absorption bands (Benning *et al.* 2004; Giordano *et al.* 2001; Keller,1986; Naumann *et al.* 1996; Stuart, 1997; Wong *et al.* 1991; Dean *et al.* 2007; Sigee *et al.* 2002; Maquelin and Kirchner, 2002). This data helped narrowing the selection range of microalgae to *C. vulgaris* 211/111 and *C. reinhardtii* 11/45. This is because both *C. vulgaris* 211/111 and *C. reinhardtii* 11/45 showed a high content of functional groups that play a vital role in the biosorption and bioremediation processes, as reported by Javanbakht *et al.* (2014).

CHAPTER FOUR

HEAVY METAL TOXICITY ON MICROALGAE AND *DAPHNIA MAGNA*

Summary

This chapter provides the presentation, analysis, and interpretation of data from experiments investigating toxicity of selected heavy metals: Cu, Zn, Ni, Hg, Co, and Cd and mixtures on selected green microalgae (*Chlorella vulgaris* 211/111, *Chlorella vulgaris* 211/21A, and *Chlamydomonas reinhardtii* 11/45) and *Daphnia magna*. For the microalgae, five concentrations of each of the metals were used: 0 mg/l (control), 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l. The matrixes used to prepare metal solutions are 3-NBBM and f/2 medium for microalgae toxicity tests. The concentrations used for *Daphnia magna* toxicity experiments were 0 mg/l (control), 0.001 mg/l, 0.01 mg/l, 0.1 mg/l, 1 mg/l, and 10 mg/l. Metals were added to a nutritious solution that has nutritious palate used to feed *Daphnia magna.* The aim of this chapter is to measure the LC_{50} of each metal for each of the organisms used in the study.

4.1 The impact of heavy metals on the growth of *Chlorella vulgaris* 211/111

4.1.1 The impact of cadmium on the growth of *Chlorella vulgaris* 211/111

C. vulgaris was incubated with five concentrations of cadmium 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Cd on the growth of *C. vulgaris* represented by LC_{50}. The growth was monitored for 5 days up to the beginning of the exponential phase (Figure 4.1a). At 100 mg/l of cadmium, *C. vulgaris* growth was totally inhibited. However, at the other three concentrations, 0.1 mg/l, 1 mg/l, and 10 mg/l, the culture was less impacted and maintained growth levels at around the same values as in the control. Towards the end of the incubation, growth rates significantly reduced, compared to the control, for both 10 and 1 mg/l. However, the concentration of 0.1 mg/l continued to grow at rates that did not differ from the control significantly (Figure 4.1b, Table 4.1) (p >0.05).

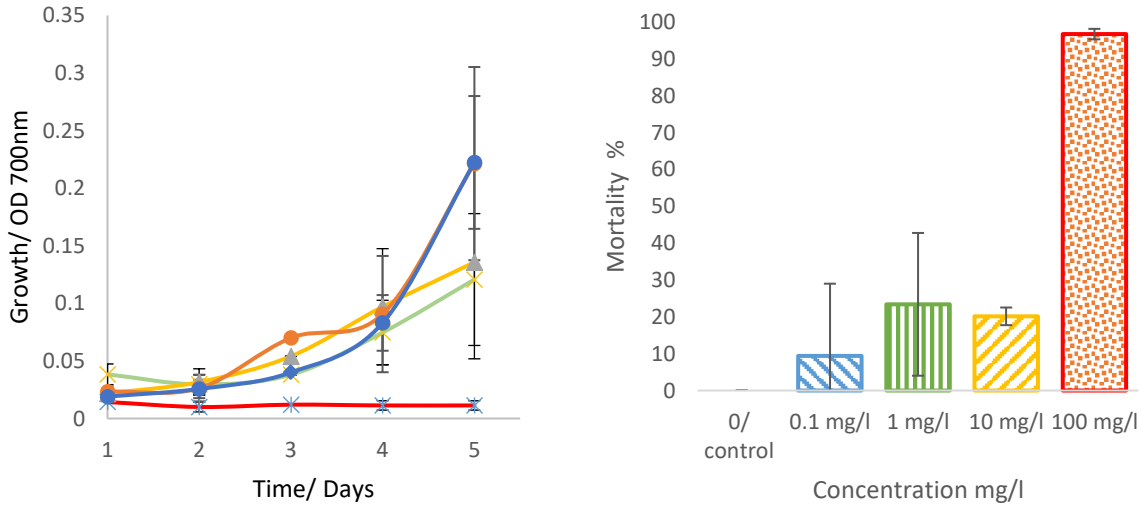

Figure 4.1. The growth of *Chlorella vulgaris* in five concentrations of cadmium: 0 mg/l, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l (**a**) Growth curves of *Chlorella vulgaris* 211/111 exposed to Cd in five different concentrations. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality ratios % of *Chlorella vulgaris* 211/111in different concentrations of Cd , in relation to the control, at day 5.

Table 4.1. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of cadmium.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	4 days	3 days	4 days	No
Day 5 growth/OD*	0.22	0.21	0.14	0.12	No
Growth rate	0.84/day	0.76/day	0.40/day	038/day	No

* Determined by absorbance at 700 nm

The mortality ratio of *C. vulgaris* in relation to the control, was found for the five conditions used in the test (Figure 4.1b). In addition, the maximum growth obtained on day five was compared with the control to calculate the population reduction at the end of the toxicity test. The biomass reductions for the concentrations 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were 9.4%, 23.39%, 20.13%, and 96.74% respectively (Figure 4.1b). The LC_{50} for cadmium for *Chlorella vulgaris* 211/12 was calculated using Probit analysis and was 27.54 mg/l (Figure 4.1b).

4.1.2 The impact of cobalt on the growth of *Chlorella vulgaris* 211/111

Five concentrations of cobalt (0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l) were used to test the impact of the metal on the growth of *C. vulgaris* and to calculate the LC_{50}. The growth was monitored for five days up to the beginning of the log phase (Figure 4.2a). Both 100 mg/l and 10 mg/l of cobalt reduced *C. vulgaris* growth by 78.26% and 74.68%, respectively: with no significant difference between the two treatments ($p > 0.05$), but a significant difference from the control ($p < 0.05$). In the cultures containing 1 mg/l and 0.1mg/l there were more than 20% decrease in growth with no significant difference between the two treatment ($p > 0.05$), but there were significant differences from the control and cultures with cobalt

concentration of 100 mg/l and 10 mg/l (p <0.05) (Figure 4.2b, Table 4.2). The concentration of cobalt that caused 50% reduction of the *C. vulgaris* population is 3.6 mg/l and was found using Probit analysis (Figure 4.2b).

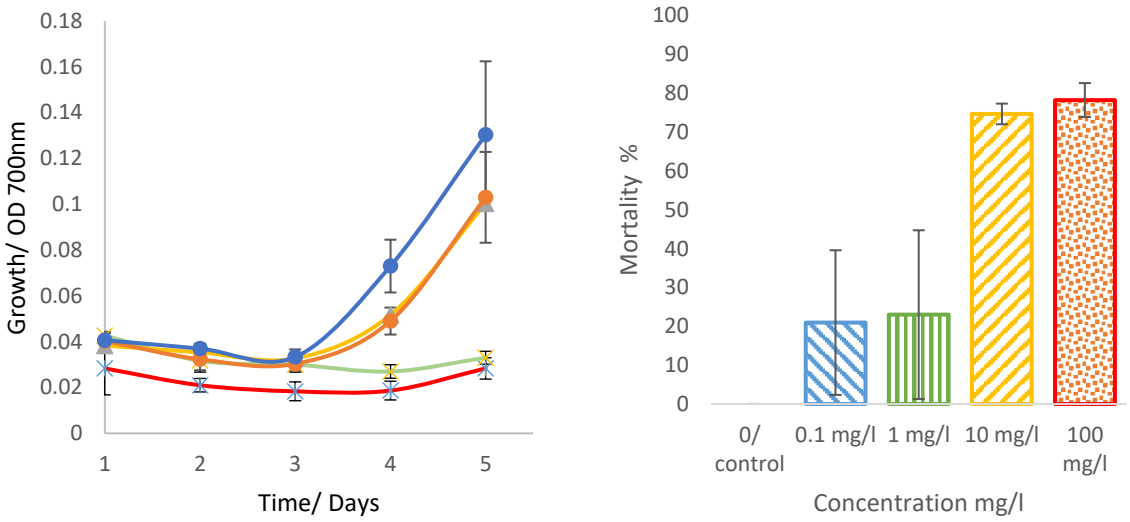

Figure 4.2. The growth of *Chlorella vulgaris* in five concentrations of cobalt: 0 mg/l, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l (**a**) Growth curves of *Chlorella vulgaris* 211/111 exposed to cobalt in five different concentrations. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) The mortality ratios % of *Chlorella vulgaris* 211/111in different concentrations of Co , in relation to the control, at day 5.

Table 4.2. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of cobalt.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.13	0.10	0.10	No	No
Growth rate	0.68/day	0.61/day	0.56/day	No	No

* Determined by absorbance at 700 nm

4.1.3 The impact of copper on the growth of *Chlorella vulgaris* 211/111

Five concentrations of copper 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. vulgaris* to test the impact of Cu on the growth of *C. vulgaris* represented by the LC_{50}. The test time was five days up to the beginning of the log phase (Figure 4.3a). Both concentrations of 0.1 and 1mg/l of copper showed improved growth rates and no signs of inhibition (p >0.05) (Figure 4.3a). The other two concentrations of copper, 100 mg/l and 10 mg/l, decreased *C. vulgaris* growth rates by 77.24% and 67.26 %, respectively. The LC_{50} of copper for *C. vulgaris* 211/12 is 8.49 mg/l (Probit) (Figure 4.3b Table 4.3).

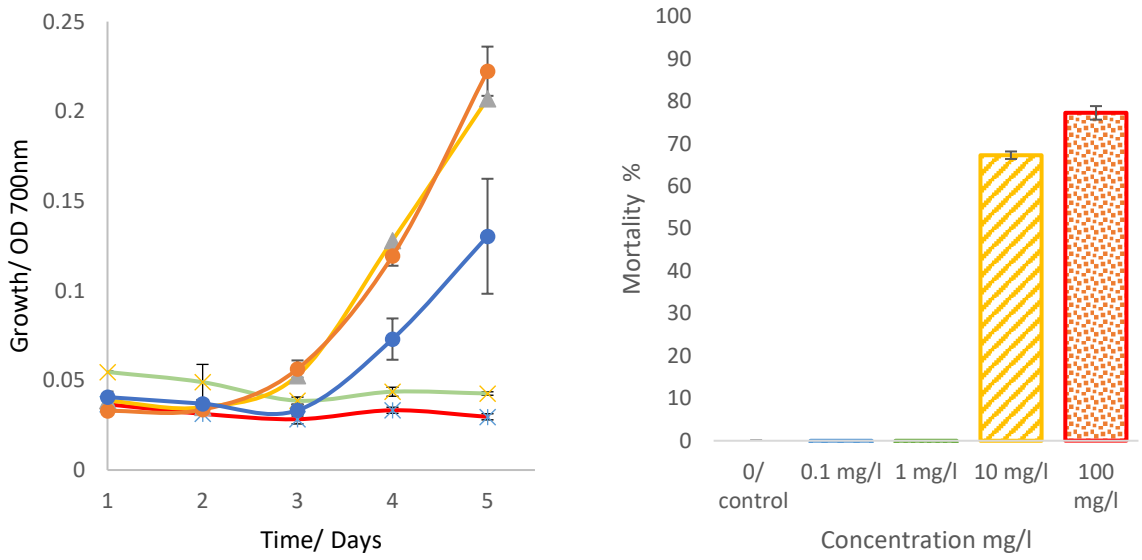

Figure 4.3. *Chlorella vulgaris* growth test with five concentrations of copper (**a**) *Chlorella vulgaris* 211/12 inhibition by Cu, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control (**b**) Mortality of *Chlorella vulgaris* 211/12 on different concentrations of Cu at the end of the inhibitory test (day 5).

Table 4.3. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of copper.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.13	0.22	0.20	No	No
Growth rate	0.68/day	0.69/day	0.68/day	No	No

* Determined by absorbance at 700 nm

4.1.4 The impact of nickel on the growth of *Chlorella vulgaris* 211/111

C. vulgaris was incubated with five concentrations of nickel 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Ni on the growth of *C. vulgaris* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the log phase (Figure 4.4a). Above 90% reduction in *C. vulgaris* population has occurred at both 100 mg/l and 10 mg/l of nickel. Improved growth at 1 mg/l has arisen and negligible inhibition at 0.1 mg/l of nickel with a 5.65% decrease ($p < 0.05$) (Figure 4.4a). The LC_{50} of nickel for the strain *C. vulgaris* 211/111 was found to be 3.77 mg/l (Probit) (Figure 4.4b).

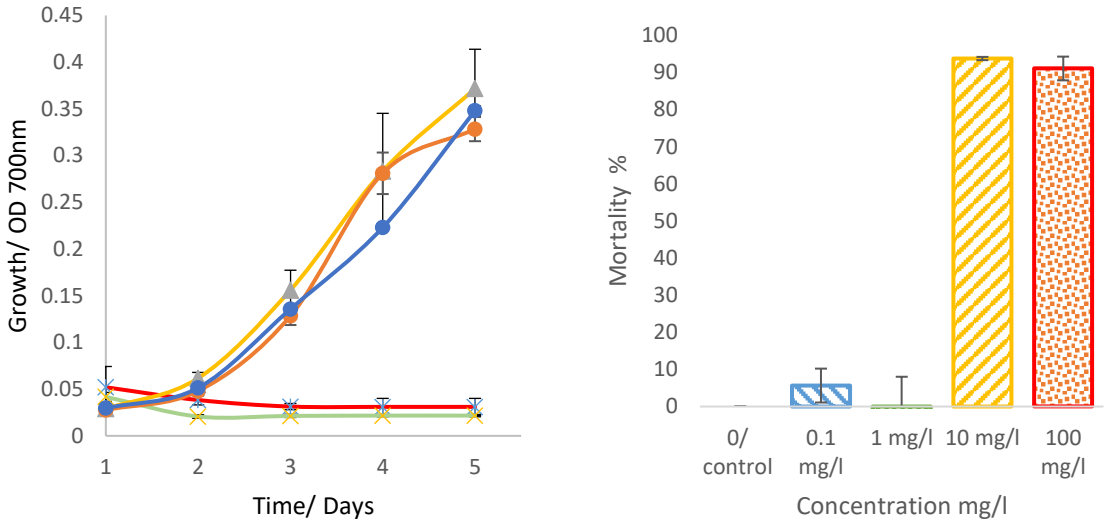

Figure 4.4. *Chlorella vulgaris* growth test with five concentrations of nickel (**a**) *Chlorella vulgaris* 211/12 inhibition by Ni, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control.(**b**) Mortality of *Chlorella vulgaris* 211/12 on different concentrations of Ni at the end of the inhibitory test (day 5).

Table 4.4. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of nickel.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	2 days	2 days	2 days	No	No
Day 5 growth/OD*	0.35	0.33	0.37	No	No
Growth rate	0.61/day	0.47/day	0.43/day	No	No

* Determined by absorbance at 700 nm

4.1.5 The impact of mercury on the growth of *Chlorella vulgaris* 211/111

C. vulgaris was incubated with five concentrations of mercury 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Hg on the growth of *C. vulgaris* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure

4.5a). Mercury was lethal for *C. vulgaris* at both 100 and 10 mg/l causing algae culture 100% inhibition. Whereas a 32.12% reduction was caused by mercury at 1 mg/l, and 47.46% at 0.1mg/l ($p < 0.05$) (Figure 4.5a). The level of Hg that caused 50% reduction of *C. vulgaris* 211/12 growth was 0.22 mg/l (Probit) (Figure 4.5b).

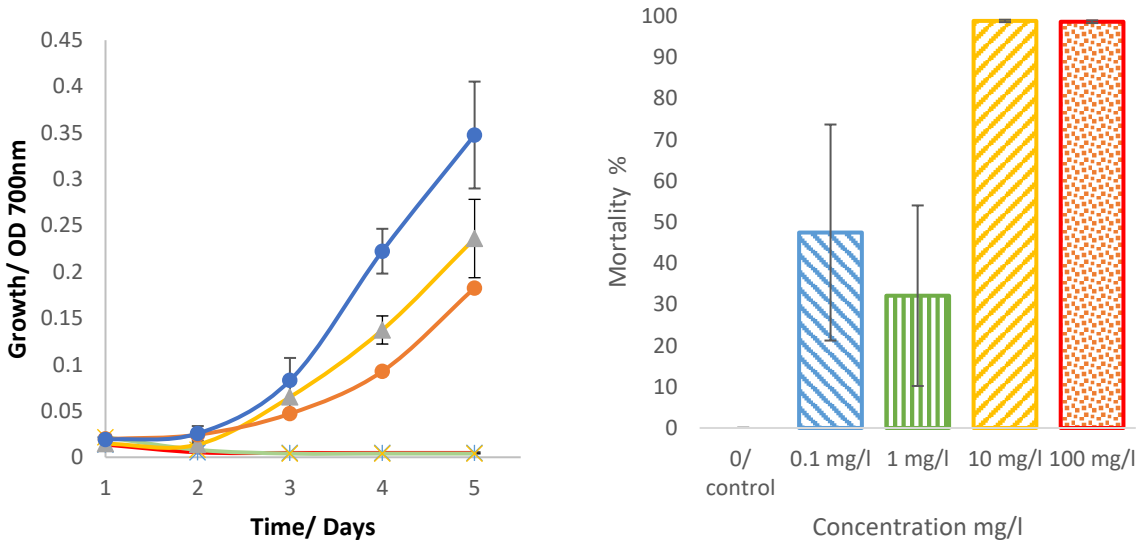

Figure 4.5. *Chlorella vulgaris* growth test with five concentrations of mercury (**a**) *Chlorella vulgaris* 211/12 inhibition by Hg, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control (**b**) Mortality of *Chlorella vulgaris* 211/12 on different concentrations of Hg at the end of the inhibitory test (day 5).

Table 4.5. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of mercury.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	2 days	No	No
Day 5 growth/OD*	0.23	0.10	0.14	No	No
Growth rate	0.72/day	0.68/day	0.64/day	No	No

* Determined by absorbance at 700 nm

4.1.6 The impact of zinc on the growth of *Chlorella vulgaris* 211/111

Five concentrations of zinc: 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. vulgaris* to test the impact of Zn on the growth of *C. vulgaris* represented by the LC_{50}. The growth was observed for five days up to the beginning of the exponential phase (Figure 4.6a). At concentration 100 mg/l of zinc, *C. vulgaris* showed 75.05% reduction in the growth rate compared to the control. While at the other three concentrations; 10 mg/l, 1 mg/l, and 0.1mg/l, the culture showed a significant enhancement in growth and maintained growth levels at around the same values as in the control (p <005) (Figure 4.6a; Table 4.6). The LC_{50} of zinc for *C. vulgaris* 211/111 was 25.4 mg/l (Probits) (Figure 4.6b).

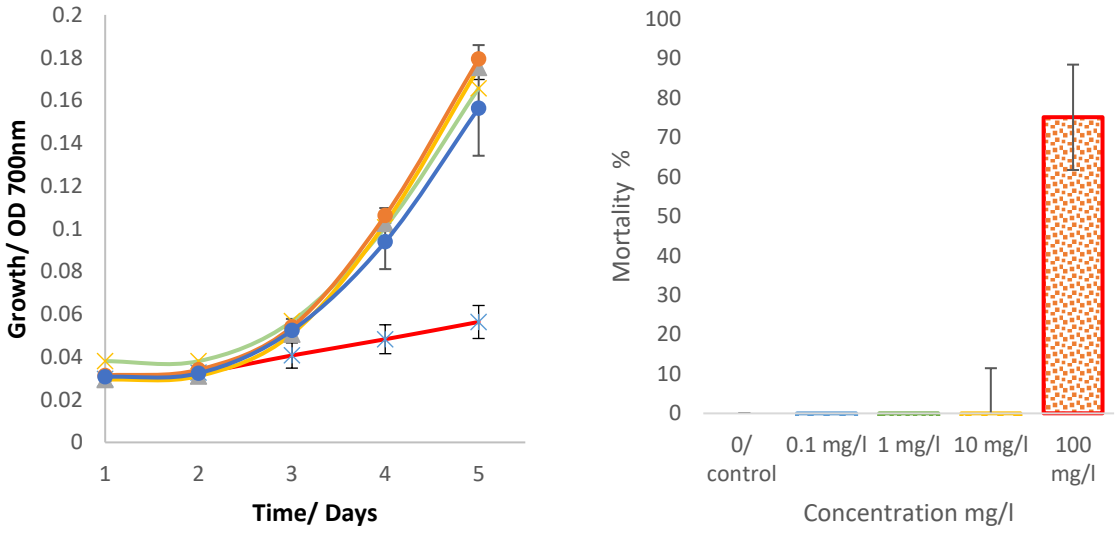

Figure 4.6. *Chlorella vulgaris* growth test with five concentrations of zinc **(a)** *Chlorella vulgaris* 211/12 inhibition by Zn, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control **(b)** Mortality of *Chlorella vulgaris* 211/12 on different concentrations of Zn at the end of the inhibitory test (day 5).

Table 4.6. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of zinc.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	3 days	No
Day 5 growth/OD*	0.15	0.17	0.18	0.18	No
Growth rate	0.55/day	0.61/day	0.62/day	0.62/day	No

* Determined by absorbance at 700 nm

4.1.7 The impact of mixture of heavy metals on the growth of *Chlorella vulgaris* 211/111

C. vulgaris was incubated with five concentrations of a mixture of heavy metals 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of metals mixture on the growth of *C. vulgaris* represented by the LC_{50}. The mixture was prepared by adding equal amounts of each metal in the mixture (Cu, Zn, Ni, Hg, Co, and Cd) to prepare the required mixture concentration. For example, to prepare 100 mg/l of the metal mixture, an amount of 16.67 mg/l of each metal (Cu, Zn, Ni, Hg, Co, and Cd) were mixed in one litre of the growth medium to reach a final concentration of 100 mg/l of the metal mixture. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.7a). The mixture of metals resulted in over 70% reduction of the growth rates of *C. vulgaris* for both concentrations of 100 and 10 mg/l with 77.6% and 74.83% decrease in the populations, respectively (p <0.05). However, growth rates at 1 mg/l and 0.1 mg/l showed a slight improvement compared to the control (Figure 4.7a). On day five, end of the test, algae mortality was calculated at each concentration and from there, the LC_{50} was found to be 15.08 mg/l for the metals mixture (Probit) (Figure 4.7b; Table 4.7).

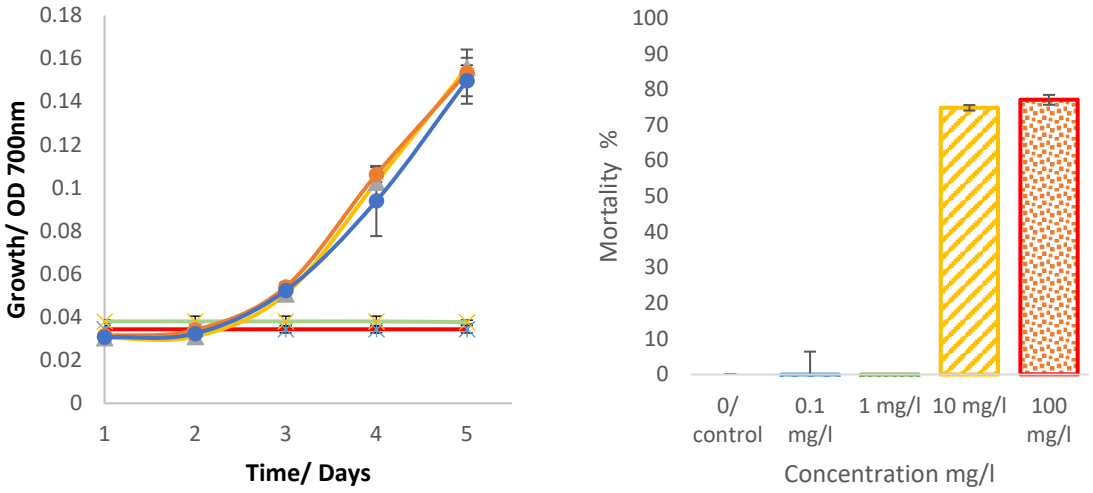

Figure 4.7. *Chlorella vulgaris* growth test with five concentrations of mixture of metals (**a**) *Chlorella vulgaris* 211/12 inhibition by mix of heavy metals, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control (**b**) Mortality of *Chlorella vulgaris* 211/12 on different concentrations of mix of heavy metals at the end of the inhibitory test (day 5).

Table 4.7. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of mixture of metals.

Chlorella Vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.15	0.15	0.15	No	No
Growth rate	0.53/day	0.54/day	0.53/day	No	No

* Determined by absorbance at 700 nm

4.1.8 The impact of manganese on the growth of *Chlorella vulgaris* 211/111

C. vulgaris was incubated with five concentrations of manganese 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Mn on the growth of *C. vulgaris* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.8).The tested concentrations of manganese showed no inhibition effect on *C. vulgaris* 211/111.

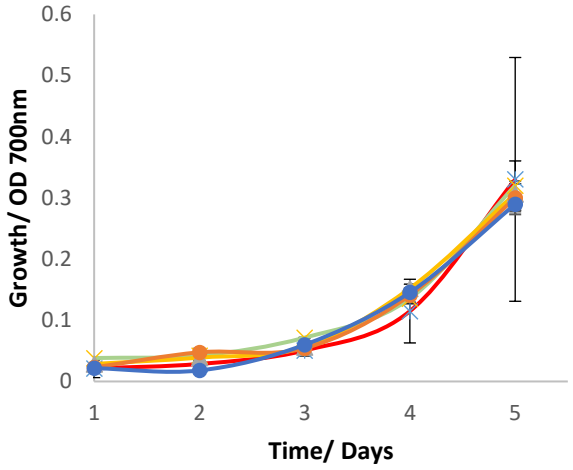

Figure 4.8. *Chlorella vulgaris* growth test with five concentrations of manganese, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control

4.1.9 The impact of aluminium on the growth of *Chlorella vulgaris* 211/111

C. vulgaris was incubated with five concentrations of aluminium 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Al on the growth of *C. vulgaris* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase. Aluminium caused the growth rate of *C. vulgaris* 211/111 to be totally inhibited at concentration100 mg/l of Al and maintained growth levels at similar rates as in the control for both 1 mg/l and 0.1 mg/l. The concentration of 10 mg/l of Al caused a slight reduction in the strain population (p >0.05) (Figure 4.9). The LC_{50} was not calculated for Al as it was eliminated from the study.

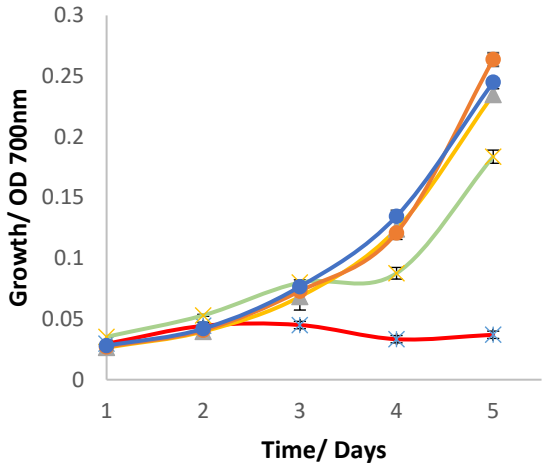

Figure 4.9. *Chlorella vulgaris* growth test with five concentrations of aluminium, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control

4.1.10 The impact of arsenic on the growth of *Chlorella vulgaris* 211/111

C. vulgaris was incubated with five concentrations of arsenic 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of As on the growth of *C. vulgaris* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the log phase. The tested concentrations of arsenic showed no significant inhibition of *C. vulgaris* 211/111 (Figure 4.10).

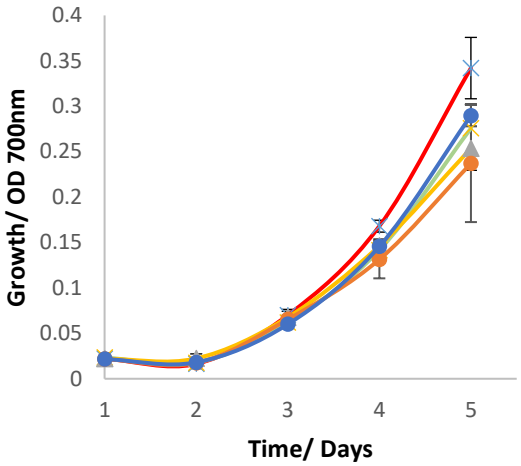

Figure 4.10. *Chlorella vulgaris* growth test with five concentrations of arsenic, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control

4.2 The impact of heavy metals on the growth of *Chlorella vulgaris* 211/21A

4.2.1 The impact of cadmium on the growth of *Chlorella vulgaris* 211/21A

Five concentrations of Cd 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. vulgaris* to test the impact of Cd on the growth of *C. vulgaris* 211/21A represented by the LC_{50}. The test time was five days up to the beginning of the log phase (Figure4.11a). The growth curve of *C. vulgaris* at concentration 0.1mg/l showed improved growth rates and no signs of inhibition (Figure 4.11a). The effect of cadmium concentrations on *C. vulgaris* growth rates was as follows; 78.11%, 50.05%, 4.14%, 0, and 0 decrease for the concentrations 100 mg/l, 10 mg/l, 1 mg/l, 0.1 mg/l, and 0 mg/l, respectively (p <0.05). Therefore, the LC_{50} of cadmium for *C. vulgaris* 211/21A was 17.8 mg/l (Probit) (Figure 4.11b; Table 4.8).

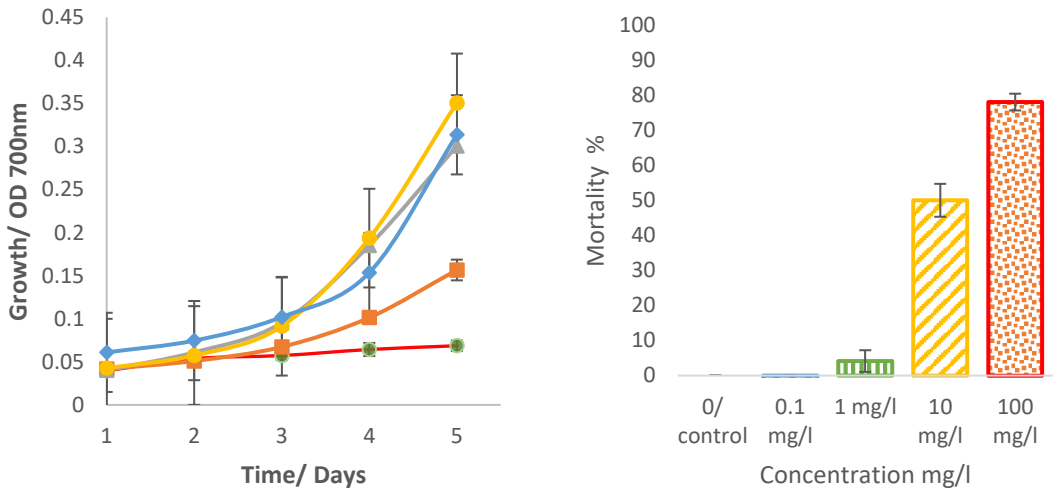

Figure 4.11. *Chlorella vulgaris* growth test with five concentrations of cadmium (**a**) *Chlorella vulgaris* 211/21A inhibition by Cd, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control (**b**) Mortality of *Chlorella vulgaris* 211/21A on different concentrations of Cd at the end of the inhibitory test (day 5).

Table 4.8. Growth parameters for *Chlorella vulgaris* 211/21A grown in five concentrations of mixture of cadmium.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	3 days	No
Day 5 growth/OD*	0.31	0.35	0.30	0.16	No
Growth rate	0.56/day	0.67/day	0.57/day	0.42/day	No

* Determined by absorbance at 700 nm

4.2.2 The impact of cobalt on the growth of *Chlorella vulgaris* 211/21A

C. vulgaris was incubated with five concentrations of Co 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of cobalt on the growth of *C. vulgaris* 211/21A represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.12a). The inhibition effect of cobalt on *C. vulgaris* showed relative relation to the increased concentration of the metal in the solution; 94.5%, 85.98%, 51.51%, 41.64%, and 0 reduction for the concentrations 100 mg/l, 10 mg/l, 1 mg/l, 0.1 mg/l, and 0 mg/l, respectively

(p <0.05) (Figure 4.12a). The level of cobalt that causes a 50% reduction of *C. vulgaris* growth is 0.33 mg/l (Probits) (Figure 4.12b; Table 4.9).

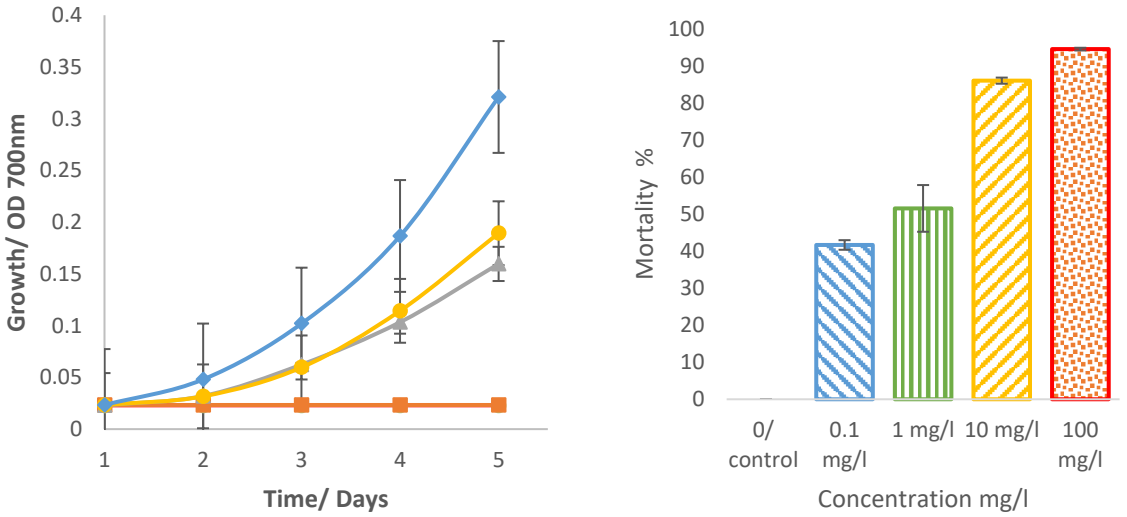

Figure 4.12. *Chlorella vulgaris* growth test with five concentrations of cobalt (**a**) *Chlorella vulgaris* 211/21A inhibition by Co, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlorella vulgaris* 211/21A on different concentrations of Co at the end of the inhibitory test (day 5).

Table 4.9. Growth parameters for *Chlorella vulgaris* 211/21A grown in five concentrations of cobalt.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.32	0.19	0.16	No	No
Growth rate	0.57/day	0.58/day	0.47/day	No	No

* Determined by absorbance at 700 nm

4.2.3 The impact of copper on the growth of *Chlorella vulgaris* 211/21A

Five concentrations of copper 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. vulgaris* to test the impact of Cu on the growth of *C. vulgaris* 211/21A

represented by the LC_{50}. The test time was five days up to the beginning of the log phase (Figure4.13a). *C. vulgaris* responded to copper in the solution in a relative relation to the increased concentration of the Cu; 95.43%, 93.87%, 55.24%, 39.67%, and 0 reduction for the concentrations 100 mg/l, 10 mg/l, 1 mg/l, 0.1 mg/l, and 0 mg/l, respectively (Figure 4.13a). The level of copper that caused a 50% reduction of *C. vulgaris* growth was 0.26 mg/l (Probit) (Figure 4.13b; Table 4.10).

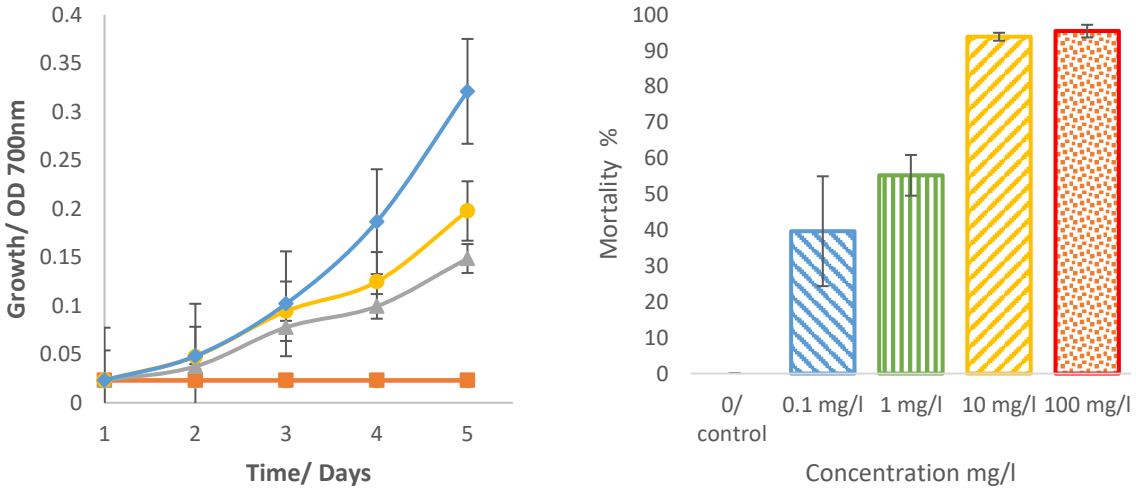

Figure 4.13. *Chlorella vulgaris* growth test with five concentrations of copper (**a**) *Chlorella vulgaris* 211/21A inhibition by Cu, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlorella vulgaris* 211/21A on different concentrations of Cu at the end of the inhibitory test (day 5).

Table 4.10. Growth parameters for *Chlorella vulgaris* 211/21A grown in five concentrations of copper.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.32	0.20	0.15	No	No
Growth rate	0.57/day	0.37/day	0.33/day	No	No

* Determined by absorbance at 700 nm

4.2.4 The impact of nickel on the growth of *Chlorella vulgaris* 211/21A

C. vulgaris was incubated with five concentrations of Ni 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Ni on the growth of *C. vulgaris* 211/21A represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.14a) The growth curve of *C. vulgaris* showed a dramatic decrease with the increase of nickel concentration. Ratios of reduction for 100 mg/l,10 mg/l,1 mg/l, and 0.1 mg/l were 87.02%, 53.27%, 16.3%, and 5.71%, respectively (p <0.05) (Figure 4.14a). The LC_{50} of nickel for *C. vulgaris* was 7.32 mg/l (Probit) (Figure 4.14b; Table 4.11).

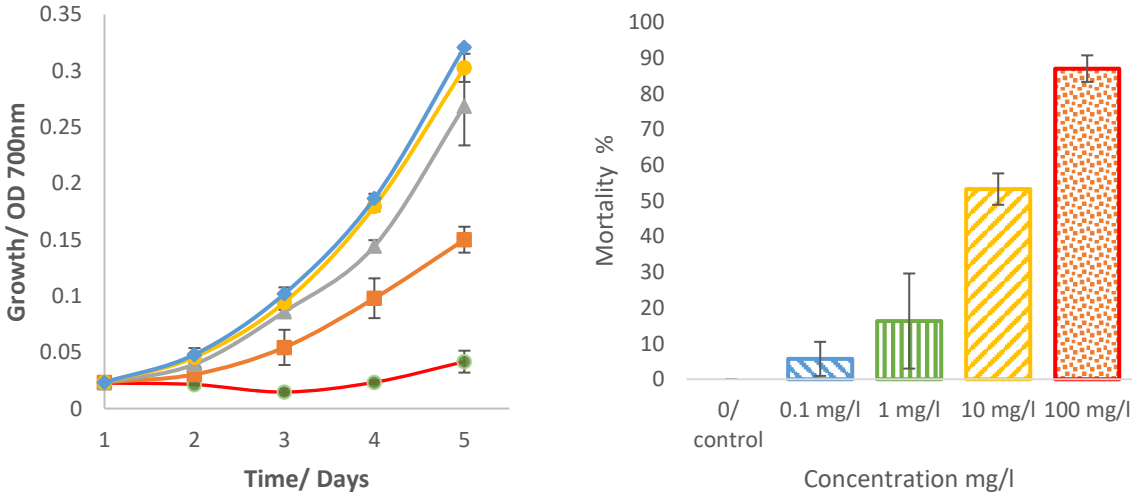

Figure 4.14. *Chlorella vulgaris* growth test with five concentrations of nickel *Chlorella vulgaris* 211/21A inhibition by Ni, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlorella vulgaris* 211/21A on different concentrations of Ni at the end of the inhibitory test (day 5).

Table 4.11. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of nickel.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	3 days	No
Day 5 growth/OD*	0.32	0.30	0.27	0.15	No
Growth rate	0.57/day	0.58/day	0.57/day	0.51/day	No

* Determined by absorbance at 700 nm

4.2.5 The impact of mercury on the growth of *Chlorella vulgaris* 211/21A

C. vulgaris was incubated with five concentrations of mercury 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Hg on the growth of *C. vulgaris* 211/21A represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.15a) The three concentrations of mercury: 100 mg/l,10 mg/l and 1 mg/l had a fatal impact on *C. vulgaris*, causing above 90% inhibition of algal culture (Figure 4.15a). In addition, at concentration 0.1mg/l of Hg, a reduction of 45.06% of the growth had occurred (p <0.05). The concentration of Hg that caused 50% decrease in *C. vulgaris* population was 0.01 mg/l (Probit) (Figure 4.15b; Table 4.12).

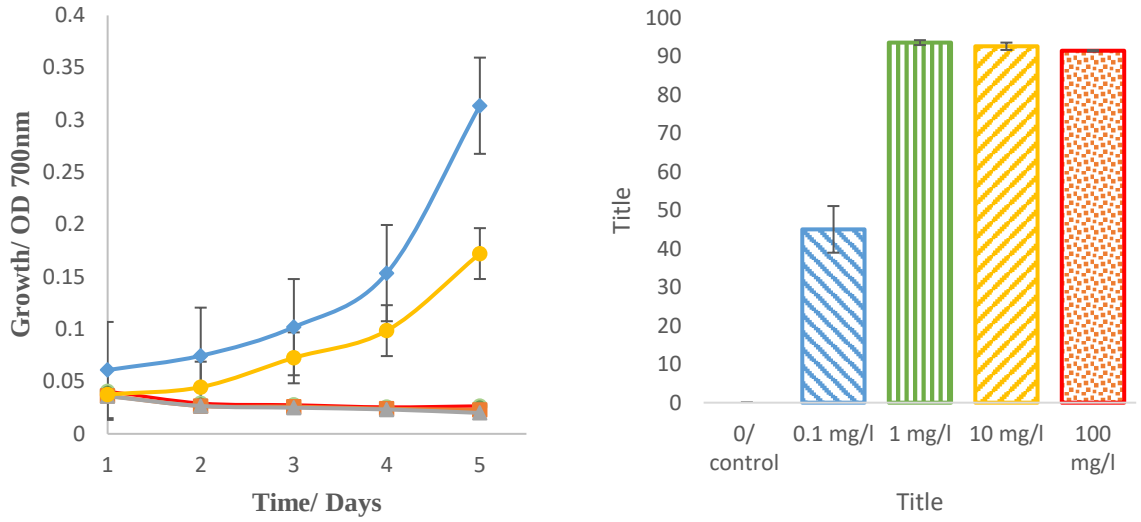

Figure 4.15. *Chlorella vulgaris* growth test with five concentrations of mercury (a) *Chlorella vulgaris* 211/21A inhibition by Hg, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlorella vulgaris* 211/21A on different concentrations of Hg at the end of the inhibitory test (day 5).

Table 4.12. Growth parameters for *Chlorella vulgaris* 211/21A grown in five concentrations of mercury.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	No	No	No
Day 5 growth/OD*	0.31	0.17	No	No	No
Growth rate	0.56/day	0.43/day	No	No	No

* Determined by absorbance at 700 nm

4.2.6 The impact of zinc on the growth of *Chlorella vulgaris* 211/21A

Five concentrations of Zn 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. vulgaris* to test the impact of zinc on the growth of *C. vulgaris* 211/21A represented by the LC_{50}. The test time was five days up to the beginning of the log phase (Figure 4.16a). *C. vulgaris* responded to Zn in the solution in an intolerance behaviour with a reduction of; 68.11%, 69.18%, 52.7%, 48.88%, and 0 for the concentrations 100 mg/l, 10 mg/l, 1 mg/l, 0.1

mg/l, and 0 mg/l, respectively (p <0.05) (Figure 4.16a). The level of Zn that caused a 50% decrease of *C. vulgaris* population was 0.15 mg/l (Probit) (Figure 4.16b; Table 4.13).

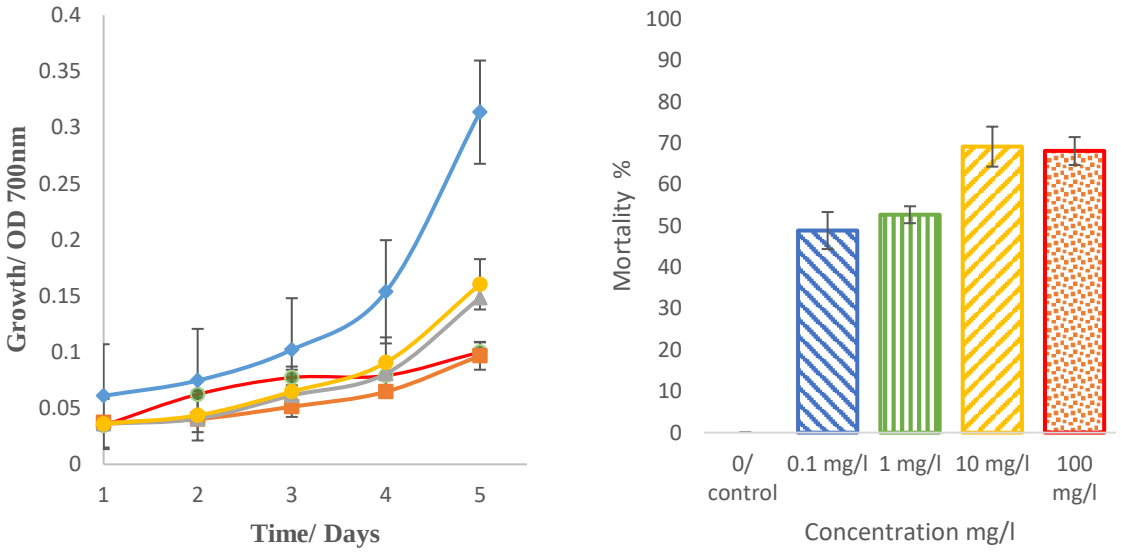

Figure 4.16. *Chlorella vulgaris* growth test with five concentrations of zinc (**a**) *Chlorella vulgaris* 211/21A inhibition by Zn, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlorella vulgaris* 211/21A on different concentrations of Zn at the end of the inhibitory test (day 5).

Table 4.13. Growth parameters for *Chlorella vulgaris* 211/111 grown in five concentrations of mixture of metals.

Chlorella vulgaris	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.31	0.16	0.15	No	No
Growth rate	0.56/day	0.45/day	0.41/day	No	No

* Determined by absorbance at 700 nm

4.3 The impact of heavy metals on the growth of *Chlamydomonas reinhardtii* 11/45

4.3.1 The impact of cadmium on the growth of *Chlamydomonas reinhardtii* 11/45

Five concentrations of Cd 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. reinhardtii* to test the impact of Cd on the growth of *C. reinhardtii* represented by the LC_{50}. The test time was five days up to the beginning of the log phase (Figure4.21a). Upon exposure to Cd, the growth rate of *C. reinhardtii* was significantly inhibited at 100 mg/l and significantly reduced at 10 mg/l, 91.41%, and 60.02%, respectively (p <0.05). Whereas at the lowest concentrations, 1 mg/l and 0.1mg/l, of Cd, growth had a reduction average of 17.37% and 15.7%, respectively (Figure 4.17a). On day five, algae mortality had been calculated at each concentration, and from there, the LC_{50} was found to be 4.04 mg/l of cadmium for *C. reinhardtii* (Probit) (Figure 4.217b; Table 4.14)

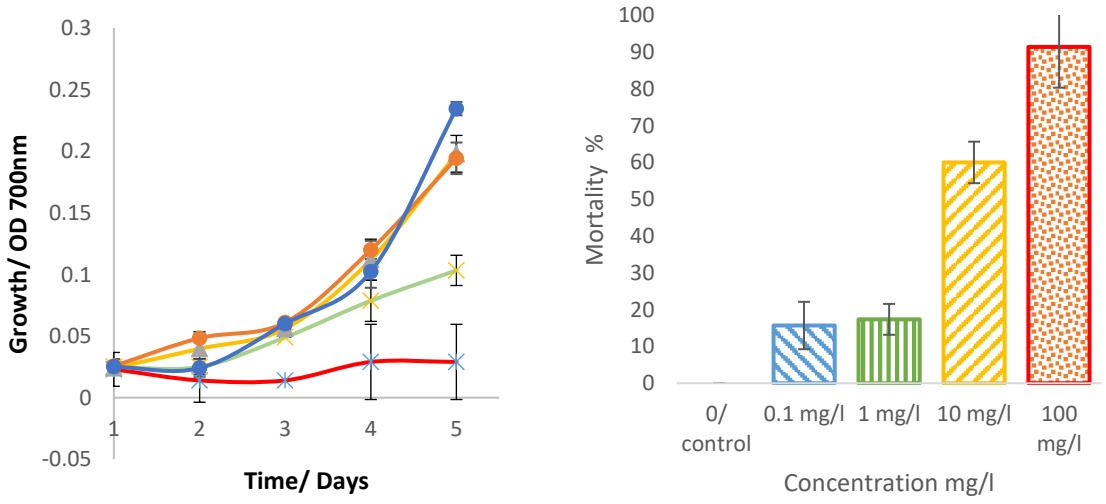

Figure 4.17. *Chlamydomonas reinhardtii* growth test with five concentrations of cadmium (**a**) *Chlamydomonas reinhardtii* 11/45 inhibition by Cd, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlamydomonas reinhardtii* 11/45 on different concentrations of Cd at the end of the inhibitory test (day 5).

Table 4.14. Growth parameters for *Chlamydomonas reinhardtii* grown in five concentrations of cadmium.

C. reinhardtii	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	3 days	No
Day 5 growth/OD*	0.23	0.19	0.20	0.10	No
Growth rate	0.68/day	0.58/day	0.63/day	0.37/day	No

* Determined by absorbance at 700 nm

4.3.2 The impact of cobalt on the growth of *Chlamydomonas reinhardtii* 11/45

C. reinhardtii was incubated with five concentrations of cobalt 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Co on the growth of *C. reinhardtii* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.17a).The growth rate of *C. reinhardtii* was inhibited by 80% at 100 mg/l of cobalt, 74.87% at 10 mg/l, 2.67% at 1 mg/l, and 20.32% at 0.1 mg/l. both growth curves at concentrations 1 mg/l and 0.1 mg/l showed no significant difference in growth from the control treatment ($p > 0.05$) (Figure 4.18a). At day five, the end of the incubation, The LC_{50} of cobalt for *C. reinhardtii* was found to be 5.91 mg/l (Probit) (Figure 4.17b; Table 4.15).

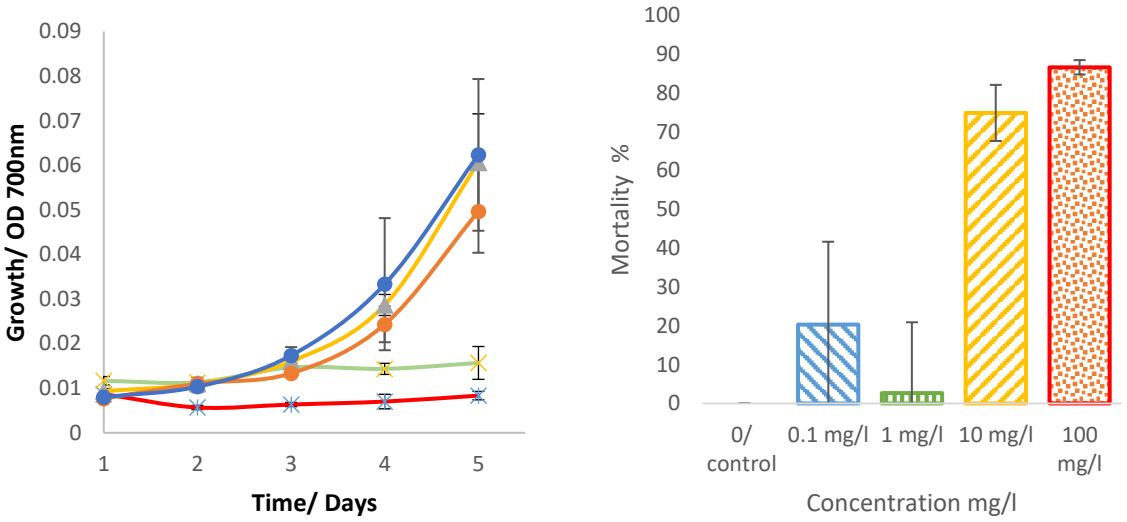

Figure 4.18. *Chlamydomonas reinhardtii* growth test with five concentrations of cobalt (**a**) *Chlamydomonas reinhardtii* 11/45 inhibition by Co, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlamydomonas reinhardtii* 11/45 on different concentrations of Co at the end of the inhibitory test (day 5).

Table 4.15. Growth parameters for *Chlamydomonas reinhardtii* grown in five concentrations of cobalt.

C. reinhardtii	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.06	0.05	0.06	No	No
Growth rate	0.64/day	0.66/day	0.67/day	No	No

* Determined by absorbance at 700 nm

4.3.3 The impact of copper on the growth of *C. reinhardtii* 11/45

C. reinhardtii was incubated with five concentrations of copper 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Cu on the growth of *C. reinhardtii* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.19a).The inhibition of *C. reinhardtii* growth caused by copper showed relative relation to the increased levels of the metal in the solution in both 100 and 10 mg/l. On the other hand,

concentrations of 1 mg/l,0.1 mg/l had a positive impact on the growth rates of *C. reinhardtii* ($p < 0.05$) (Figure 4.19a). The LC_{50} of copper on *C. reinhardtii* was 0.33 mg/l (Probit) (Figure 4.19b; Table 4.16).

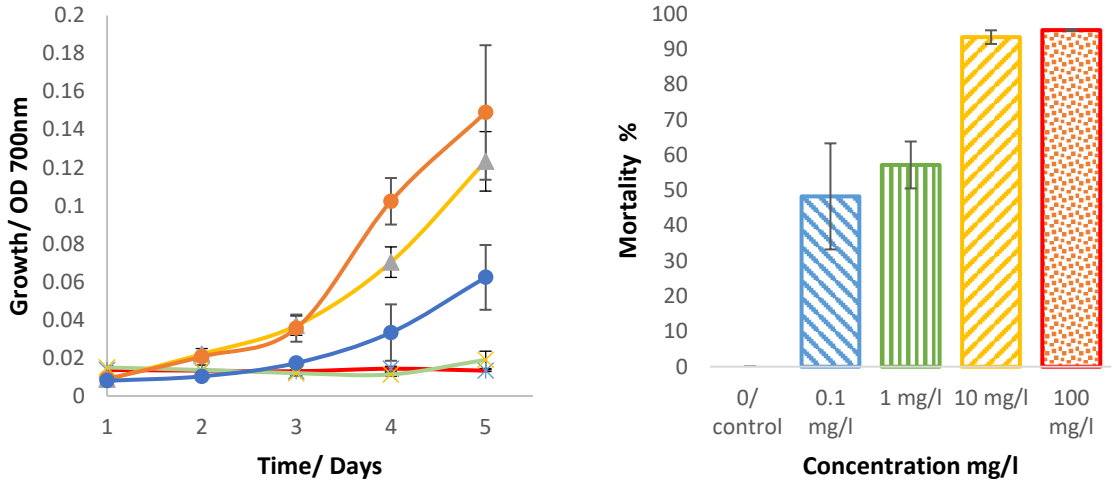

Figure 4.19. *Chlamydomonas reinhardtii* growth test with five concentrations of copper **(a)** *Chlamydomonas reinhardtii* 11/45 inhibition by Cu, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control **(b)** Mortality of *Chlamydomonas reinhardtii* 11/45 on different concentrations of Cu at the end of the inhibitory test (day 5).

Table 4.16. Growth parameters for *Chlamydomonas reinhardtii* grown in five concentrations of copper.

C. reinhardtii	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.06	0.15	0.12	No	No
Growth rate	0.64/day	0.71/day	0.60/day	No	No

* Determined by absorbance at 700 nm

4.3.4 The impact of nickel on the growth of *Chlamydomonas reinhardtii* 11/45

C. reinhardtii was incubated with five concentrations of nickel 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of Ni on the growth of *C. reinhardtii* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the exponential phase (Figure 4.20a).When exposed to Ni, the growth rate of *C. reinhardtii* was highly affected by both concentrations of 100 mg/l and 10 mg/l with 87.04%, and 84.84% decreased populations, respectively. At the same time, levels of both 1 mg/l and 0.1 mg/l of Ni showed a negligible impact on *C. reinhardtii* growth rates, 6.48% and 5.79%, respectively, with no significant difference from the control (p >0.05) (Figure 4.20a). On day five, end of the incubation, algae mortality was calculated at each concentration, and from there, the LC_{50} was found to be 5.23 mg/l (Probit) (Figure 4.20b; Table 4.17).

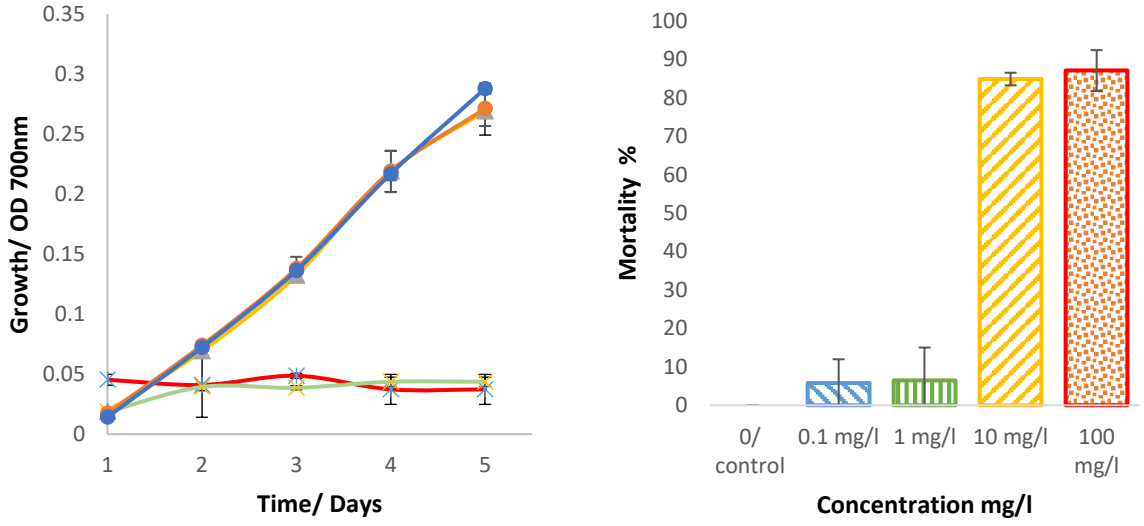

Figure 4.20. *Chlamydomonas reinhardtii* growth test with five concentrations of nickel (**a**) *Chlamydomonas reinhardtii* 11/45 inhibition by Ni, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlamydomonas reinhardtii* 11/45 on different concentrations of Ni at the end of the inhibitory test (day 5).

Table 4.17. Growth parameters for *Chlamydomonas reinhardtii* grown in five concentrations of nickel.

C. reinhardtii	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	2 days	2 days	2 days	No	No
Day 5 growth/OD*	0.29	0.28	0.27	No	No
Growth rate	0.75day	0.69/day	0.66/day	No	No

* Determined by absorbance at 700 nm

4.3.5 The impact of mercury on the growth of *Chlamydomonas reinhardtii* 11/45

Five concentrations of Hg 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. reinhardtii* to test the impact of mercury on the growth of *C. reinhardtii* represented by the LC_{50}. The test time was five days up to the beginning of the exponential phase (Figure 4.21a). Upon exposure to Hg, the growth of *C. reinhardtii* was completely inhibited at 100 mg/l, 10 mg/l, and 1 mg/l. Whereas at the lowest concentration (0.1mg/l) of mercury, growth has a very low reduction of 4.34% (p <0.05) (Figure 4.21a). On day five, end of the test, algae mortality founded and then LC_{50} was calculated to be 0.59 mg/l (Probit) (Figure 4.21b; Table 4.18).

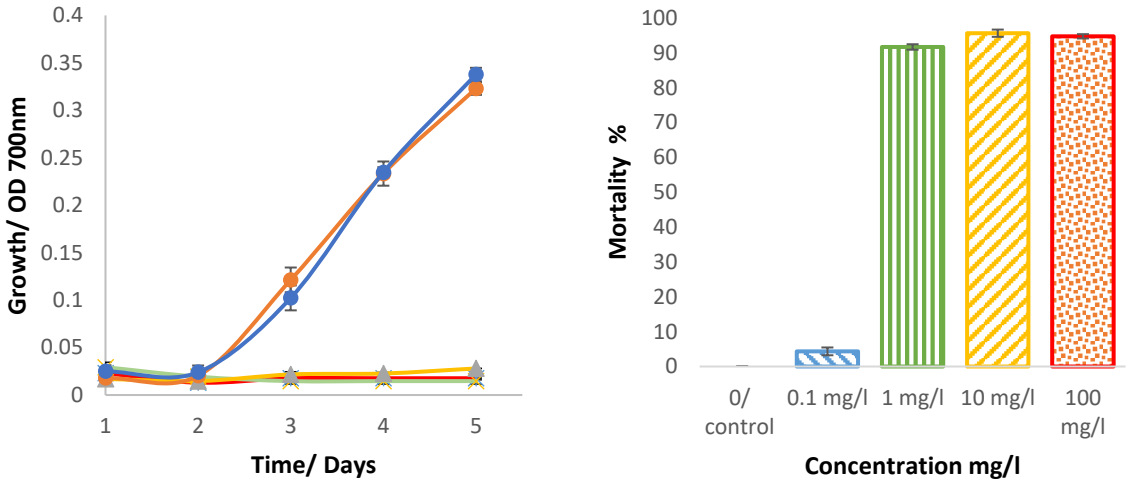

Figure 4.21. *Chlamydomonas reinhardtii* growth test with five concentrations of mercury (**a**) *Chlamydomonas reinhardtii* 11/45 inhibition by Hg, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlamydomonas reinhardtii* 11/45 on different concentrations of Hg at the end of the inhibitory test (day 5).

Table 4.18. Growth parameters for *Chlamydomonas reinhardtii* grown in five concentrations of mercury.

C. reinhardtii	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	2 days	2days	No	No	No
Day 5 growth/OD*	0.34	0.32	No	No	No
Growth rate	0.60/day	0.49/day	No	No	No

* Determined by absorbance at 700 nm

4.3.6 The impact of zinc on the growth of *Chlamydomonas reinhardtii* 11/45

C. reinhardtii was incubated with five concentrations of zinc 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of zinc on the growth of *C. reinhardtii* represented by the LC_{50}. The growth was monitored for five days up to the beginning of the log phase (Figure 4.22a). Above 80% reduction in *C. reinhardtii* population has occurred at 100 mg/l of zinc. Decrease of 26.3% at concentration 10 mg/l was found, an Improved growth rate at 1 mg/l has arisen

and a negligible inhibition at 0.1 mg/l of zinc with 1.53% decrease (p <0.05) (Figure 4.22a). The LC_{50} of Zn for the strain *C. reinhardtii* is 24.19 mg/l (Probit) (Figure 4.22b; Table 4.19).

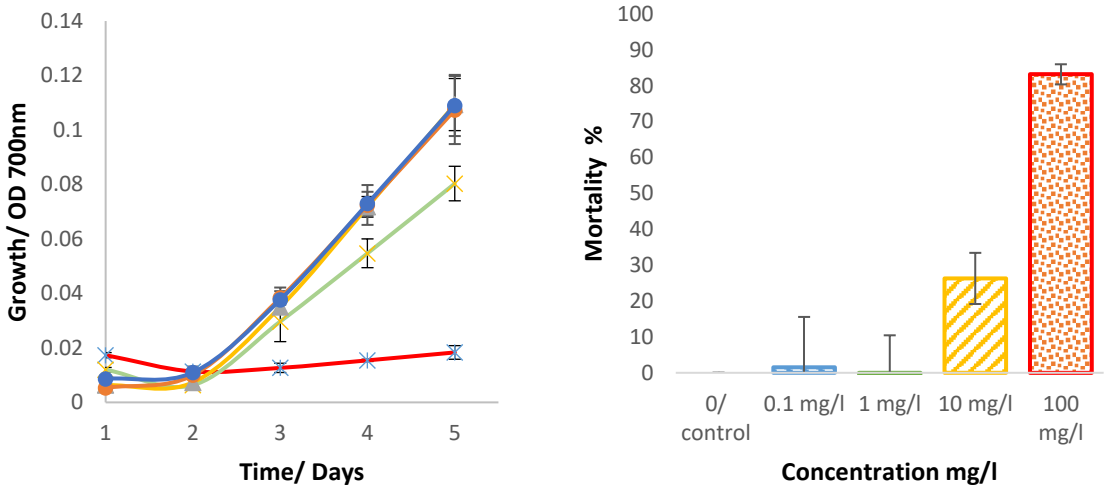

Figure 4.22. *Chlamydomonas reinhardtii* growth test with five concentrations of zinc (**a**) *Chlamydomonas reinhardtii* 11/45 inhibition by Zn, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlamydomonas reinhardtii* 11/45 on different concentrations of Zn at the end of the inhibitory test (day 5).

Table 4.19. Growth parameters for *Chlamydomonas reinhardtii* grown in five concentrations of zinc.

C. reinhardtii	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	2 days	2 days	2 days	2 days	No
Day 5 growth/OD*	0.10	0.11	0.1	0.08	No
Growth rate	0.53/day	0.54/day	0.53/day	0.41/day	No

* Determined by absorbance at 700 nm

4.1.3.7 The impact of mixture of heavy metals on the growth of *Chlamydomonas reinhardtii* 11/45

C. reinhardtii was incubated with five concentrations of a mixture of heavy metals 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l to test the impact of metals mixture on the growth of *C. reinhardtii* represented by the LC_{50}. The mixture was prepared by adding equal amounts of each metal in the mixture (Cu, Zn, Ni, Hg, Co, and Cd) to prepare the required mixture concentration. For example, to prepare 100 mg/l of the metal mixture, an amount of 16.67 mg/l of each metal (Cu, Zn, Ni, Hg, Co, and Cd) were mixed in one litre of the growth medium to reach a final concentration of 100 mg/l of the metal mixture. The growth was monitored for five days up to the beginning of the log phase (Figure 4.23a). When exposed to equally mixed metals, the growth rate of *C. reinhardtii* was highly affected by both concentrations of 100 mg/l and 10 mg/l with 83.41%, and 75.12% decreased in the populations, respectively. At the same time, the level of 1 mg/l of the mixture showed a negligible impact on *C. reinhardtii* growth rates and 3.46% improved growth rates at concentration 0.1 mg/l (p <0.05) (Figure 4.23a). On day five, end of the incubation, algae mortality was calculated at each concentration and from there, the LC_{50} was found to be 10.66 mg/l for the metals mixture for *C. reinhardtii* (Probit) (Figure 4.23b; Table 4.20).

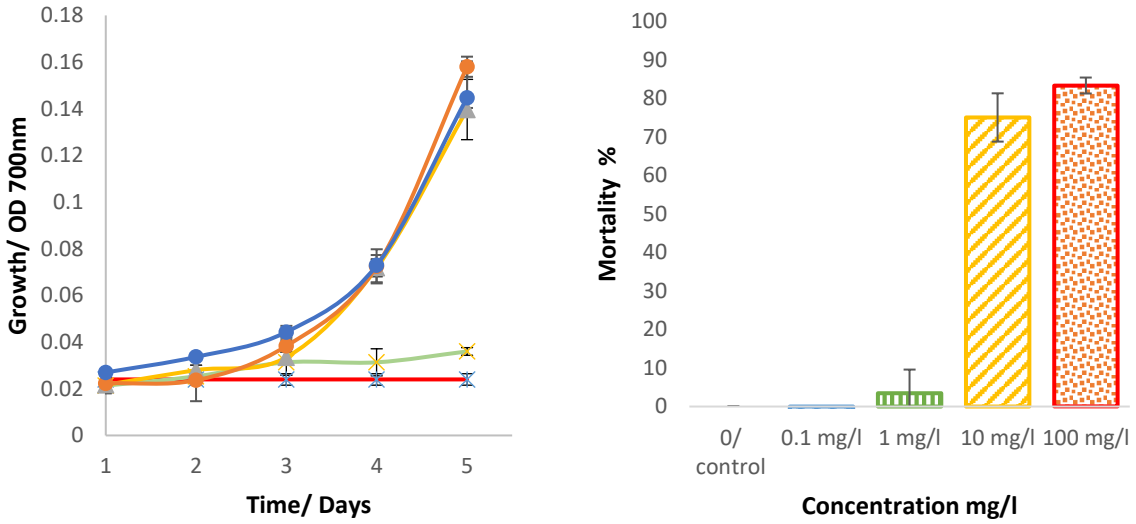

Figure 4.23. *Chlamydomonas reinhardtii* growth test with five concentrations of mix of metals (**a**) *Chlamydomonas reinhardtii* 11/45 inhibition by mix of heavy metals, growth curves of each test exposure. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control. (**b**) Mortality of *Chlamydomonas reinhardtii* 11/45 on different concentrations of mix of heavy metals at the end of the inhibitory test (day 5).

Table 4.20. Growth parameters for *Chlamydomonas reinhardtii* grown in five concentrations of mixture of metals.

C. reinhardtii	Control	0.1 mg/l	1 mg/l	10 mg/l	100 mg/l
Lag phase	3 days	3 days	3 days	No	No
Day 5 growth/OD*	0.15	0.16	0.15	No	No
Growth rate	0.59/day	0.71/day	0.71/day	No	No

* Determined by absorbance at 700 nm

4.3.8 The impact of manganese on the growth of *Chlamydomonas reinhardtii* 11/45

Five concentrations of Mn 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. reinhardtii* to test the impact of Mn on the growth of *C. reinhardtii* represented by the LC_{50}. The test time was five days up to the beginning of the log phase. The tested concentrations of manganese showed no inhibitory effect on *C. reinhardtii* 11/45 (Figure 4.24).

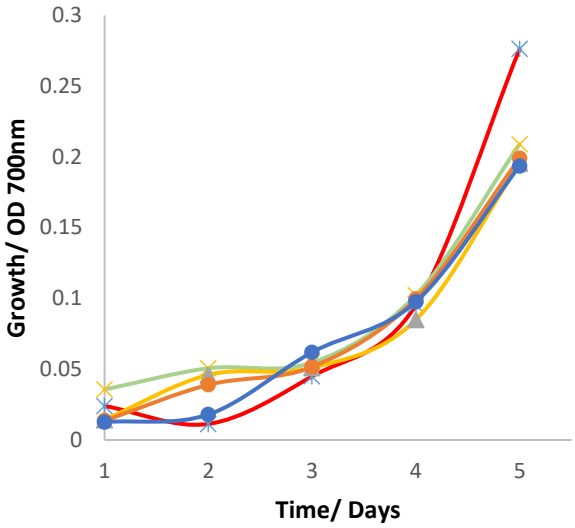

Figure 4.24. *Chlamydomonas reinhardtii* growth test with five concentrations of manganese. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control.

4.3.9 The impact of aluminium on the growth *Chlamydomonas reinhardtii* 11/45

Five concentrations of Al 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. reinhardtii* to test the impact of Al on the growth of *C. reinhardtii* represented by the LC_{50}. The test time was five days up to the beginning of the log phase. Aluminium caused the growth rate of *C. reinhardtii* to be totally inhibited at 100 mg/l and significantly reduced at both 10 and 1 mg/l. Whereas at the lowest concentration (0.1mg/l) of Al, the growth rate has a slight decrease (p >0.05) (Figure 4.25). The LC_{50} was not calculated for Al for being eliminated from the study.

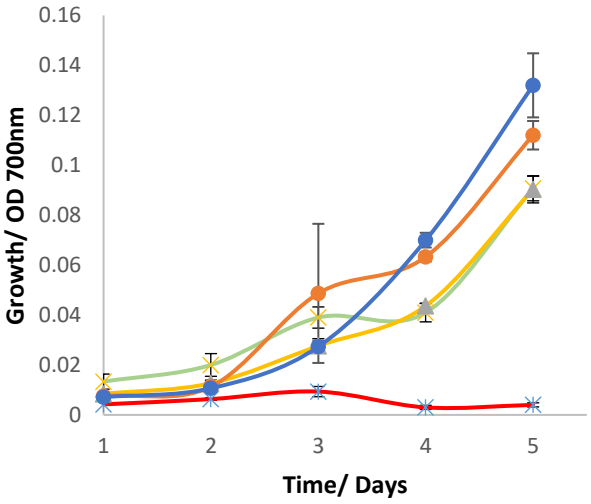

Figure 4.25. *Chlamydomonas reinhardtii* growth test with five concentrations of aluminium. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●) 0.1 mg/l, and (♦) the control

4.3.10 The impact of arsenic on the growth of *Chlamydomonas reinhardtii* 11/45

Five concentrations of As 0, 0.1 mg/l, 1 mg/l, 10 mg/l, and 100 mg/l were used to incubate *C. reinhardtii* to test the impact of As on the growth of *C. reinhardtii* represented by the LC_{50}. The test time was five days up to the beginning of the log phase. The tested concentrations of arsenic showed no significant inhibition of *C. reinhardtii* 11/45 (Figure 4.26).

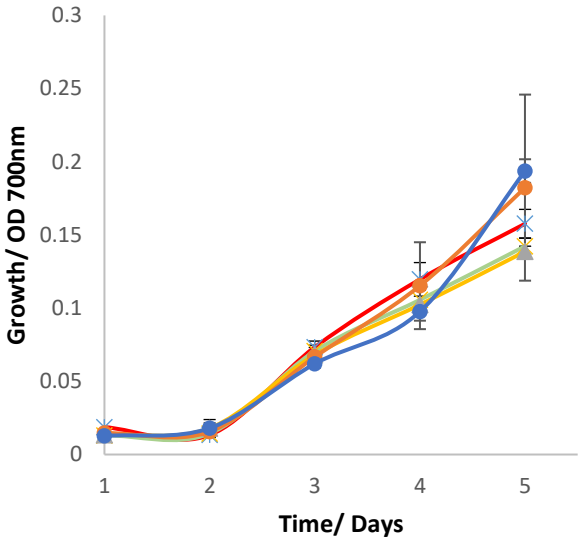

Figure 4.26. *Chlamydomonas reinhardtii* growth test with five concentrations of arsenic. Treatments are: (∗) 100 mg/l, (×)10 mg/l, (▲)1 mg/l, (●)0.1 mg/l, and (♦) the control

Table 4.21. Concentration of heavy metal (mg/l) that result in LC_{50} of microalgae

	strain	Zn	Hg	Co	Cd	Cu	Ni	Mixture
LC_{50}	C.V	25.4	0.22	3.6	27.5	8.49	3.76	15.08
	C.R	24.19	0.59	5.91	4.21	0.33	5.2	10.66

C.V: *Chlorella vulgaris*. C.R: *Chlamydomonas reinhardtii*

4. The impact of heavy metals on the growth of *Daphnia magna*

4.1 The impact of zinc on the growth *Daphnia magna*

D. magna was incubated in six concentrations of zinc 0 mg/l (control) 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l to measure the toxicity of the metal (Figure 4.27). The growth was observed for 48 hours, and a count of living *Daphnia* was taken every two hours. Analysis of variance with one-way ANOVA was performed to detect any significant

differences from the control (p <0.05). Concentrations of 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l of Zn caused a significant impact on the growth of *D. magna* (p <0.05). At the concentration of 0.01mg/l, 60% reduction of the number of *D. magna* in the solution has occurred within the first 12 hours of the test. However, at the end of incubation the population of *D. magna* reduced to 66.7%. On the other hand, the concentrations of 0.1mg/l, 1mg/l, and 10 mg/l of Zn caused 100% reduction of *D. magna* numbers. The concentration of 0.001 mg/l Zn did not show a considerable differentiation from the control (p >0.05). The LC_{50} of zinc was below 0.01 mg/l.

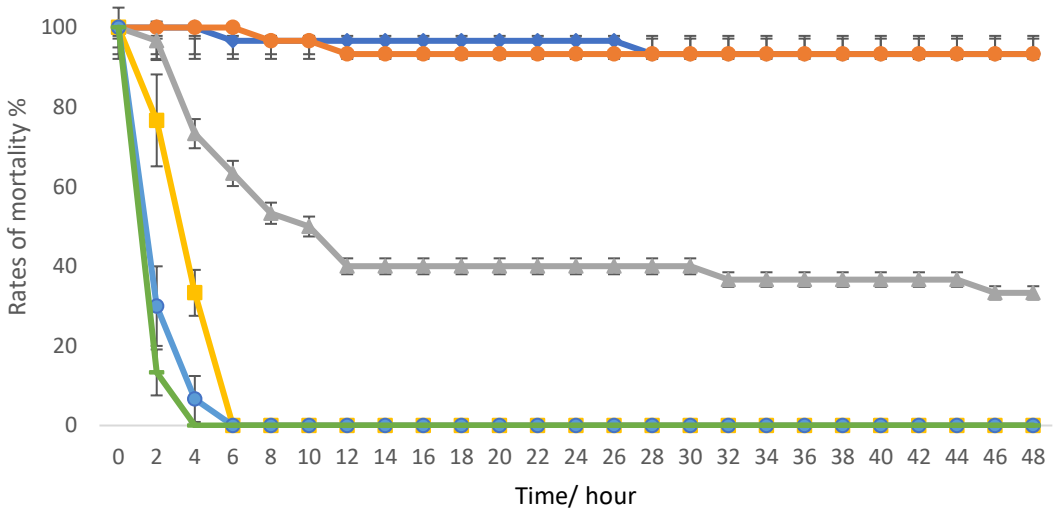

Figure 4.27. *Daphnia magna* response to zinc at different concentrations: (–) 10 mg/l, (∗)1 mg/l, (■) 0.1 mg/l, (▲)0.01 mg/l, (●)0.001 mg/l, and (♦) the control.

4.2 The impact of copper on the growth of *Daphnia magna*

Six concentrations of copper were used to measure the toxicity on *D. magna* 0 mg/l, 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l (Figure 4.28). The growth was monitored for 48 hours, and a count of living *Daphnia* was taken every two hours. All the concentrations used

in the toxicity test of copper on *D. magna:* 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l showed a significant difference from the control with a 100% reduction of the population of *D. magna* within the first 6 hours of the test (p <0.05). Therefore, the LC_{50} of Cu was below 0.001 mg/l.

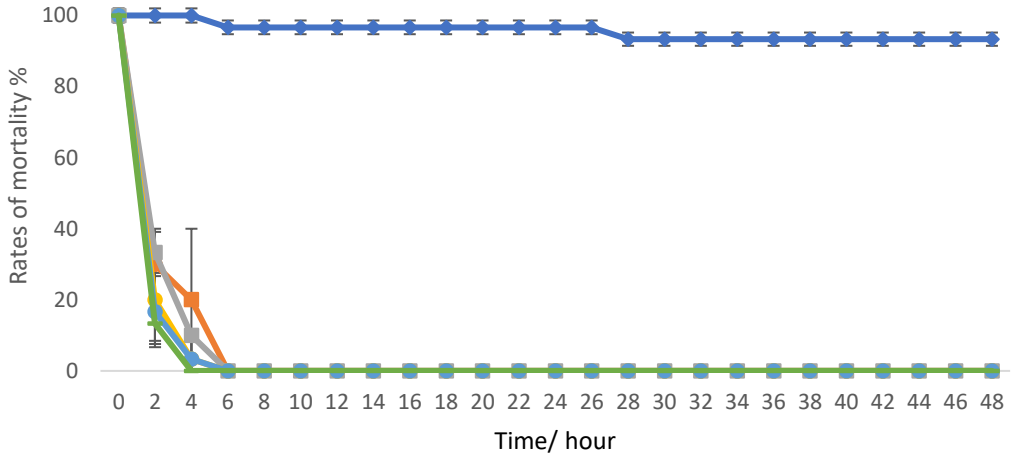

Figure 4.28. *Daphnia magna* responses to copper at different concentrations: (▬) 10 mg/l, (∗)1 mg/l, (■) 0.1 mg/l, (▲)0.01 mg/l, (●)0.001 mg/l, and (♦) the control.

4.3 The impact of nickel on the growth of *Daphnia magna*

D. magna was incubated with six concentrations of nickel: 0 mg/l, 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l as shown in Figure 4.29. The growth was monitored for 48 hours, and the number of living *Daphnia* was recorded every two hours. The concentrations of 0.001mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l showed a significance difference from the control (p <0.05). The concentration of 0.001 mg/l of Ni resulted in a reduction of 40% in the population of *Daphnia*, within the first 8 hours. The concentrations of 0.01mg/l, 0.1mg/l,

1mg/l, and 10 mg/l of Ni caused a total inhibition of *D. magna* growth. The LC_{50} of nickel for *D. magna* was below 0.01 mg/l.

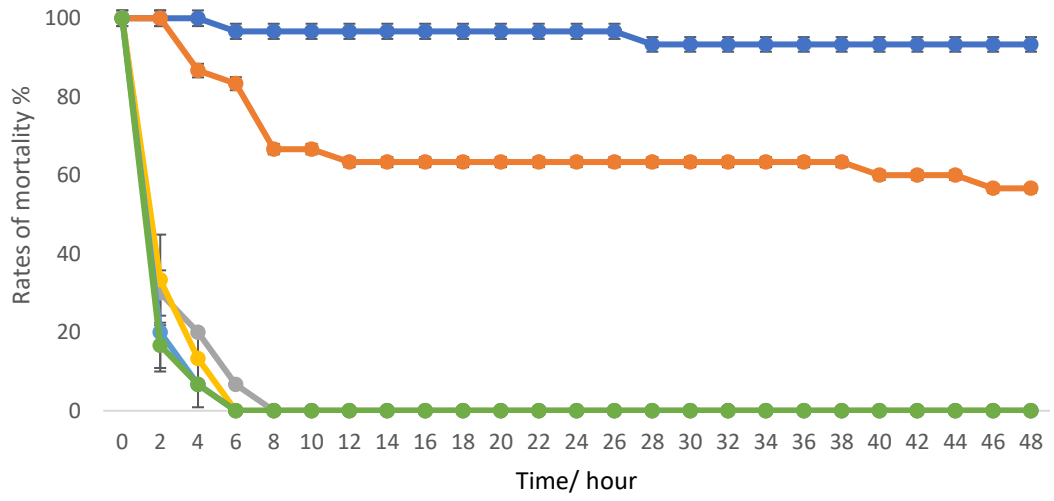

Figure 4.29. *Daphnia magna* responses to nickel at different concentrations: (–) 10 mg/l, (∗)1 mg/l, (■) 0.1 mg/l, (▲)0.01 mg/l, (●)0.001 mg/l, and (♦) the control.

4.4 The impact of mercury on the growth of *Daphnia magna*

D. magna was tested with six concentrations of mercury 0 mg/l, 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l. The results of incubating and monitoring *D. magna* for 48 hours in mercury solutions are shown in Figure 4.30. The concentrations of 0.001mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l of mercury, showed a significant difference from the control ($p < 0.05$). At concentrations of 0.01mg/l, 0.1mg/l, 1mg/l, and 10 mg/l of Hg, total inhibition of *D. magna* growth has occurred within the first 4-6 hours. The concentration of 0.001 mg/l Hg did not show a significant difference from the control. The LC_{50} of mercury was below 0.01 mg/l for *D. magna*.

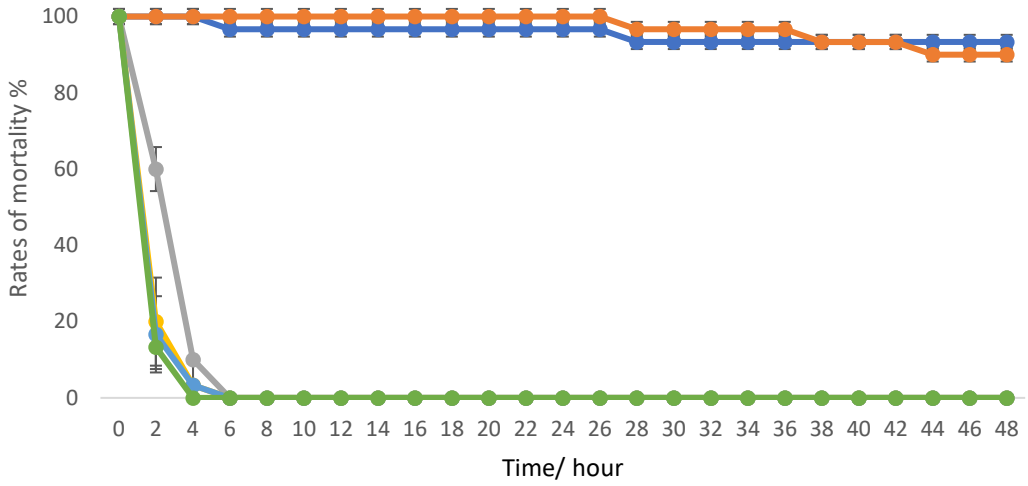

Figure 4.30. *Daphnia magna* responses to mercury at different concentrations: (–) 10 mg/l, (∗)1 mg/l, (■) 0.1 mg/l, (▲)0.01 mg/l, (●)0.001 mg/l, and (♦) the control.

4.5 The impact of cobalt on the growth of *Daphnia magna*

D. magna was exposed to five concentrations of cobalt 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, 10mg/l, in addition to the control (0 mg/l), shown in Figure 4.31. The growth was observed for 48 hours, and a count of living *Daphnia* was taken every two hours. The analysis of data showed that concentrations of 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l of cobalt were significantly different from the control. Reduction of 20% and 30% of *D. magna* population were caused by concentrations of 0.01mg/l and 0.1 mg/l, respectively within the first 24 hours (p <0.05). By the end of the incubation, mortality rates have reduced to 26.7% and 66.7% at concentrations of 0.01mg/l, 0.1mg/l after 48 hours, respectively. At levels 1 mg/l, and 10 mg/l of cobalt, *D. magna* was totally inhibited after 10 hours. The concentration of

0.001 mg/l of Co did not show a significant difference from the control (p <0.05). The LC_{50} of cobalt was below 0.1 mg/l for *D. magna*.

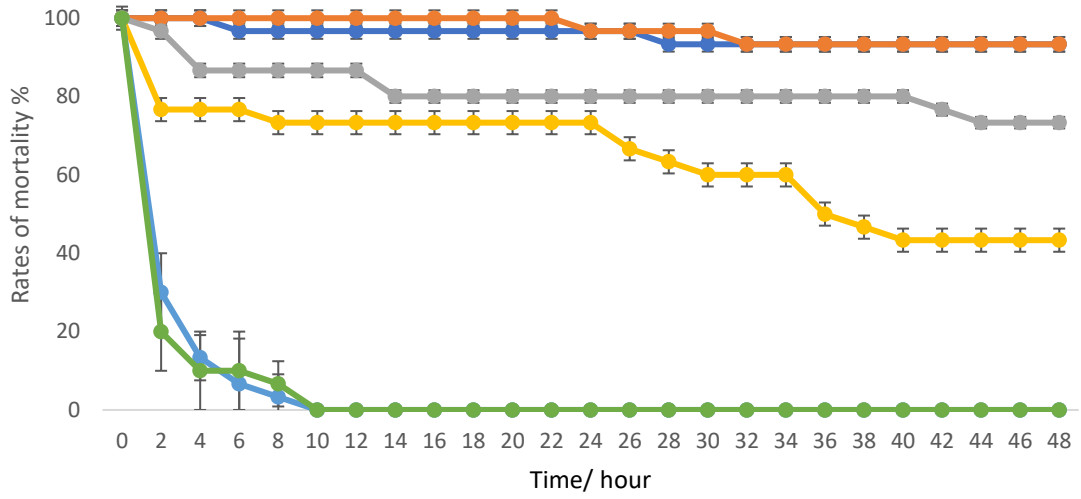

Figure 4.31. *Daphnia magna* responses to cobalt at different concentrations: (–) 10 mg/l, (∗)1 mg/l, (■) 0.1 mg/l, (▲)0.01 mg/l, (●)0.001 mg/l, and (♦) the control.

4.6 The impact of cadmium on the growth of *Daphnia magna*

Six concentrations of Cd were used to incubate *D. magna* 0 mg/l, 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l to test the toxicity of cadmium on *Daphnia* (Figure 4.32). The growth was observed for 48 hours, and a count of living *Daphnia* was taken every two hours. At the concentrations of 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l of cadmium, a significant difference from the control was appeared (p <0.05). The reduction rates of *D. magna* population were 40%, 50% and 60% at hours 10,12,30 hours, respectively, for the concentrations of 0.01mg/l of Cd. Also, 80% and then 90% reduction of *D. magna* numbers at hours 8,12, respectively, occurred up to the end of the test for the concentration of 0.1 mg/l of Cd. The concentrations 1mg/l and 10 mg/l of cadmium caused a total inhibition of *D.*

magna after 6 hours. The concentration of 0.001 mg/l started to show a significant difference from the control (p <0.05) after 28 hours of the incubation to reach 23.7% reduction in *D. magna* numbers by the end of the test. According to the results, half of *D. magna* population reduced at a concentration less than 0.01mg/ of cadmium.

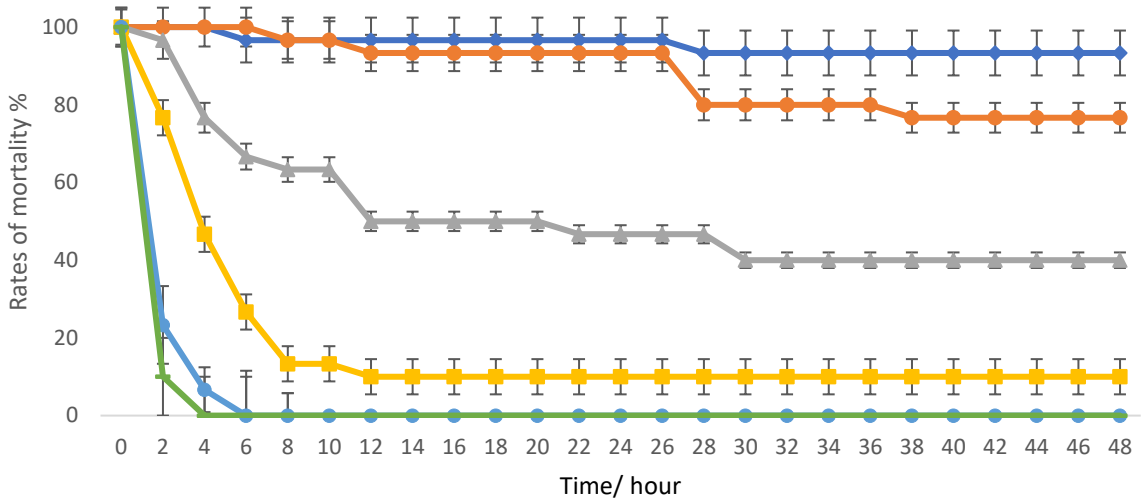

Figure 4.32. *Daphnia magna* responses to cadmium at different concentrations: (–) 10 mg/l, (∗)1 mg/l, (■) 0.1 mg/l, (▲)0.01 mg/l, (●)0.001 mg/l, and (♦) the control.

4.7 The impact of mixture of heavy metals on the growth of *Daphnia magna*

Six different concentrations, namely: 0 mg/l, 0.001 mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l of a mixture of heavy metals (Zn, Cu, Hg, Co, Cd, and Ni) were prepared by mixing equal amounts of each metal to prepare the required concentration and then used to test their toxic effect in a combination on *D. magna* (Figure 4.33). The growth was monitored for 48 hours, and the number of living *Daphnia* was recorded every two hours. The metal mixture

concentrations of 0.001mg/l, 0.01mg/l, 0.1mg/l, 1mg/l, and 10mg/l showed a significant difference from the control (p <0.05). The concentration of 0.001mg/l of the mixture caused 84% reduction of *D. magna* population after 12 hours and up to the end of the test. At concentrations of 0.01mg/l, 0.1mg/l, 1mg/l, and 10 mg/l of the metal's mixture, 100% inhibition of *D. magna* growth has occurred. The LC_{50} of the metal mixture was below 0.001 mg/l for *D. magna*.

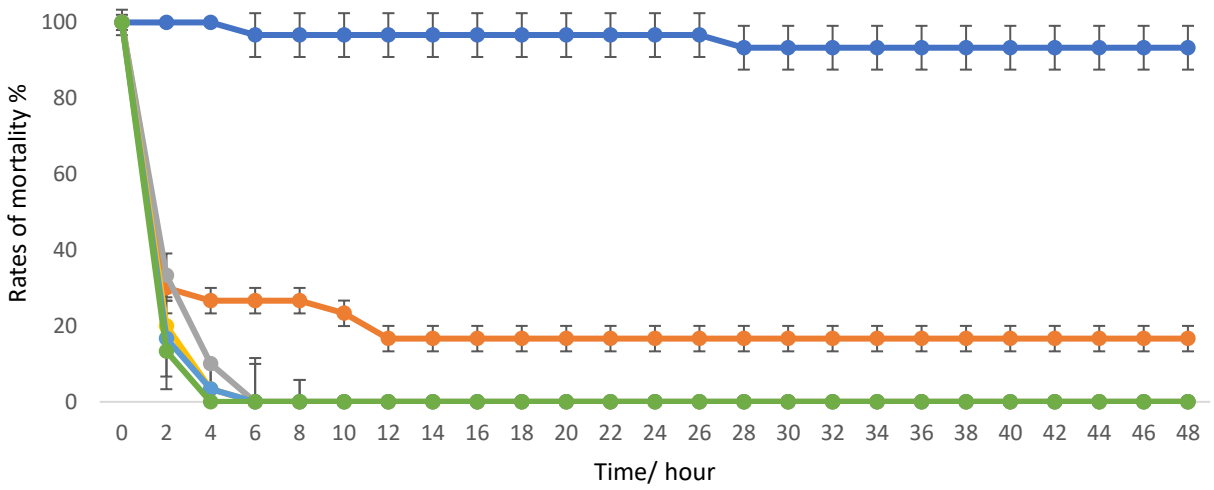

Figure 4.33. *Daphnia magna* responses to mixture of heavy metals at different concentrations: (–) 10 mg/l, (∗)1 mg/l, (■) 0.1 mg/l, (▲)0.01 mg/l, (●)0.001 mg/l, and (♦) the control.

4.8 Results discussion

The results revealed that the test of metals mixture impact on *C. vulgaris* showed a high LC_{50} value (15.08 mg/l) compared to what has been reported by other researchers (Expósito *et al.* 2021). Expósito *et al.* (2021) stated that predictive mixture effect models tend to overestimate the effects of metal mixtures in *C. vulgaris* for both growth and photosynthesis inhibition tests. This is because of the other factors that impact toxicity intensity, such as the microalgal species used, metals interactions in the mixture of metals and other physical and chemical

influences; like pH, and temperature (Hart,1982). For example, the presence of nickel, lead, and selenium in the solution reduced cadmium toxicity as found by Hotchinson and Stokes (1975); Hotchinson (1976); Dietilainin (1976); Al-Waeli *et al.* (2012); Shamshad *et al.* (2016) ;Zwolak (2020), probably because of the complexing part of the cadmium to lessen the concentration of toxic forms (Gesamp, 1987). On the other hand, *C. reinhardtii* had an LC_{50} value of 10.66 mg/l when exposed to heavy metals mixture during toxicity experiment, and *C. vulgaris* 211/21A has an LC_{50} of 1 mg/l for the mixture of metals; these results confirm what was stated by Hotchinson (1976); Hotchinson and Stokes (1975); Dietilainin (1976); Hall (2000); Arunakumara, and Zhang (2008), regarding the algal species depending response to metals toxicity. In addition, cobalt in natural water bodies, ranges from $4x10^{-6}$ to 1.7 mg/l (Abbasi et al.,1998; Tchounwou *et al.*, 2012). This suggests that a species isolated from a water body that has a concentration of $4x10^{-6}$ of cobalt will have a different LC_{50} for the same species habitats a water body that has an average concentration of 1.7 mg/l of Co. For example, *C. vulgaris* 211/111 used in the study has an LC_{50} of 3.6 mg/l, unexpectedly lower than *C. reinhardtii* (5.91 mg/l); this might be related to the original habitats these two species were originally from. *C. vulgaris* 211/21A has an LC_{50} of 0.33 mg/l, another confirmation that this species is more delicate than the other two species; these findings are similar to what has been reported by Australian and New Zealand Environment and Conservation Council (ANZECC) & Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ) (2000). The reaction of the three strains to metals toxicity strengthened the conclusion of eliminating *C. vulgaris* 211/21A from the study. Wang (1986) reported that algae collected from wastewater treatment lagoons exhibited different responses to zinc toxicity after acclimation. This suggests that toxicological response depends upon algal acclimation to the specific environment, not the origin of the algal source. Wang (1986) used algal consortium in his study and tested them after prolonged

exposure to Zn. Algae isolated from a low zinc concentration developed resistance to zinc when acclimated to higher considerations of zinc for a prolonged period of exposure. The same behaviour was obtained from the algal consortium isolated from a high concentration zinc environment adapted to a low zinc concentration environment.

CHAPTER FIVE

THE ADAPTATION OF MICROALGAE TO HEAVY METALS IN THE GROWTH MEDIUM

Summary

In this chapter, the adaptation stages of microalgae (*Chlorella vulgaris* and *Chlamydomonas reinhardtii)* to elevated concentrations of heavy metals have been presented. In addition, metal concentrations in the growth medium before and after microalgal growth have been analysed using inductively coupled plasma - optical emission spectrometry **(**ICP-OES). The obtained data were analysed and demonstrated in the second part of this chapter. Furthermore, the spectra obtained from Fourier Transform Infrared (FTIR) spectroscopy, was analysed. Scanning of algae strains before and after adaptation has been conducted to determine the structural changes after this process. Comparison through detecting the marker bands has been made in addition to determining the position of each band for both strains *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. The aim of this chapter was to monitor cell composition impacted by adaptation of microalgae to heavy metal/s.

5.1 The adaptation of microalgae to heavy metals in the growth medium

Microalgae (*Chlorella vulgaris* 211/111 and *Chlamydomonas reinhardtii* 11/45*)* were cultivated in Bold Basal Medium with 3-fold Nitrogen and Vitamins (3NBBM) for 7 days for each generation with a defined concentration of heavy metals over 10 generations (one stage), with metal concentrations increasing by 10% at each stage (Table 5.1). The individual metal additions or the addition of a mixture of metals were carried out to enhance microalgal

resistance to the individual metals or mixture metals. In cases where the increased metal concentration reduced the culture growth significantly, the metal concentration was kept at the same level for two stages to avoid losing the adapted phenotype. The initial concentrations of heavy metals used in stage 1 were derived from the toxicity experiments and represents the LC_{50} of microalgae for each metal. The adaptation process consisted of five stages over a period of 315 days to produce an adapted microalgal phenotype, capable of surviving in aqueous systems impacted by heavy metals and to explore how microalgal cultures could mitigate heavy metal toxicity.

Table 5.1. Heavy metals concentrations (mg/l) used in the adaptation of *Chlorella vulgaris*

Stages	**strain**	**Zn**	**Hg**	**Co**	**Cd**	**Cu**	**Ni**	**Mix**
Stage 1	C.V	25	0.2	3.6	27	8.4	3.7	15
Stage 2	C.V	27.5	0.3	4	29	9.2	4.1	16.5
Stage 3	C.V	30	0.3	4.4	32	10	4.1	18.1
Stage 4	C.V	33	0.4	4.8	32	11	4.4	19.9
Stage 5	C.V	33	0.5	5.2	32	12	4.4	22

5.1.1 Adaptation of *Chlorella vulgaris* to the presence of mercury in the growth medium

Chlorella vulgaris was repeatedly incubated in 3NBBM medium with mercury to enhance the microalga's resistance to the metal toxicity in the incubation medium. Mercury was added in concentrations: 0.2 mg/l, 0.3 mg/l, 0.3 mg/l, 0.4 mg/l and 0.5 mg/l, for stages 1-5 respectively (Table 5.1). The initial mercury concentration used was obtained from toxicity experiments, where LC_{50} of mercury was calculated to be 0.22 mg/l. In the sequential stages, Hg concentration increased by approximates of 10%. A significant microalgal growth was detected during stages 1, 2, and 3, where mercury concentrations were: 0.2 mg/l, 0.3 mg/l,

and 0.3 mg/l, respectively. However, when Hg concentration increased to 0.4 mg/l and 0.5 in stages 4 and 5, respectively, the growth significantly decreased (p<0.05) (Figure 5.1). The presence of increasing metal concentrations by 50% from Stages 1 to 3 led to a significant increase in tolerance of *Chlorella vulgaris* during the adaptation period of 315 days (p<0.05).

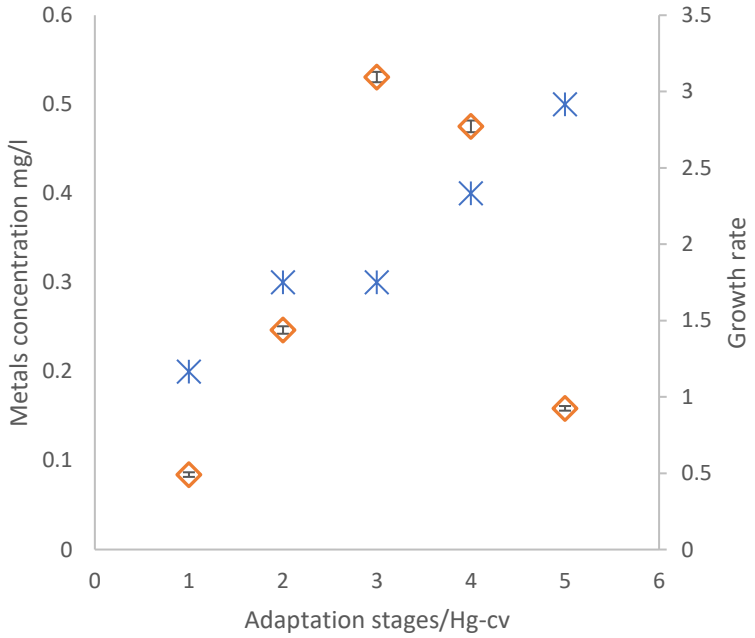

Figure 5.1. The adaptation stages of *Chlorella vulgaris* in 3NBBM with mercury in different concentrations, 0.2 mg/l, 0.3 mg/l, 0.3 mg/l, 0.4 mg/l, and 0.5 mg/l for stages 1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (*), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.1.2 Adaptation of *Chlorella vulgaris* to the presence copper in the growth medium

Copper was added to 3NBBM medium in concentrations: 8.4 mg/l, 9.2 mg/l, 10 mg/l, 11 mg/l, and 12 mg/l for Stages 1 - 5, respectively (Table 5.1). *C. vulgaris* was incubated with sequentially increasing concentrations to enhance the microalga's resistance to copper toxicity in the incubation medium. The initial copper concentration used was obtained from toxicity experiments, where LC_{50} of copper was calculated to be 8.49 mg/l. In the sequential stages, copper concentration increased by approximates of 10%. A significant improvement in the growth of *Chlorella vulgaris* was observed at the first stage of adaptation (Figure 5.2). The growth significantly increased for the following stages up to the 4th stage ($p<0.05$). In the fifth stage, in which the Cu concentration increased to 12mg/l, microalgal growth significantly decreased ($p<0.05$). The presence of increasing metal concentrations by 30% from Stages 1 to 4 led to a significant increase in tolerance of *C. vulgaris* during the adaptation period of 315 days ($p<0.05$).

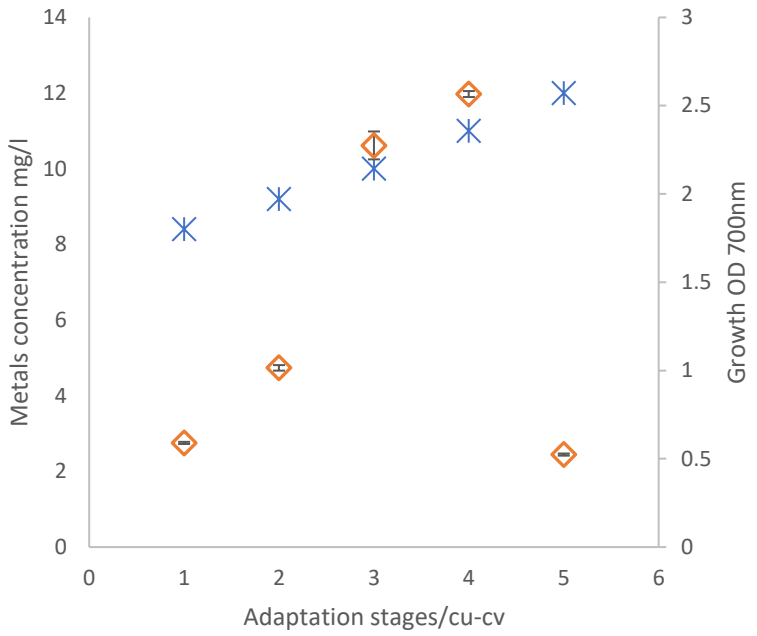

Figure 5.2. The adaptation stages of *Chlorella vulgaris* in 3NBBM with copper in different concentrations, 8.4 mg/l, 9.2 mg/l, 10 mg/l, 11 mg/l, and 12 mg/l for stages 1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (*), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.1.3 Adaptation of *Chlorella vulgaris* to the presence of zinc in the growth medium

C. vulgaris was repeatedly incubated with zinc in 3NBBM medium to enhance the microalga's resistance to the metal toxicity in the incubation medium. Zinc was added in concentrations: 25 mg/l, 27.5 mg/l, and 30 mg/l, for stages 1-3, respectively, and a concentration of 33 mg/l was used for stages 4 and 5 (Table 5.1). The initial zinc concentration used was obtained from toxicity experiments, where LC_{50} of zinc was calculated to be 25.4 mg/l. In the sequential stages, zinc concentration increased by approximates of 10%. Microalgal growth was significantly enhanced during stages 1, 2, and 3, where zinc concentrations were: 25 mg/l, 27.5 mg/l, and 30 mg/l, respectively. However, when Zn concentration increased to 33 mg/l in stage 4, the growth significantly decreased

($p<0.05$). Maintaining Zn concentration at the same level for stage 5 did not help the culture recover; the growth continued to decrease (Figure 5.3). The presence of increasing metal concentrations by 20% from Stages 1 to 3 led to a significant increase in tolerance of *C. vulgaris* during the adaptation period of 315 days ($p<0.05$).

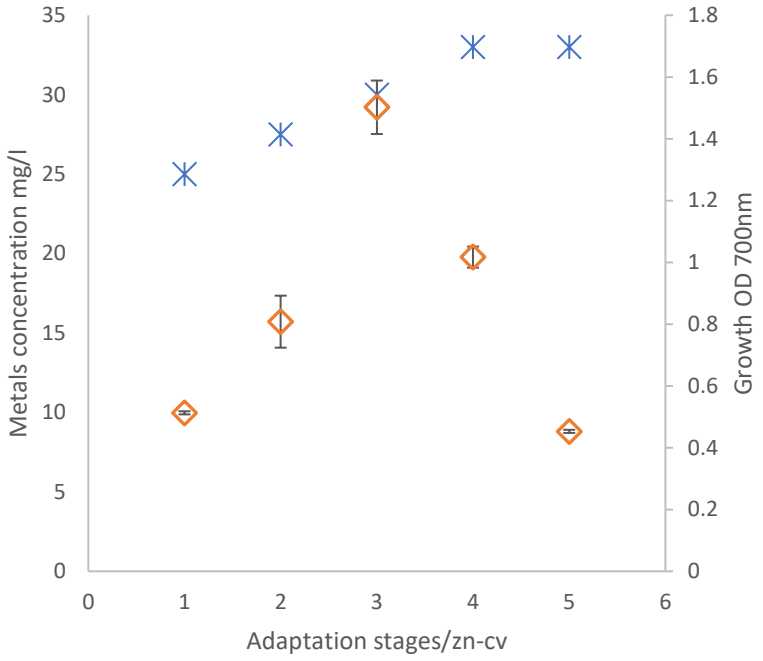

Figure 5.3. The adaptation stages of *Chlorella vulgaris* in 3NBBM with zinc in different concentrations, 25 mg/l, 27.5 mg/l, 30 mg/l, 33 mg/l, and 33 mg/l for stages 1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (*), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.1.4 Adaptation of *Chlorella vulgaris* to the presence of cobalt in the growth medium

C. vulgaris was repeatedly incubated with cobalt in the 3NBBM medium at concentrations: 3.6 mg/l, 4 mg/l, 4.4 mg/l, 4.8 mg/l, and 5.2 mg/l for stages 1 - 5, respectively (Table 5.1). *C. vulgaris* was incubated in a sequentially increasing concentrations to enhance the microalga's

resistance to cobalt toxicity in the incubation medium. The initial Co concentration used was obtained from toxicity experiments, where LC_{50} of cobalt was calculated to be 3.6 mg/l. In the sequential stages, cobalt concentration increased by 10%. A significant improvement in the growth of *C. vulgaris* was observed at the first stage of adaptation (Figure 5.4). The growth significantly increased for the following stages up to the 4th stage (p<0.05). In the fifth stage, in which the Co concentration increased to 5.2mg/l, microalgal growth significantly decreased (p<0.05). The presence of increasing metal concentrations by 34% from Stages 1 to 4 led to a significant increase in tolerance of *C. vulgaris* during the adaptation period of 315 days (p<0.05).

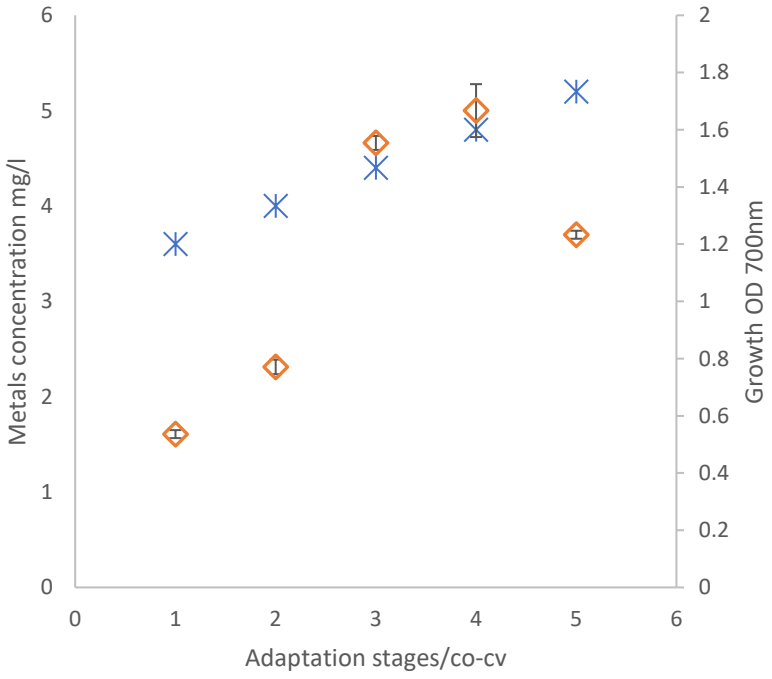

Figure 5.4. The adaptation stages of *Chlorella vulgaris* in 3NBBM with cobalt in different concentrations, 25 mg/l, 27.5 mg/l, 30 mg/l, 33 mg/l, and 33 mg/l for stages 1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (✻), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.1.5 Adaptation of *Chlorella vulgaris* to the presence of cadmium in the growth medium

C. vulgaris was repeatedly incubated in 3NBBM medium with cadmium to enhance the microalga's resistance to the metal toxicity in the incubation medium. Cadmium was added at 27 mg/l, 29 mg/l, and 32 mg/l, for stages 1-3 respectively; 32 mg/l was also used for stages 4 and 5 (Table 5.1). The initial cadmium concentration used was obtained from toxicity experiments, where LC_{50} of cadmium was calculated to be 27.5 mg/l. In the sequential stages, cadmium concentration increased by 10%. A significant increase in the growth of *C. vulgaris* occurred during stage one of adaptation to cadmium ($p<0.05$). However, the growth significantly reduced when Cd level was raised to 32 mg/l in stage 3. Furthermore, keeping the concentration at the same level in the following stages did not improve the growth in a significant way ($p<0.05$) (Figure 5.5). The presence of increasing metal concentrations by 10% from Stages 1 to 2 led to a significant increase in tolerance of *C. vulgaris* during the adaptation period of 315 days ($p<0.05$).

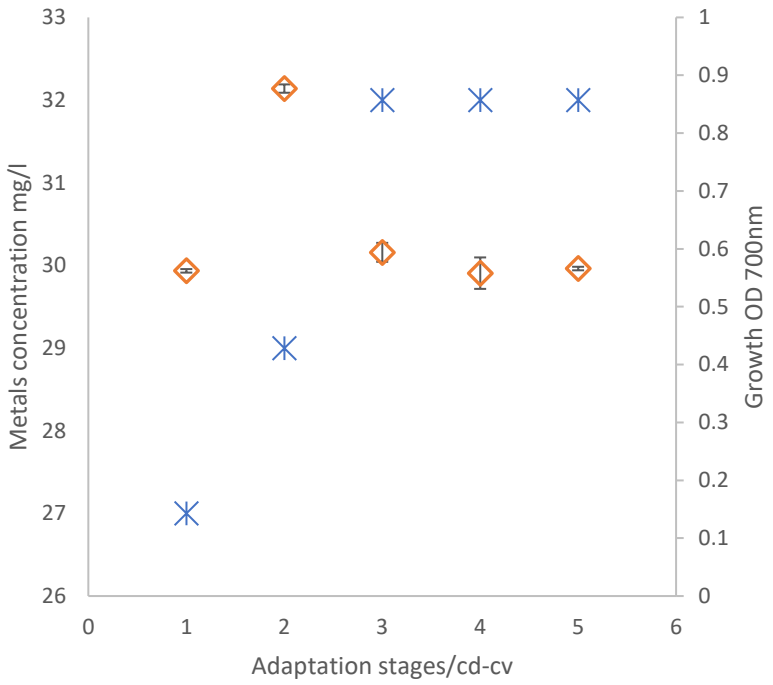

Figure 5.5. The adaptation stages of *Chlorella vulgaris* in 3NBBM with cadmium in different concentrations, 27 mg/l, 29 mg/l, 32 mg/l, 32 mg/l, and 32 mg/l for stages 1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (⁎), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.1.6 Adaptation of *Chlorella vulgaris* to the presence of nickel in the growth medium

C. vulgaris was incubated in the sequentially increasing concentrations to enhance the microalga's resistance to nickel toxicity in the incubation medium: 3.7 mg/l, 4.1 mg/l, 4.1 mg/l, 4.4 mg/l, and 4.4 mg/l for stages 1 - 5, respectively (Table 5.1). The initial nickel concentration used was obtained from toxicity experiments, where LC_{50} of nickel was calculated to be 3.76 mg/l. In the sequential stages, the nickel concentration increased by 10%. The increase of Ni concentration from 3.7 mg/l to 4.1 mg/l significantly reduced microalgal growth at stage 2 (p<0.05); however, maintaining nickel levels at 4.1 mg/l for the next stage helped the culture to recover and enhance its growth. The concentration increased

to 4.4 mg/l at stage 4 and kept the same at stage 5, to maintain the growth enhancement. The presence of increasing metal concentrations by 19 % from Stages 1 to 5 led to a significant increase in the tolerance of *C. vulgaris* during the adaptation period of 315 days (p<0.05) (Figure 5.6).

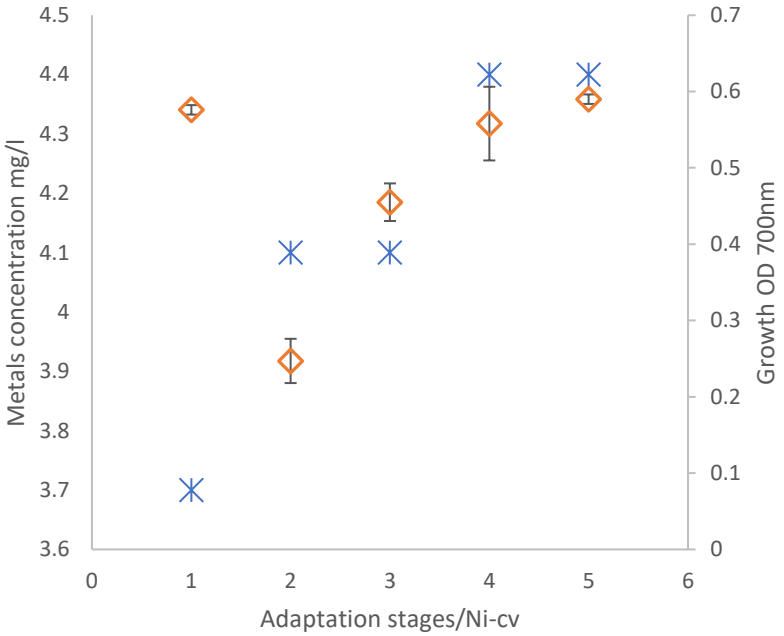

Figure 5.6. The adaptation stages of *Chlorella vulgaris* in 3NBBM with nickel in different concentrations, 3.7 mg/l, 4.1 mg/l, 4.1 mg/l, 4.4 mg/l, and 4.4 mg/l for stages 1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (∗), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage**.**

5.1.7 Adaptation of *Chlorella vulgaris* to the presence of a mixture of metals in the growth medium

C. vulgaris was repeatedly incubated in 3NBBM with a mixture of metals (Cu, Zn, Ni, Hg, Co, and Cd) to enhance its resistance to heavy metals; thereafter, the stress caused by heavy

metals pollution in the environment. The mixture was prepared by adding equal amounts of each metal in the mixture to prepare the required mixture concentration. For example, to prepare 15 mg/l of the metal mixture, an amount of 2.5 mg/l of each metal (Cu, Zn, Ni, Hg, Co, and Cd) were mixed in one litre of the growth medium. At the first stage of the adaptation process, the initial heavy metal concentrations used were obtained from toxicity experiments, where LC_{50} was calculated for the mixture of heavy metals for *C. vulgaris* and was 15.08 mg/l (Table 5.1). In the sequential stage incubations, the mixture concentration increased by approximates of 10%. The concentration used in the adaptation of *C. vulgaris* to heavy metals mixture were: 15 mg/l, 16.5 mg/l, 18.1 mg/l, 19.9 mg/l, and 22 mg/l for Stages 1 - 5, respectively. A significant improvement in the growth of *C. vulgaris* was observed at the first stage of adaptation (Figure 5.7); however, when the concentration of metals increased to 16.5mg/l in stage 2, the growth was significantly reduced ($p<0.05$). In the following stages, 3 and 4, the growth was back to increase. In stage 5, when metals concentration reached 22 mg/l, microalgal growth significantly reduced ($p<0.05$). The mixture concentration increased by 30% from stage 1 to stage 4; however, the growth did not differ between the two stages, suggesting that *C. vulgaris* has developed resistance to the metal mixture presence during the adaptation period of 315 days ($p<0.05$).

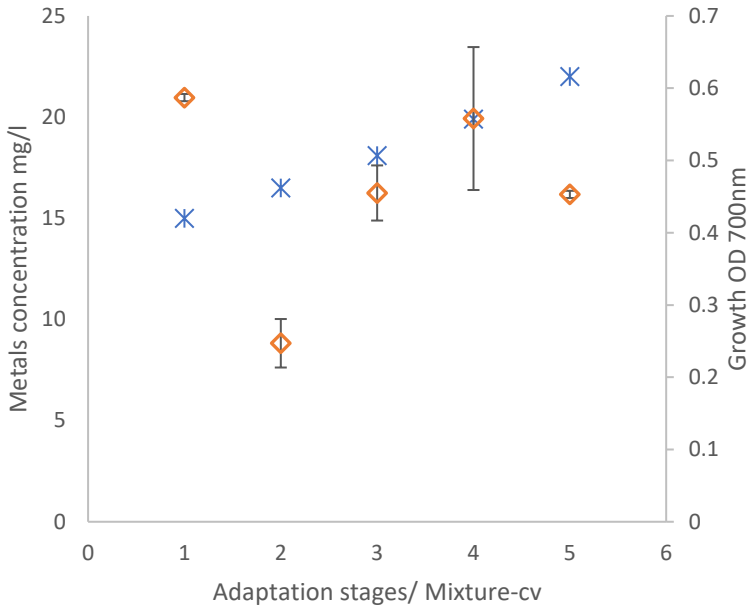

Figure 5.7. The adaptation stages of *Chlorella vulgaris* in 3NBBM with a mixture of metals, Cu, Zn, Ni, Hg, Co, and Cd, at different concentrations, 15 mg/l, 16.5 mg/l, 18.1 mg/l, 19.9 mg/l, and 22 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (✳), and microalgal growth is measured by optical density 700nm and represented on the z-axis(◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2. Adaptation stages of *Chlamydomonas reinhardtii* to elevated metals concentrations

5.2.1 Adaptation of *Chlamydomonas reinhardtii* to the presence of mercury in the growth medium

C. reinhardtii was repeatedly incubated in 3NBBM medium with Hg to enhance the microalga's resistance to the metal toxicity in the incubation medium. Mercury was added in concentrations: 0.5 mg/l, 0.6 mg/l, 0.6 mg/l, 0.7 mg/l and 0.7 mg/l, for stages 1-5 respectively (Table 5.2). The initial mercury concentration used was obtained from toxicity experiments, where LC_{50} of mercury was calculated to be 0.59 mg/l. In the sequential stages, Hg concentration increased by approximates of 10%. Improvement of *C. reinhardtii* growth was observed between stages 1 and 3; however, when metal concentration was increased in stage 2, the culture started to form aggregates. Therefore, concentration was kept the same for stage

3. The growth continued to improve up to stage 3. In stage 4, when Hg concentration increased to 0.7 mg/l, the growth significantly decreased (p <0.05) (Figure 5.8). The presence of increasing metal concentrations by 20% from stages 1 to 3 led to a significant increase in tolerance of *C. reinhardtii* during the adaptation period of 315 days (p<0.05).

Table 5.2. Heavy metals concentrations (mg/l) used in the adaptation of *Chlamydomonas reinhardtii*

Stages	strain	Zn	Hg	Co	Cd	Cu	Ni	Mix
Stage 1	C.R	24	0.5	5.9	4.2	0.3	5	10.7
Stage 2	C.R	26.4	0.6	6.5	4.6	0.4	5.5	11.8
Stage 3	C.R	30	0.6	7.1	5	0.5	5.5	13
Stage 4	C.R	33	0.7	7.8	5.5	0.6	6	14.3
Stage 5	C.R	33	0.7	7.8	5.5	0.6	6	14.3

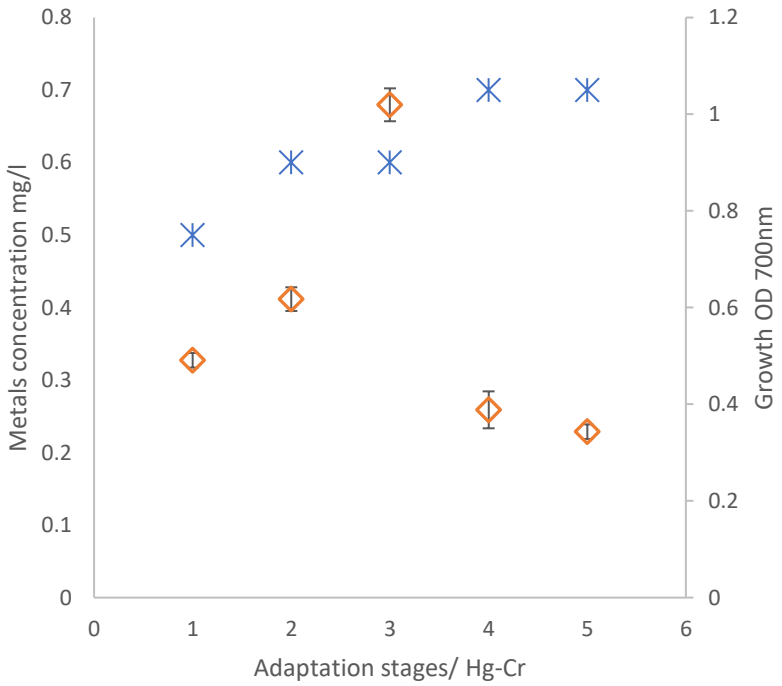

Figure 5.8. The adaptation stages of *Chlamydomonas reinhardtii* in 3NBBM with mercury at different concentrations, 15 mg/l, 16.5 mg/l, 18.1 mg/l, 19.9 mg/l, and 22 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (∗), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2.2 Adaptation of *C. reinhardtii* to the presence of copper in the growth medium

Copper was added to the 3NBBM medium in concentrations: 0.3 mg/l, 0.4 mg/l, 0.5 mg/l, 0.6 mg/l, and 0.6 mg/l for Stages 1 - 5, respectively (Table 5.2). *C. reinhardtii* was incubated in the sequentially increasing concentrations to enhance the microalga's resistance to copper toxicity in the incubation medium. The initial Cu concentration used was obtained from toxicity experiments, where LC_{50} of Cu was calculated to be 0.33 mg/l. In the sequential stages, copper concentration increased by approximates of 10%. The increase of Cu concentration from 0.3 mg/l to 0.4 mg/l reduced microalgae growth in stage 2; however, the difference was not significant between growth in stage 1 and 2. In stage 3 the growth improved even though there was an increase in Cu level to 0.5mg/l. When concentration increased to 0.6mg/l in stage 4 the growth significantly reduced (p <0.05). Maintaining Cu levels at 0.6 mg/l in the next stage helped the culture recover and enhanced growth (Figure 5.9). The tolerance of *C. reinhardtii* to copper significantly increased during the adaptation period of 315 days, where copper presented in an increasing concentration by 50% from stage 1 to stage 3 (p <0.05).

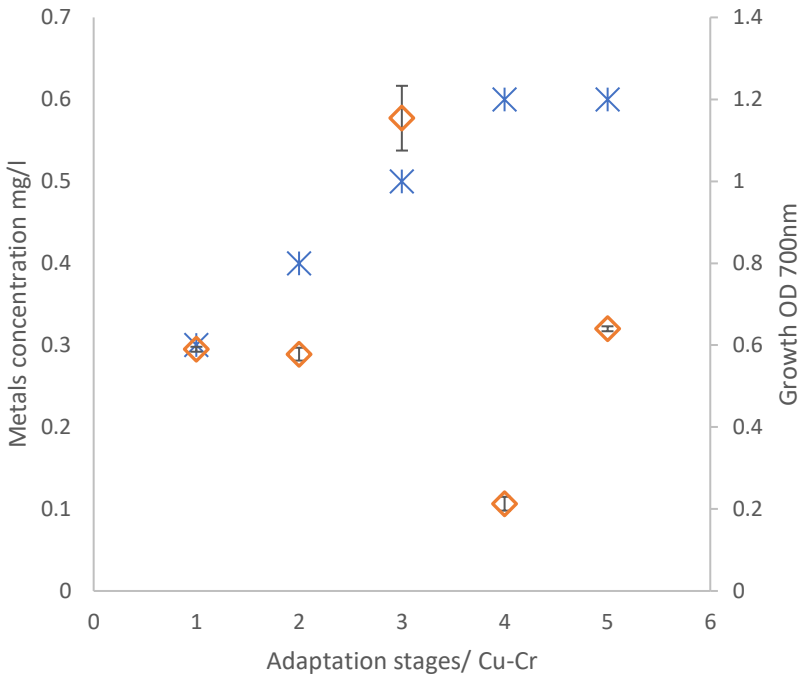

Figure 5.9. The adaptation stages of *C. reinhardtii* in 3NBBM with copper at different concentrations, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l, 0.6 mg/l, and 0.6 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (∗), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2.3 Adaptation of *C. reinhardtii* to the presence of zinc in the growth medium

C. reinhardtii was repeatedly incubated in 3NBBM medium with zinc to enhance the microalga's resistance to the metal toxicity in the incubation medium. Zn was added in concentrations: 24 mg/l, 26.4 mg/l, 30 mg/l, 33 mg/l and 33 mg/l, for stages 1-5 respectively (Table 5.2). The initial Zn concentration used was obtained from toxicity experiments, where LC_{50} of zinc was calculated to be 24.19 mg/l. In the sequential stages, zinc concentration increased by approximates of 10%. The growth of *C. reinhardtii* significantly decreased when zinc concentration increased from 24 mg/l to 26.4 mg/l at stage 2 and from 30 mg/l to 33 mg/l at stage 4; however, the growth increased for the subsequence stage (Figure 5.10). In

addition, the tolerance of *C. reinhardtii* to zinc significantly increased during the adaptation period of 315 days, where Zn presented in an increasing concentration by 37.5 % from stage 1 to stage 5 (p <0.05).

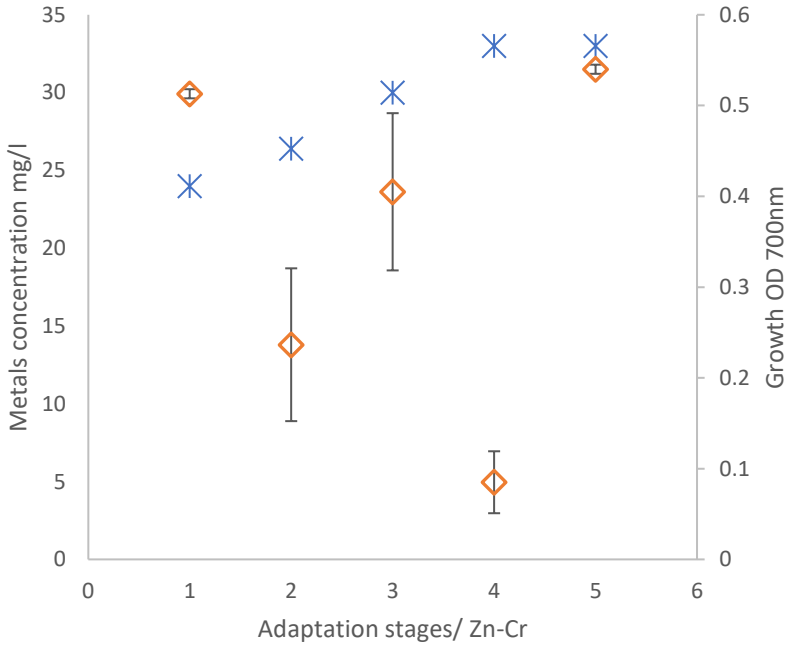

Figure 5.10. The adaptation stages of *C. reinhardtii* in 3NBBM with zinc at different concentrations, 24 mg/l, 26.4 mg/l, 30 mg/l, 33 mg/l, and 33 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (*), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2.4 Adaptation of *C. reinhardtii* to the presence of cobalt in the growth medium

Cobalt was added to 3NBBM medium in concentrations: 5.9 mg/l, 6.5 mg/l, 7.1 mg/l, 7.8 mg/l, and 7.8 mg/l for Stages 1 - 5, respectively (Table 5.2). *C. reinhardtii* was incubated in the sequentially increasing concentrations to enhance the microalga's resistance to cobalt toxicity in the incubation medium. The initial Co concentration used was obtained from toxicity experiments, where LC_{50} of Co was calculated to be 5.91 mg/l. In the sequential stages, cobalt concentration increased by approximates of 10% (Figure 5.11). There was an insignificant decrease in *C. reinhardtii* growth in stage 2 when cobalt concentration increased to 6.5 mg/l ($p > 0.05$). At stage 3, *C. reinhardtii* growth increased significantly when cobalt concentration increased from 6.5 mg/l to 7.1 mg/l at stage 3 ($p<0.05$). The tolerance of *C. reinhardtii* to Co significantly increased during the adaptation period of 315 days, where cobalt presented in an increasing concentration by 20 % from stage 1 to stage 3 ($p < 0.05$).

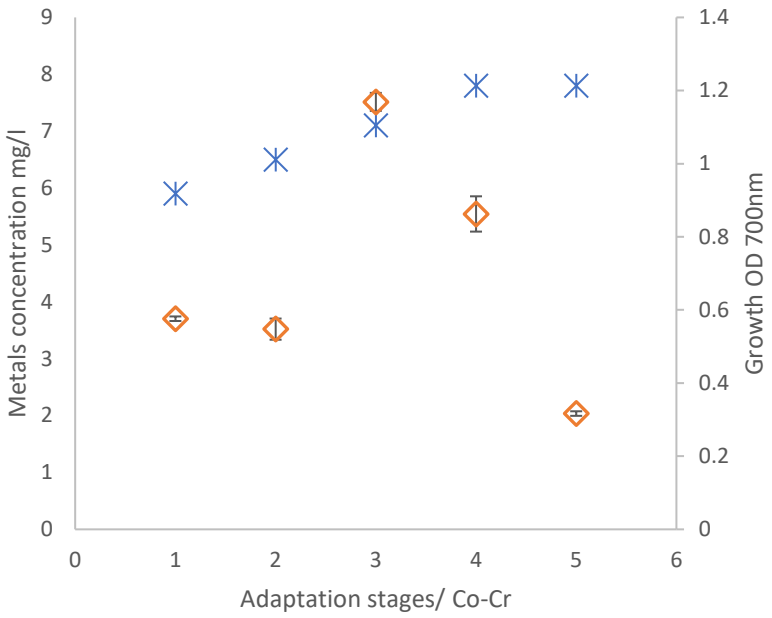

Figure 5.11. The adaptation stages of *C. reinhardtii* in 3NBBM with cobalt at different concentrations, 5.9 mg/l, 6.5 mg/l, 7.1 mg/l, 7.8 mg/l, and 7.8 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (∗), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2.5 Adaptation of *C. reinhardtii* to the presence of cadmium in the growth medium

C. reinhardtii was repeatedly incubated in 3NBBM medium with Cd to enhance the microalga's resistance to the metal toxicity in the incubation medium. Cadmium was added in concentrations: 4.2 mg/l, 4.6 mg/l, 5 mg/l, 5.5 mg/l, and 5.5 mg/l, for stages 1-5 respectively (Table 5.2). The initial Cd concentration used was obtained from toxicity experiments, where LC_{50} of cadmium was calculated to be 4.21 mg/l. In the sequential stages, Cd concentration increased by approximates of 10%. A significant increase in the growth of *C. reinhardtii* occurred during the second stage of adaptation to cadmium ($p<0.05$). However, the growth significantly reduced when Cd level was increased to 5.5 mg/l in stage 4 ($p<0.05$). Furthermore, keeping the concentration at the same level in the following stages did not improve the growth of *C. reinhardtii* (Figure 5.12). The presence of increasing metal

concentrations by 19% from Stages 1 to 3 led to a significant increase in tolerance of *C. reinhardtii* during the adaptation period of 315 days (p<0.05).

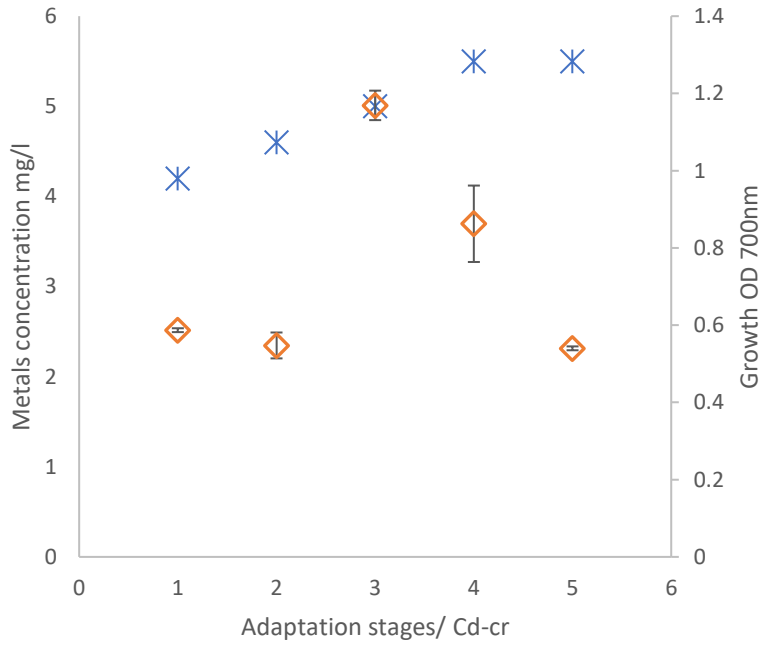

Figure 5.12. The adaptation stages of *C. reinhardtii* in 3NBBM with cadmium at different concentrations, 4.2 mg/l, 4.6 mg/l, 5 mg/l, 5.5 mg/l, and 5.5 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (∗), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2.6 Adaptation of *C. reinhardtii* to the presence of nickel in the growth medium

Nickel was added to 3NBBM medium in concentrations: 5 mg/l, 5.5 mg/l, 5.5 mg/l, 6 mg/l, and 6 mg/l for Stages 1 - 5, respectively (Table 5.2). *C. reinhardtii* was incubated in the sequentially increasing concentrations to enhance the microalga's resistance to Ni toxicity in the incubation medium. The initial nickel concentration used was obtained from toxicity experiments, where LC_{50} of nickel was calculated to be 5.2 mg/l. In the sequential stages, Ni concentration increased by approximates of 10%. There was no significant improvement in

the growth of *C. reinhardtii* at the first stage of adaptation (Figure 5.13). However, the growth significantly increased for the following stage when metal concentration kept at the same level (p<0.05). In the fourth stage, in which the Ni concentration increased to 6 mg/l, microalgal growth significantly decreased (p<0.05). The presence of increasing metal concentrations by 10% from Stages 1 to 3 led to a significant increase in tolerance of *C. reinhardtii* during the adaptation period of 315 days (p<0.05).

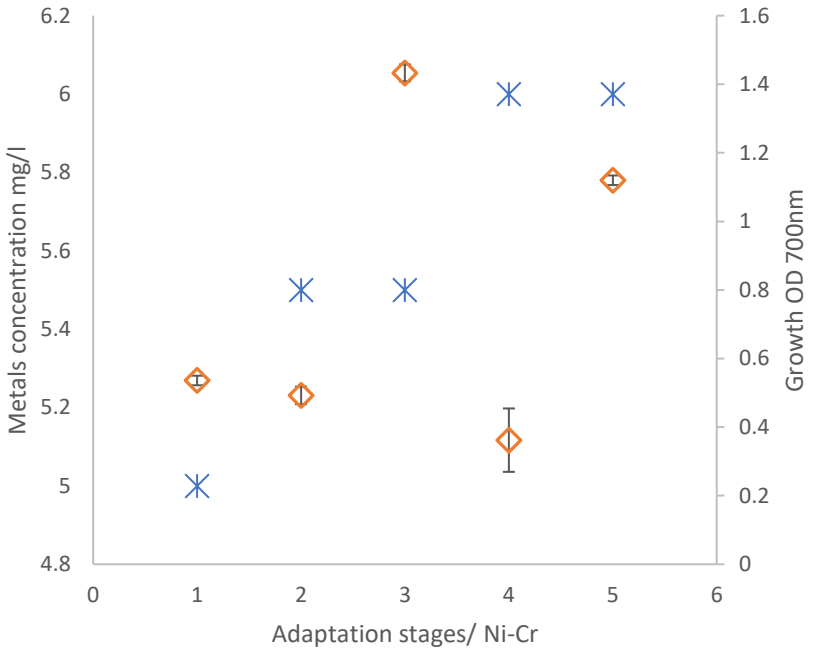

Figure 5.13. The adaptation stages of *C. reinhardtii* in 3NBBM with nickel at different concentrations, 5 mg/l, 5.5 mg/l, 5.5 mg/l, 6 mg/l, and 6 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (∗), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2.7 Adaptation of *C. reinhardtii* to the presence of metals mixture in the growth medium

C. reinhardtii was repeatedly incubated in 3NBBM with a mixture of metals (Cu, Zn, Ni, Hg, Co, and Cd) to enhance its resistance to heavy metals; thereafter, the stress caused by heavy metals pollution in the environment. At the first stage of the adaptation process, the initial heavy metal concentrations used were obtained from toxicity experiments, where LC_{50} was calculated for the mixture of heavy metals for *C. reinhardtii* and was 10.66 mg/l (Table 5.2). In the sequential stage incubations, the mixture concentration increased by approximates of 10%. Concentration used in the adaptation of *C. reinhardtii* to heavy metals mixture were: 10.7 mg/l, 11.8 mg/l, 13 mg/l, 14.3 mg/l, and 14.3 for Stages 1 - 5, respectively. A significant improvement in the growth of *C. reinhardtii* was observed at the first stage of adaptation up to the third stage (Figure 5.14). In stage 4 and 5, when metals concentration raised to 14.3 mg/l, microalgal growth significantly reduced ($p<0.05$). The mixture concentration increased by 21% from stage 1 to stage 3, suggesting that *C. vulgaris C. reinhardtii* has developed resistance to the metals mixture presence during the adaptation period of 315 days ($p<0.05$).

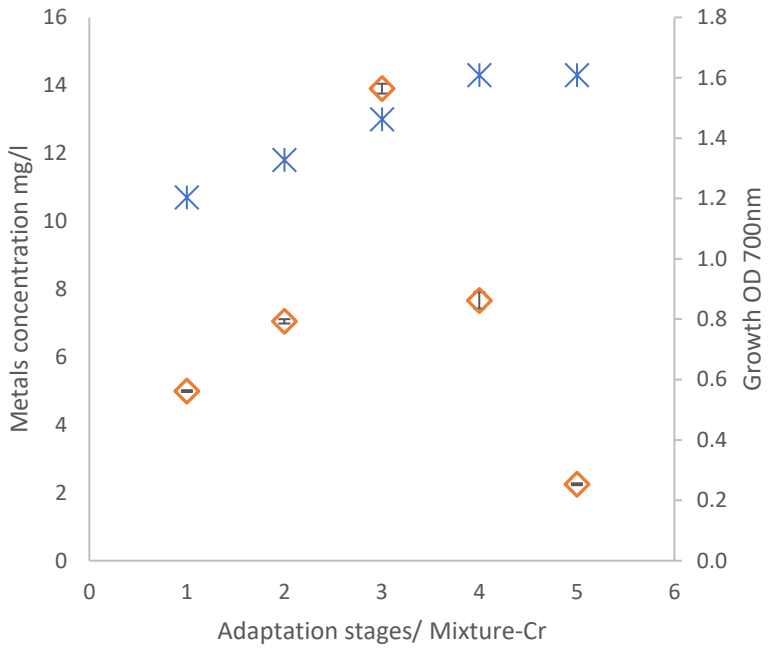

Figure 5.14. The adaptation stages of *C. reinhardtii* in 3NBBM with a mixture of metals, Cu, Zn, Ni, Hg, Co, and Cd, at different concentrations, 10.7 mg/l, 11.8 mg/l, 13 mg/l, 14.3 mg/l, and 14.3 mg/l for stage1 to 5, respectively: metal concentrations are presented on y-axis as mg/l (*), and microalgal growth is measured by optical density 700nm and represented on the z-axis (◊). The X-axis represents the adaptation stages over time, where each stage consists of ten generations and each generation last for seven days for a total adaptation period of 315 days. The growth of each stage is an average of generations of that stage.

5.2.8 A comparison between *Chlorella vulgaris* and *Chlamydomonas reinhardtii* in response to adaptation to heavy metals

The LC_{50} of zinc for *Chlorella vulgaris* was 25 mg/l, however, it increased to 30 mg/l by the end of the adaptation period, this means that the produced phenotype increased resistance to zinc by 20%. On the other hand, the LC_{50} of Zn for *Chlamydomonas reinhardtii* was 24.19 mg/l and then reached 33 mg/l at the end of the adaptation experiments. The phenotype produced was 37.5% more tolerant to zinc as a result (Table 5.3).

Table 5.3. A comparison between *Chlorella vulgaris* and *Chlamydomonas reinhardtii* in response to adaptation to heavy metals

Metal	Concentration before adaptation mg/l *Chlorella vulgaris*	Concentration after adaptation mg/l	The increase in the metal resistance	Concentration before adaptation mg/l *Chlamydomonas reinhardtii*	Concentration after adaptation mg/l	The increase in the metal resistance
Zn	25	30	20%	24.19	33	37.5%
Hg	0.22	0.3	50%	0.5	0.6	20%
Co	3.6	4.8	34%	5.9	7.7	20%
Cd	27.5	29	10%	4.2	5	19%
Cu	8.4	12	30%	0.3	0.5	50%
Ni	3.76	4.1	19%	5	5.5	10%
Mixture	15.08	19	30%	10.7	13	21%

The tolerance of *Chlorella vulgaris* phenotype to mercury was increased by 50% from 0.22 mg/l at the beginning of the adaptation to 0.3 mg/l by the end of the experiments. However, this rate was 20% in the case of *Chlamydomonas reinhardtii* when the concentration of Hg increased from 0.5 mg/l to 0.6 mg/l through the adaptation stages. The tolerance of *Chlorella vulgaris* phenotype to Co was increased by 34% from 3.6 mg/l at the beginning of the adaptation to 4.8 mg/l by the end of the adaptation experiments. However, this rate was 20% in the case of *Chlamydomonas reinhardtii* when the concentration of Cobalt increased from 5.6 mg/l to 7.7 mg/l through the adaptation stages. The increase of resistance to cadmium was 10% in the produced phenotype in comparison to the original strain of *C. vulgaris*. But the phenotype produced from adaptation experiments was more tolerant to Cd by 19% in the case of *C. reinhardtii.*

The LC_{50} of Cu for *C. vulgaris* was 8.4 mg/l, however, it was increased to 12 mg/l by the end of the adaptation period, this means that the produced phenotype increased resistance to Cu by 30%. On the other hand, the LC50 of copper for *C. reinhardtii* was 0.3 mg/l and then reached 0.5 mg/l at the end of the adaptation experiments. The phenotype produced was 50% more tolerant to Cu as a result. The increase of resistance to nickel was 19% in the produced

phenotype in comparison to the original strain of *C. vulgaris*. But the phenotype produced from adaptation experiments was more tolerant to Ni by 10% in the case of *C. reinhardtii.* The tolerance of *C. vulgaris* phenotype to the metal mixture was increased by 30% from 15.08 mg/l at the beginning of the adaptation to 19 mg/l by the end of the adaptation experiments. However, this rate was 21% in the case of *C. reinhardtii*.

5.3 Analysis of growth medium using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) for metal concentration before and after microalgal growth

In this part of the chapter, the removal of metal from the solution has been evaluated by filtering the growth medium used to grow the adapted phenotype at the last stage of adaptation. The filtered medium solution was analysed using ICP-OES, and the removal percentage was calculated for each metal-algal strain.

5.3.1 Analysis of growth medium using ICP-OES for metal removal by *Chlorella vulgaris*

During the last stage of adaptation, *C. vulgaris* bioactivity in cadmium solution significantly reduced the concentration of Cd from 32mg/l to 6.45mg/l (p <0.05). Figure 5.15 shows the level of cadmium in the solution before adding microalgae and the concentration of Cd after microalgal growth for one generation (7 days). The total removal of cadmium by *C. vulgaris* was 81.55%.

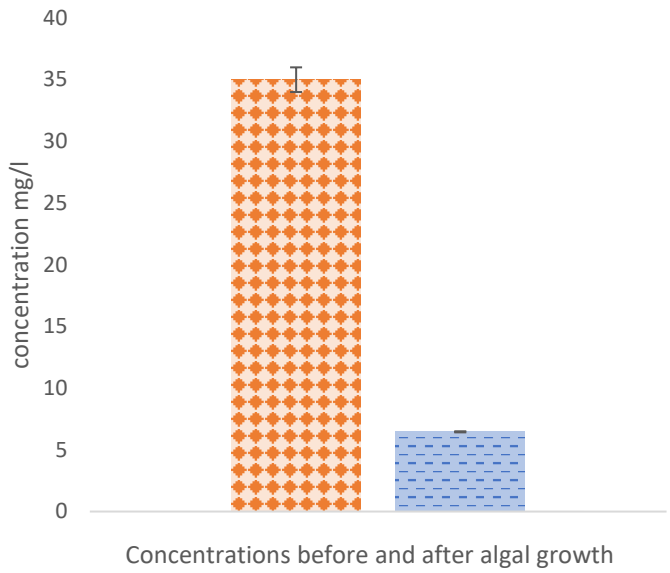

Figure 5.15. Cadmium concentration before and after *Chlorella vulgaris* bioactivity (♦) the original concentration of Cd solution mg/l. (▬) the concentration of Cd solution after microalgae bioactivity mg/l.

Cobalt solution in the last adaptation stage was 5.2mg/l; this was significantly reduced by the end of the incubation period of *C. vulgaris*, which was 7 days, in the final generation of the stage (p <0.05). The concentration of Co was 1.34mg/l after microalgal bioactivity (Figure 5.16). The total removal of cobalt by *C. vulgaris* was 74.23%.

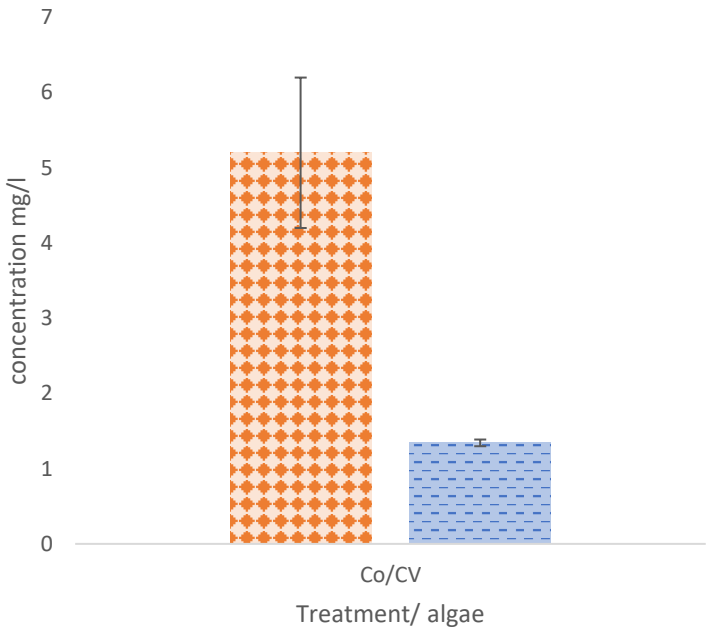

Figure 5.16. Cobalt concentration before and after *Chlorella vulgaris* bioactivity (◆) the original concentration of Co solution mg/l. (▬) the concentration of Co solution after microalgae bioactivity mg/l.

In the last stage of adaptation, *C. vulgaris* bioactivity in copper solution significantly decreased the concentration of Cu from 12mg/l to 1.55mg/l (p <0.05). Figure 5.17 demonstrates the variance of copper levels in the solution before adding microalgae and the concentration of Cu after microalgal growth for one generation (7 days). The total removal of Cu by *C. vulgaris* was 87.08%.

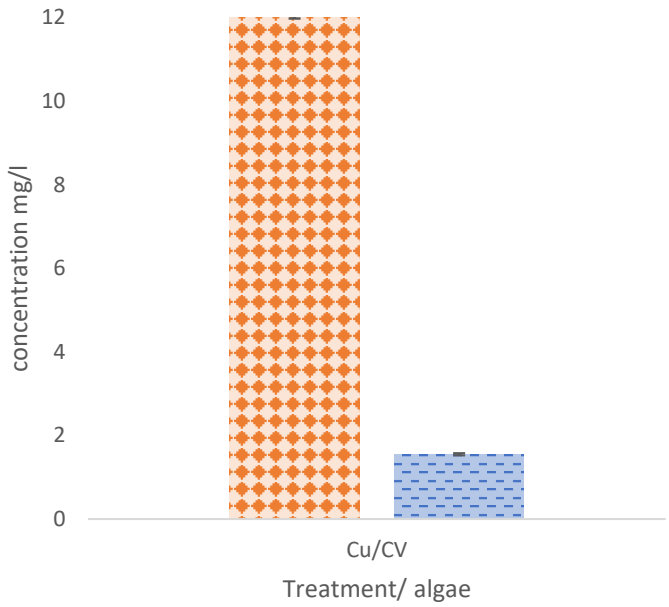

Figure 5.17. Copper concentration before and after *Chlorella vulgaris* bioactivity (♦) the original concentration of Cu solution mg/l. (▬) the concentration of Cu solution after microalgae bioactivity mg/l.

During the last stage of adaptation, *C. vulgaris* bioactivity in nickel solution significantly reduced the concentration of Ni from 4.4mg/l to 2.09mg/l (p <0.05). Figure 5.18 shows the Ni level in the solution before adding microalgae and the concentration of Ni after microalgal growth for one generation (7 days). The total removal of Ni by *C. vulgaris* was 52.73%.

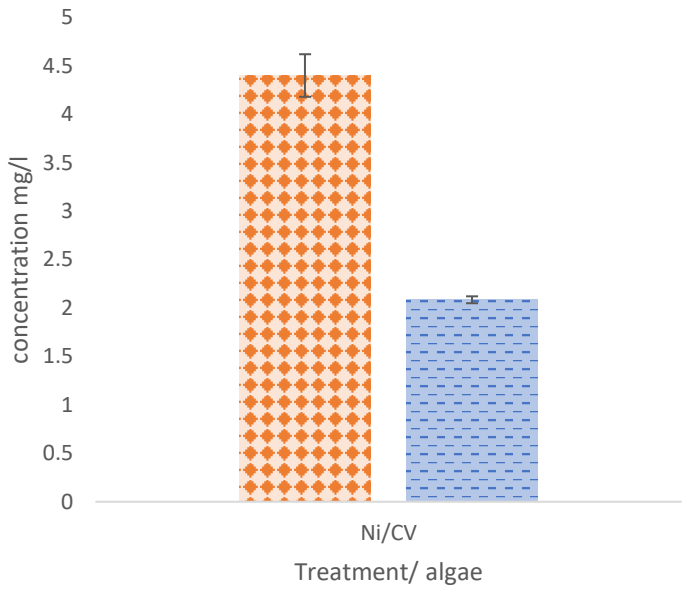

Figure 5.18. Nickel concentration before and after *Chlorella vulgaris* bioactivity (◆) the original concentration of Ni solution mg/l. (▬) the concentration of Ni solution after microalgae bioactivity mg/l.

Figure 5.19 shows the variance between zinc concentration in the solution before adding *C. vulgaris* and the concentration of Zn after microalgal growth for one generation (7 days). In the last stage of adaptation, *Chlorella vulgaris* bioactivity in zinc solution significantly decreased the concentration of Zn from 33mg/l to 4.19mg/l (p <0.05). The total removal of zinc by *C. vulgaris* was 87.27%.

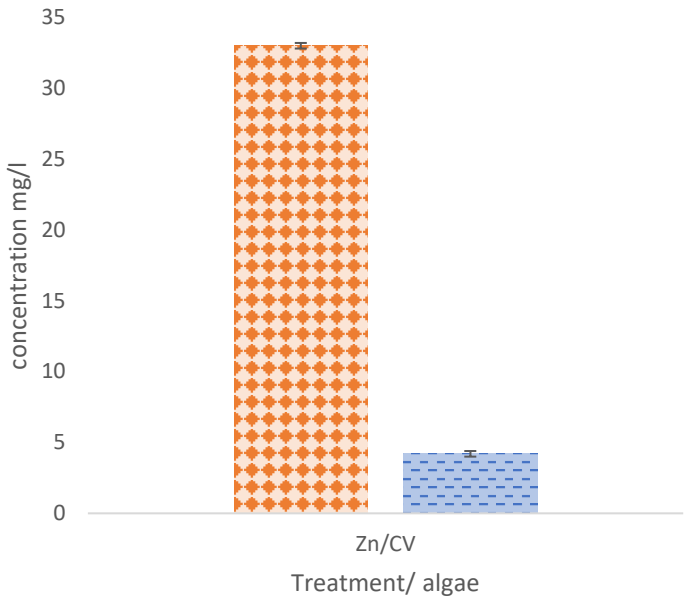

Figure 5.19. Zinc concentration before and after *Chlorella vulgaris* bioactivity (◆) the original concentration of Zn solution mg/l. (▬) the concentration of Zn solution after microalgae bioactivity mg/l.

C. vulgaris bioactivity in mercury solution significantly reduced the concentration of Hg from 0.5 mg/l to 0.06mg/l (p <0.05) in the last stage of adaptation. Figure 5.20 shows the difference in Hg level in the solution before adding microalgae and mercury concentration after microalgal growth for one generation (7 days). The total removal of Hg by *C. vulgaris* was 87.8%.

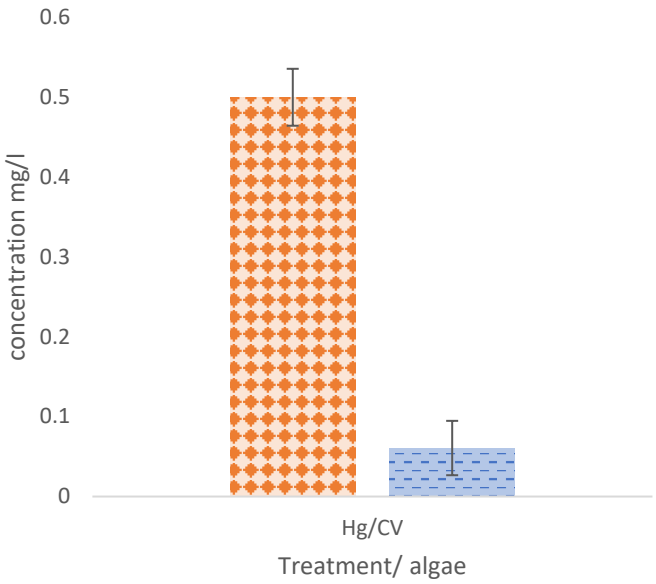

Figure 5.20. Mercury concentration before and after *Chlorella vulgaris* bioactivity (♦) the original concentration of Hg solution mg/l. (▬) the concentration of Hg solution after microalgae bioactivity mg/l.

Figure 5.21 shows the concentration of metals in the solution before adding *C. vulgaris* and the concentration of the metals mixture after microalgal growth for one generation (7 days). In the last stage of adaptation, *C. vulgaris* bioactivity in a mixture of metals solution significantly decreased zinc concentration from 3.66 mg/l to 1.59 mg/l (p <0.05). The same was observed for cobalt which reduced from 3.66 mg/l to 1.57 mg/l (p <0.05). A significant reduction in concentration of copper from 3.66 mg/l to 1.42 mg/l was also noticed (p <0.05). Microalgal bioactivity successfully reduced Ni, Hg, and Cd levels in the metals mix from 3.66 mg/l to 1.46mg/l, 2.08 mg/l, and 1.66 mg/l, respectively (p <0.05). The total removal of metals mixture by *C. vulgaris* was 55.54%. The removal of each metal in the mixture was: 56.56%, 57.1%, 61.2%, 60.1%, 43.2%, and 54.64% of Zn, Co, Cu, Ni, Hg, and Cd, respectively.

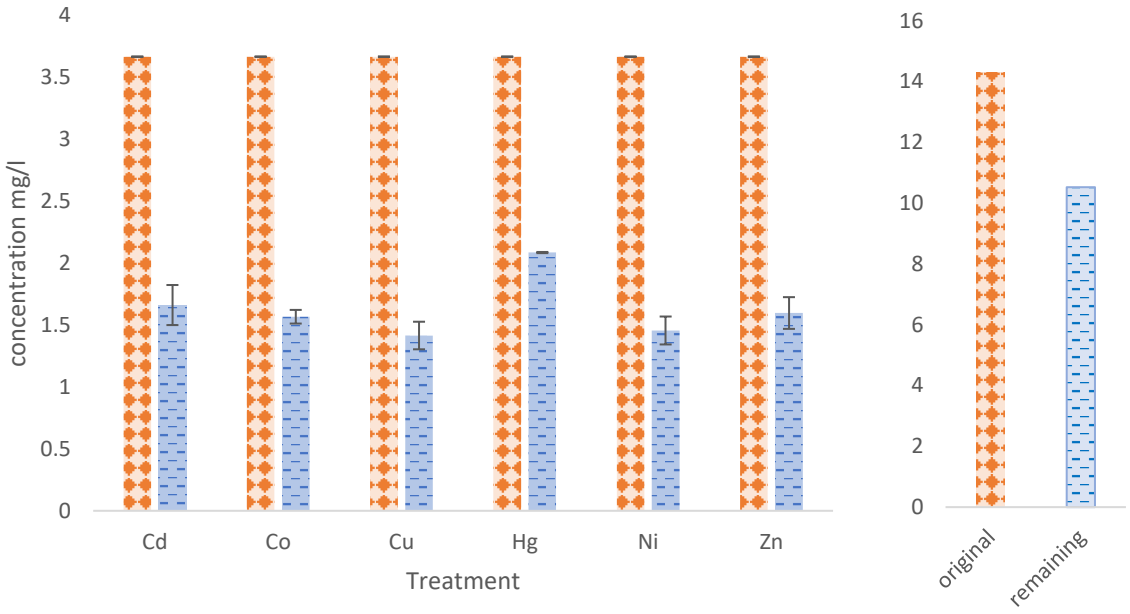

Figure 5.21. (a) Metals mixture concentration before and after *Chlorella vulgaris* bioactivity (♦) the original concentration of metals in the mixed solution mg/l. (▬) the concentration of metals in the mixed solution after microalgae bioactivity mg/l.(b) the sum of metals concentrations in the mixture before and after *Chlorella vulgaris* bioactivity.

5.3.2 Analysis of growth medium using ICP-OES for metal removal by *Chlamydomonas reinhardtii*

In the last stage of adaptation, bioactivity of *C. reinhardtii* in copper solution significantly decreased the concentration of Cu from 0.6 mg/l to 0.38 mg/l (p <0.05). Figure 5.22 demonstrates the variance of in the level of copper in the solution before adding microalgae and the concentration of Cu after microalgal growth for one generation (7 days). The total removal of copper by *C. reinhardtii* was 36.67%.

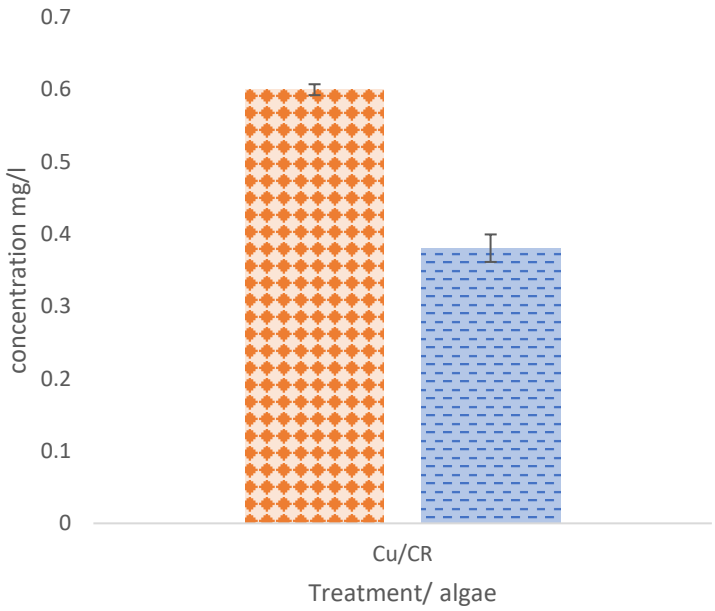

Figure 5.22. Copper concentration before and after *Chlamydomonas reinhardtii* bioactivity (♦) the original concentration of Cu solution mg/l. (▬) the concentration of cu solution after microalgae bioactivity mg/l.

During the last stage of adaptation, *C. reinhardtii* bioactivity in nickel solution significantly reduced the concentration of Ni from 6 mg/l to 0.61mg/l (p <0.05). Figure 5.22 shows the difference of Ni level in the solution before adding microalgae and the concentration of Ni after microalgal growth for one generation (7 days). The total removal of nickel by *C. reinhardtii* was 90%.

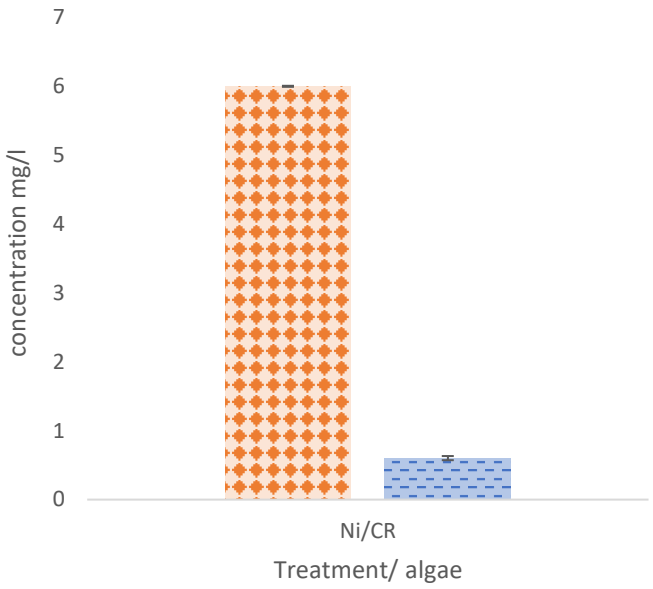

Figure 5.23. Nickel concentration before and after *Chlamydomonas reinhardtii* bioactivity (◆) the original concentration of Ni solution mg/l. (▬) the concentration of Ni solution after microalgae bioactivity mg/l.

Figure 5.24 shows the concentration (+/- s.d.) of zinc in the solution before and after adding *C. reinhardtii* microalgal growth for one generation (7 days). In the last stage of adaptation, *C. reinhardtii* bioactivity in zinc solution significantly decreased the concentration of Zn from 33mg/l to 3.36mg/l (p <0.05). The total removal of Zn by *C. reinhardtii* was 89.82%.

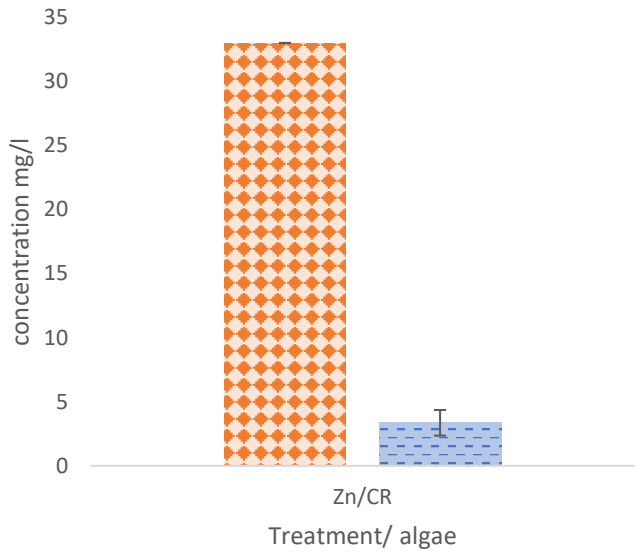

Figure 5.24. Zinc concentration before and after *Chlamydomonas reinhardtii* bioactivity (♦) the original concentration of Zn solution mg/l. (▬) the concentration of Zn solution after microalgae bioactivity mg/l.

Figure 5.25 shows the difference of cadmium level in the solution before adding *C. reinhardtii* and Cd concentration after microalgae growth for one generation (7 days). In the last stage of adaptation, Microalgal bioactivity in cadmium solution significantly reduced the concentration of Cd from 5.5mg/l to 1.34mg/l (p <0.05). The total removal of Cd by *C. reinhardtii* was 75.64%.

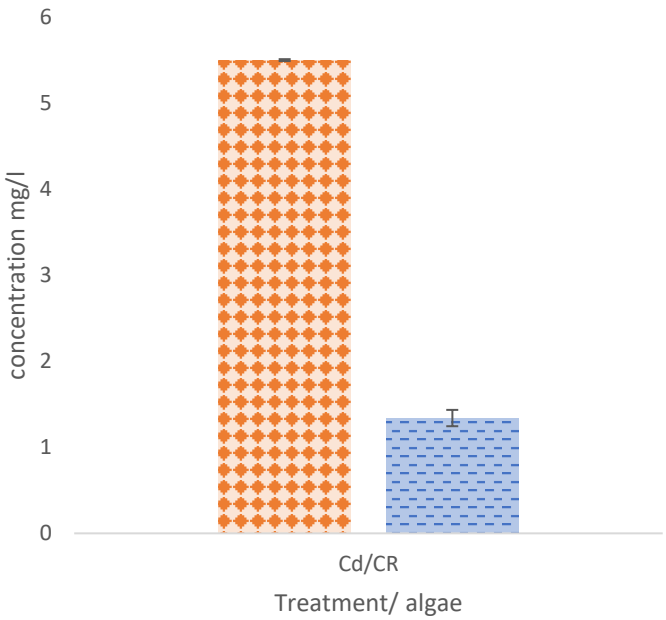

Figure 5.25. Cadmium concentration before and after *Chlamydomonas reinhardtii* bioactivity (♦) the original concentration of Cd solution mg/l. (▬) the concentration of Cd solution after microalgae bioactivity mg/l.

Mercury solution in the last adaptation stage was 0.7mg/l; this was significantly reduced by the end of the incubation period of *C. reinhardtii* which is 7 days, in the final generation of the stage (p <0.05). The concentration of Hg was 0.45mg/l after microalgal bioactivity (Figure 5.26). The total removal of mercury by *C. reinhardtii* was 35%.

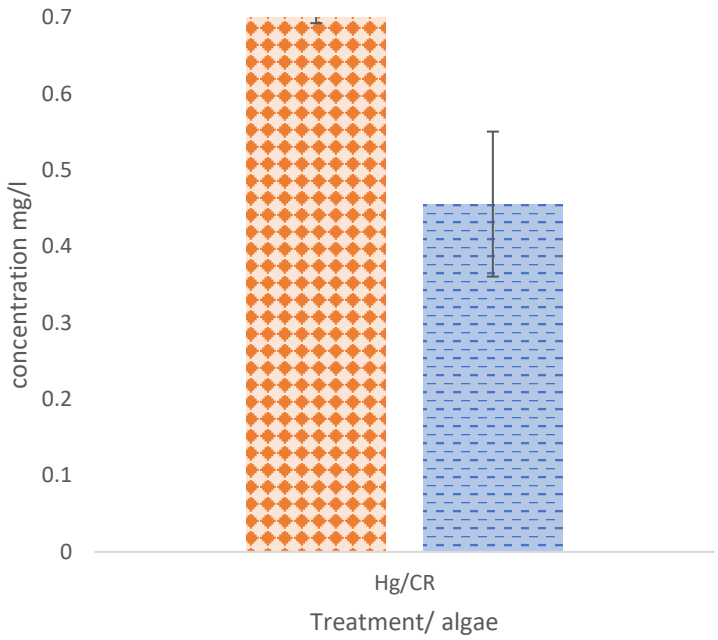

Figure 5.26. Mercury concentration before and after *Chlamydomonas reinhardtii* bioactivity (♦) the original concentration of Hg solution mg/l. (▬) the concentration of Hg solution after microalgae bioactivity mg/l.

C. reinhardtii bioactivity in cobalt solution significantly reduced the concentration of Co from 7.8 mg/l to 5.73 mg/l (p <0.05) in the last stage of adaptation. Figure 5.27 shows the difference of Co level in the solution before adding *C. reinhardtii* and the concentration of cobalt after microalgal growth for one generation (7 days). The total removal of cobalt by *C. reinhardtii* was 26.54%.

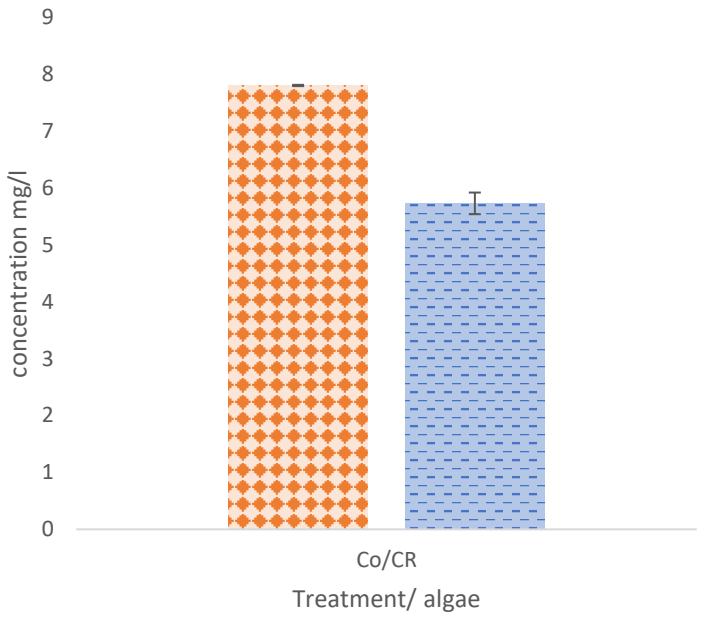

Figure 5.27. Cobalt concentration before and after *Chlamydomonas reinhardtii* bioactivity (◆) the original concentration of Co solution mg/l. (▬) the concentration of Co solution after microalgae bioactivity mg/l.

Figure 5.28 shows the variance of metals concentrations in the solution before adding *C. reinhardtii* and the concentration of the metals mixture after microalgal growth for one generation (7 days). In the last stage of adaptation, *C. reinhardtii* bioactivity in a mixture of metals solution significantly reduced zinc concentration from 2.38 mg/l to 1.75mg/l (p <0.05). Similarly, cobalt decreased from 2.38 mg/l to 1.69mg/l (p <0.05). A significant reduction was also noticed in copper concentration from 2.38 mg/l to 1.37mg/l (p <0.05). *C. reinhardtii* bioactivity successfully reduced Ni and Cd levels in the metals mix from 2.38 mg/l to 1.53mg/l and 1.9mg/l, respectively (p <0.05). On the other hand, mercury levels did not significantly differ before and after microalgae bioactivity in the mixed metal solution. The total removal of metals mixture by *C. reinhardtii* was 26.43%. The removal of each metal in the mixture was: 26.47%, 28.99%, 42.44%, 35.71%, 4.2%, and 20.17% of Zn, Co, Cu, Ni, Hg, and Cd, respectively.

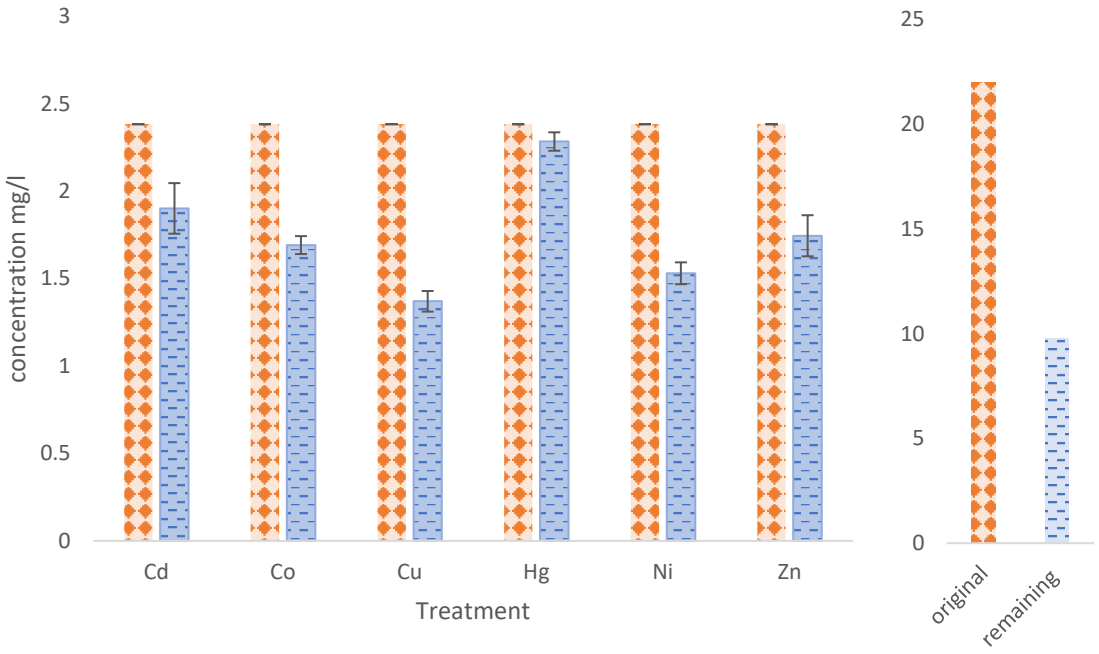

Figure 5.28. (a) Metals' mixture concentration before and after *Chlamydomonas reinhardtii* bioactivity (◆) the original concentration of metals in the mixed solution mg/l. (▬) the concentration of metals in the mixed solution after microalgae bioactivity mg/l. (b) the sum of metals concentrations in the mixture before and after *Chlamydomonas reinhardtii* bioactivity.

5.4 Fourier transform infrared (FTIR) spectroscopy of microalgae

5.4.1 Fourier transform infrared (FTIR) spectroscopy of *Chlorella vulgaris*

General identification of absorption bands was obtained from published information (Benning *et al.* 2004; Giordano *et al.* 2001; Keller,1986; Naumann *et al.* 1996; Stuart, 1997; Wong *et al.* 1991; Dean *et al.* 2007; Sigee *et al.* 2002; Maquelin and Kirchner, 2002). In addition, supportive information for band assignment of protein, nucleic acid, soluble carbohydrate, and fatty acids were obtained from Sigee *et al.* (2002). Table 5.4 contains the band assignments, which were issued FTIR spectra to a specific functional group. Band contributions were as follows: residual water (band 1), proteins (bands 1, 4, 5, 6 and 7), lipids

(bands 2, 3, 6 and 7), carbohydrate (bands 2, 9, 10, 11), cellulose (band 3), and nucleic acids (bands 8, 10).

Each peak in the scans was assigned a functional group. Protein spectra were characterized by a peak at 3270 cm^{-1} (amide A); lipid spectra were categorized by three sets of strong vibrations, the C-H at 2974 cm^{-1}, 2907 cm^{-1}, and 2897 cm^{-1}; protein also featured at 1645 cm^{-1} (amide I) and 1552 (amide II). Position 1380 cm^{-1} represent protein and lipids bending of methyl. Band of position 1242 cm^{-1} stands for nucleic acids. Carbohydrate absorption bands due to C-O-C of polysaccharides found at 1148 cm^{-1}, 1045 cm^{-1} up to 883 cm^{-1}.

Table 5.4 General band assignment of FTIR spectrum of *Chlorella vulgaris*

Band	Main peak (cm^{-1})	Typical band assignment from the literature	Wavenumber range (cm^{-1})
1	3327	Water v(O-H) stretching Protein v(N-H) stretching (amide A)	3029-3639
2	2928	Lipid – carbohydrate Mainly vas(CH2) and vs(CH2) stretching	2809-3012
3	1737	Cellulose–Fatty Acids v(C=O) stretching of esters	1763-1712
4	1649	Protein amide I band Mainly v(C=O) stretching	1583-1709
5	1543	Protein amide II band mainly δ(N-H) bending and v(C-N) stretching	1481-1585
6	1452	Protein δas(CH2) and δas(CH3) bending of methyl, Lipid δas(CH2) bending of methyl	1425-1477
7	1389	Protein δs(CH2) and δs(CH3) bending of methyl Carboxylic Acid vs(C-O) of COOgroups of carboxylates Lipid δs(N(CH3)3) bending of methyl	1357-1423
8	1248	Nucleic Acid (other phosphate-containing compounds) vas(>P=O) stretching of phosphodiesters	1191-1356
9	1149	Carbohydrate v(C-O-C) of Polysaccharides	1134-1174
10	1082	Carbohydrate v(C-O-C) of polysaccharides Nucleic Acid (and other phosphate-containing compounds) vs(>P=O) stretching of phosphodiesters	1072-1099
11	1036	Carbohydrate v(C-O-C) of polysaccharides	880-1072

Band assignment based on Benning et al. (2004), Giordano et al. (2001), Keller (1986), Naumann et al. (1996), Stuart (1997), Wong et al. (1991), Dean et al. (2007), Sigee et al. (2002), Maquelin and Kirchner (2002). FTIR, Fourier transform infrared. 3270

The biological print of *Chlorella vulgaris* strain was observed using FTIR before and after exposure to each metal used in the study. Variations in peak positions upon exposure to

specific stress, (in this case, metals) are essential to obtain valuable structural and functional information about the studied system.

Figure 5.29 shows the cell's molecular groups of *Chlorella vulgaris*, a phenotype obtained from exposure to cadmium during the adaptation experiment. The figure shows a clear shift in the absorbance of some bands, indicating the changes occurred due to the presence of cadmium on microalgae.

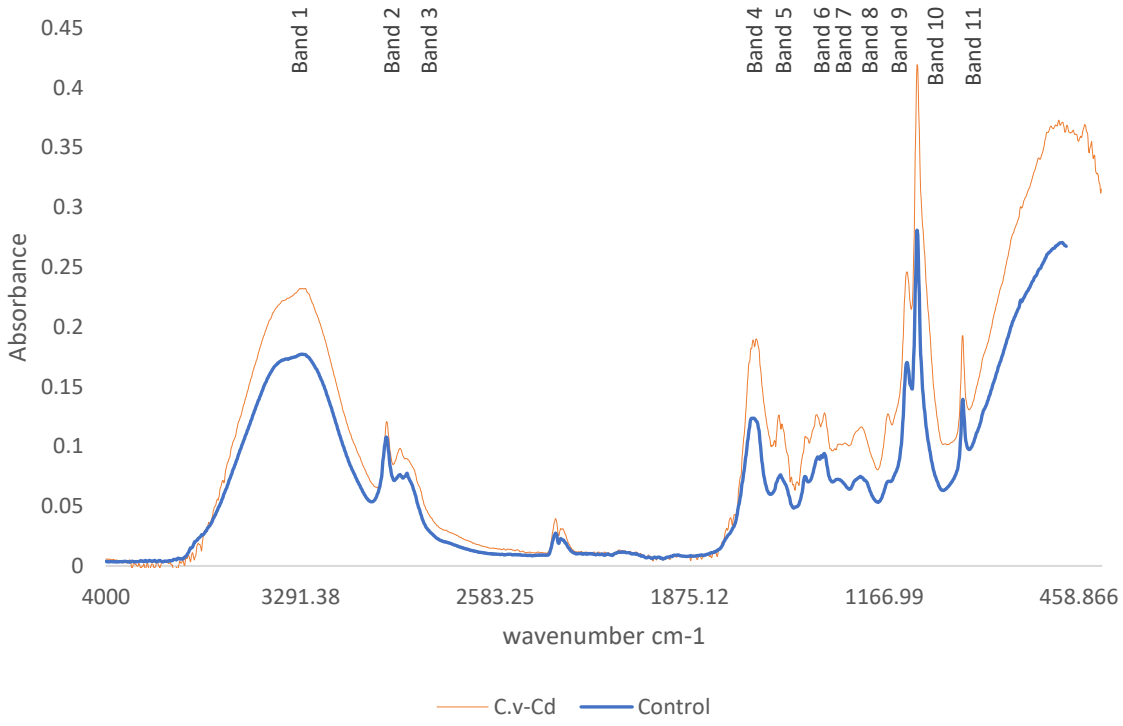

Figure 5.29. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)*, the control, and *Chlorella vulgaris* phenotype obtained from exposure to cadmium (C.v-Cd). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Band assignment is presented in Table 5; the bands 1 to 11 have been listed with the absorbance data for *Chlorella vulgaris* before adaptation (control) and the phenotype

obtained after adaptation at the accompanying position. The significance of difference is also included in the table at both 99% and 95% probabilities.

Table 5.5 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. vulgaris* before	*C. vulgaris* After	N	Position	Significancy
1	0.176	0.231	30	3270	n
2	0.105	0.113	30	2974	*
3	0.075	0.082	30	2907	**
4	0.074	0.188	30	2897	**
5	0.124	0.185	30	1645	n
6	0.073	0.122	30	1552	n
7	0.093	0.127	30	1380	n
8	0.074	0.114	30	1242	n
9	0.071	0.126	30	1148	n
10	0.281	0.419	30	1045	n
11	0.126	0.178	30	883	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

There was a significant difference between the control and the Cd-adapted phenotype of *Chlorella vulgaris* on band 2 (p <0.05). Likewise, bands 3 and 4 showed a significant difference in their absorbance (p <0.01). All other bands: 1, 5, 6, 7, 8, 9, 10 and 11 did not present a significant difference from the control (p >0.05).

The *Chlorella vulgaris* phenotype produced from the zinc adaptation experiment was scanned, and FTIR spectra were obtained and shown in Figure 5.30. Comparison to the control was also conducted to find the difference in the main peaks absorbance that reflects

the changes that might have occurred during adaptation. Each peak in the scans represents one of the cell's molecular groups.

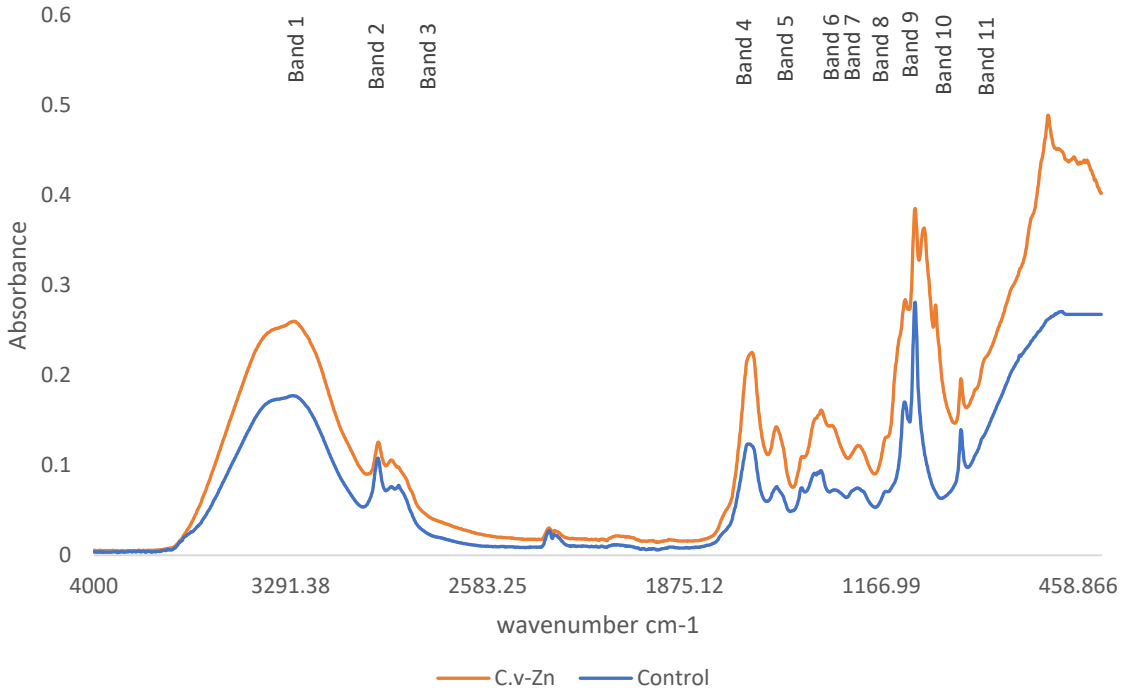

Figure 5.30. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)* , the control, and *Chlorella vulgaris* phenotype obtained from exposure to zinc (C.v-Zn). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Table 6 presents the bands assignment of the spectra attained from *Chlorella vulgaris* -Zn phenotype, in comparison to the control. bands 1 to 11 have been listed with the accompanying position of each band. The significance of difference is also comprised in the table at both 99% and 95% probabilities.

Table 5.6 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. vulgaris* before	*C. vulgaris* After	N	Position	Significancy
1	0.176	0.257	30	3270	n
2	0.105	0.125	30	2974	*
3	0.075	0.097	30	2907	**
4	0.074	0.105	30	2897	**
5	0.124	0.218	30	1645	*
6	0.073	0.135	30	1552	n
7	0.093	0.161	30	1380	**
8	0.074	0.121	30	1242	n
9	0.071	0.131	30	1148	**
10	0.281	0.382	30	1045	*
11	0.126	0.178	30	883	**

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Bands 2, 5, and 10 showed a significant difference from the control (p <0.05). Similarly, bands 3, 4, 7, 9, and 11 were significantly different compared to the control (p <0.01). On the other hand, 1, 6, and 8 did not differ from the original strain.

Cobalt-adaptation impact on the cell structure as a function of FTIR spectra is shown in Figure 5.31. Bands 1 to 11 representing the molecular groups of algal cells of both the control and the treated strain, are clearly illustrated.

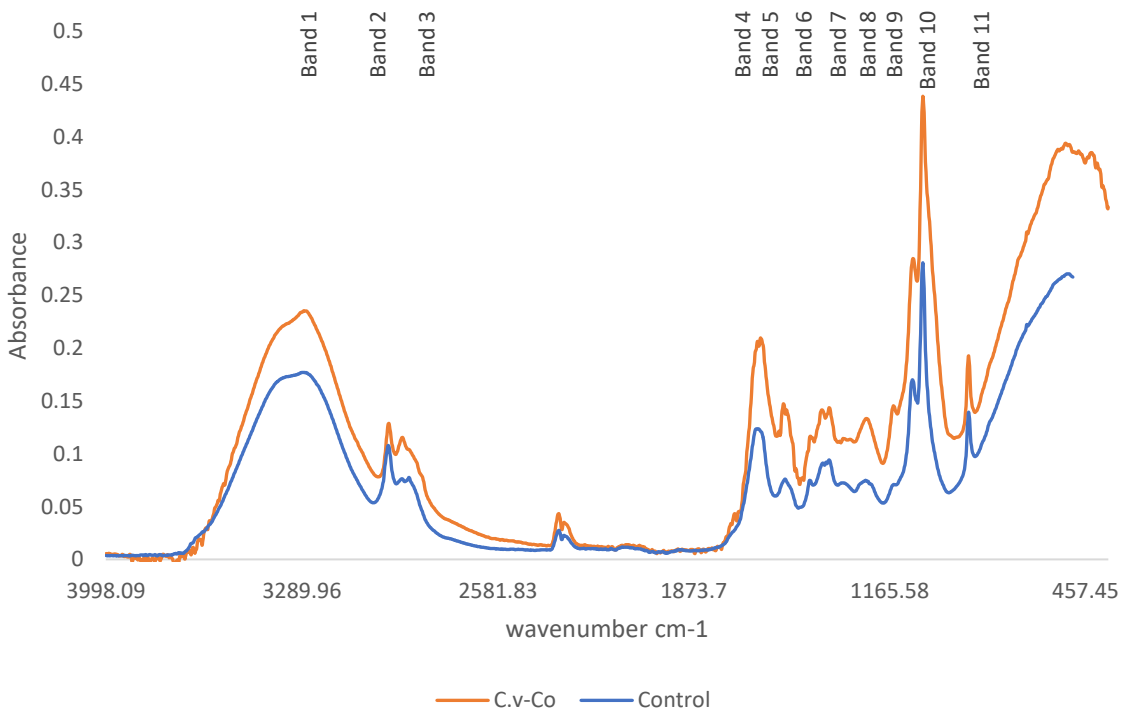

Figure 5.31. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)*, the control, and *Chlorella vulgaris* phenotype obtained from exposure to cobalt (C.v-Co). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Assignment of each band is shown in Table 7; the bands 1 to 11 have been listed with the absorbance data for *Chlorella vulgaris* before adaptation (control) and the phenotype obtained after adaptation to cobalt. Bands 2, 6, 7, 8, and 9 showed a significant difference from the control (p <0.05). Similarly, bands 3, 4, and 10 were significantly different compared to the control (p <0.01). On the other hand, 1, 5, and 11 did not differ from the control (p >0.05).

Table 5.7 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. vulgaris* before	*C. vulgaris* After	N	Position	Significancy
1	0.176	0.235	30	3270	n
2	0.105	0.129	30	2974	*
3	0.075	0.105	30	2907	**
4	0.074	0.103	30	2897	**
5	0.124	0.199	30	1645	n
6	0.073	0.140	30	1552	*
7	0.093	0.144	30	1380	*
8	0.074	0.133	30	1242	*
9	0.071	0.145	30	1148	*
10	0.281	0.434	30	1045	**
11	0.126	0.171	30	883	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Figure 5.32 demonstrates the cell's functional groups of *Chlorella vulgaris*, a phenotype obtained from exposure to mercury during adaptation attempt, compared to the control.

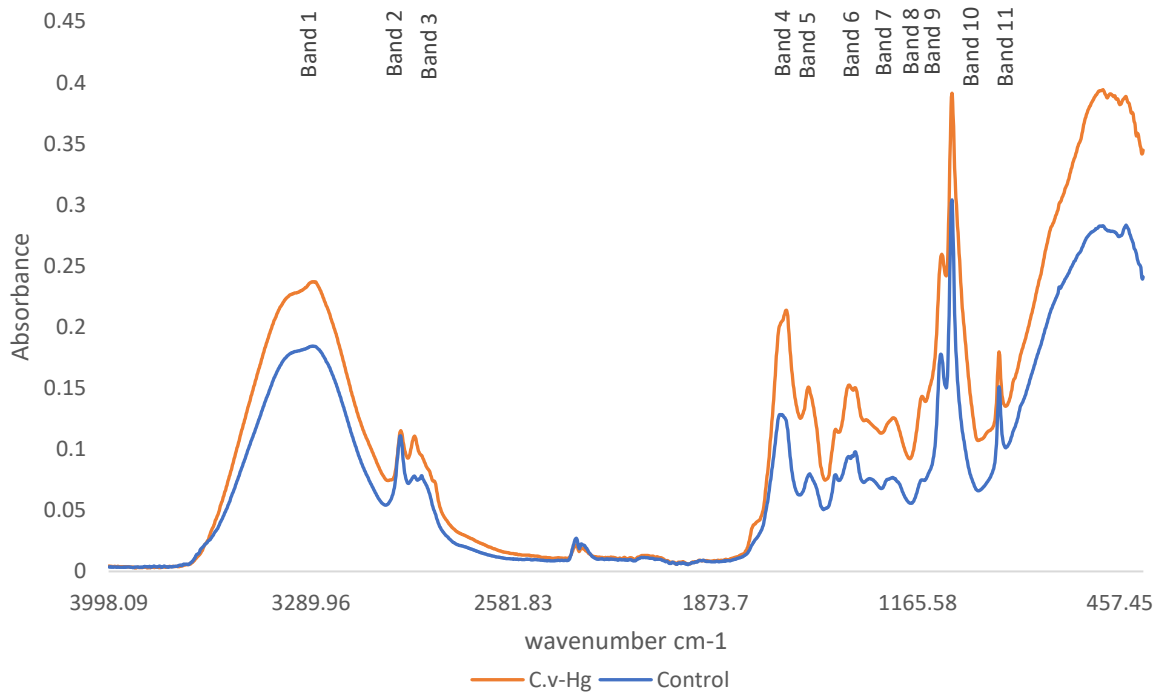

Figure 5.32. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)*, the control, and *Chlorella vulgaris* phenotype obtained from exposure to mercury (C.v-Hg). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

The absorbance of some bands shows a clear effect on the amount of some functional groups in the cell, indicating the changes occurred due to the stress applied on microalgae by mercury.

Table 8 shows the assignment of the spectra achieved from FTIR scans to *Chlorella vulgaris* -Hg phenotype and the control (original strain). bands 1 to 11 have been listed with the associated position of each band. The significance of difference is also covered in the table at both 99% and 95% probabilities.

Table 5.8 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. vulgaris* before	*C. vulgaris* After	N	Position	Significancy
1	0.176	0.236	30	3270	n
2	0.105	0.115	30	2974	n
3	0.075	0.101	30	2907	**
4	0.074	0.093	30	2897	*
5	0.124	0.202	30	1645	*
6	0.073	0.147	30	1552	*
7	0.093	0.15	30	1380	**
8	0.074	0.125	30	1242	n
9	0.071	0.143	30	1148	**
10	0.281	0.333	30	1045	*
11	0.126	0.160	30	883	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

There was a significant difference between the control and the phenotype of *Chlorella vulgaris* that exposed to Hg in some bands, such as band 4, 5, 6, and 10 (p <0.05). Likewise, bands 3, 7, and 9 showed a significant difference in their absorbance (p <0.01). On the other hand, band 1, 2, 8, and 11 did not show a significant difference from the control.

The produced phenotype of *Chlorella vulgaris* obtained from the copper adaptation experiment was scanned, and FTIR spectra were originated, processed, and shown in Figure 5.33. Comparison to the control was also made to find the difference in the main peaks absorbance that reflects the changes that might have occurred during adaptation. Each peak in the scans represents one or more of the cell's molecular groups.

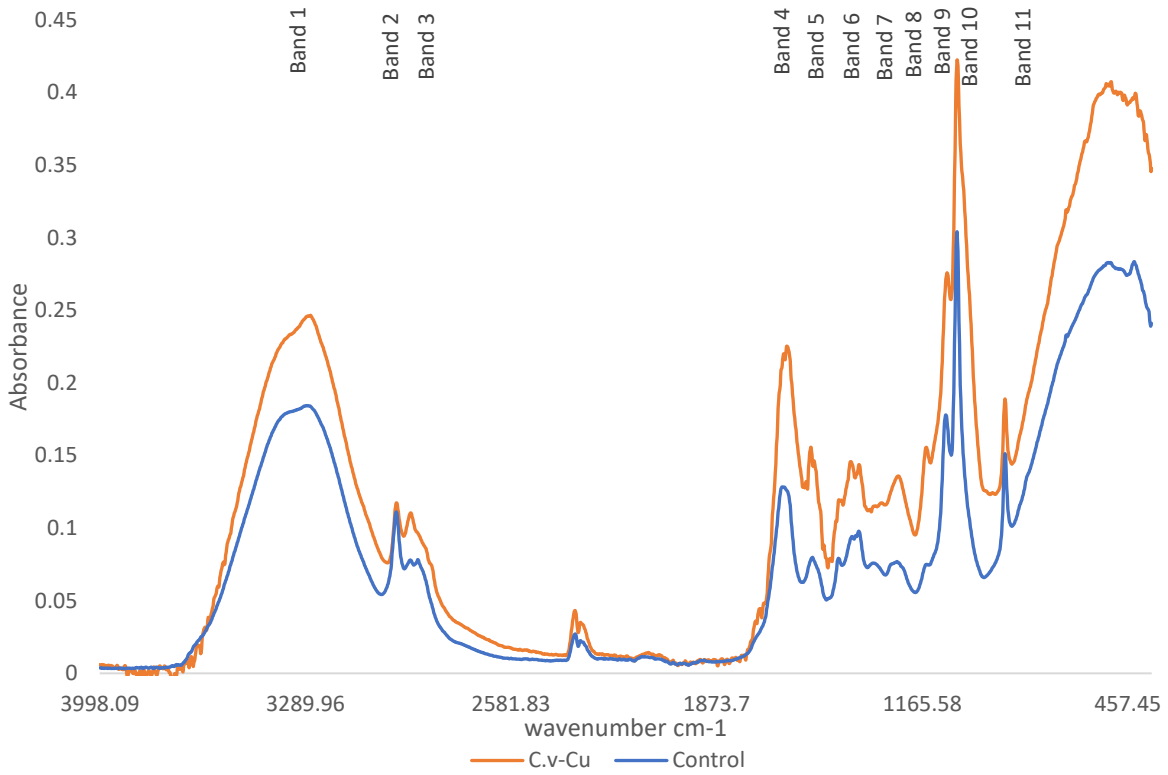

Figure 5.33. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)*, the control, and *Chlorella vulgaris* phenotype obtained from exposure to copper (C.v-Cu). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Assignment of each band is shown in Table 8. bands have been listed with the absorbance data for *Chlorella vulgaris* before adaptation (control) and the phenotype obtained after adaptation to copper. Bands 6 and 7 showed a significant difference from the control (p <0.05). Alike, bands 3, 4, 8, 9, and 10 were significantly different compared to the control (p <0.01). While bands 1, 2, 5, and 11 did not differ from the original strain.

Table 5.9 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. vulgaris* before	*C. vulgaris* After	N	Position	Significancy
1	0.176	0.261	30	3270	n
2	0.105	0.117	30	2974	n
3	0.075	0.098	30	2907	**
4	0.074	0.095	30	2897	**
5	0.124	0.212	30	1645	n
6	0.073	0.149	30	1552	*
7	0.093	0.117	30	1380	*
8	0.074	0.127	30	1242	**
9	0.071	0.139	30	1148	**
10	0.281	0.389	30	1045	**
11	0.126	0.160	30	883	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

The structure of *Chlorella vulgaris* shaped after adaptation to heavy metals mixture consisting of Cd, Co, Cu, Zn, Ni, and Hg at an equal concentration of each, is presented in Figure 5.34 as a function of FTIR spectra. The collected spectra were then compared to the molecular content of the original strain to find the difference in the cell structure associated with the adaptation process.

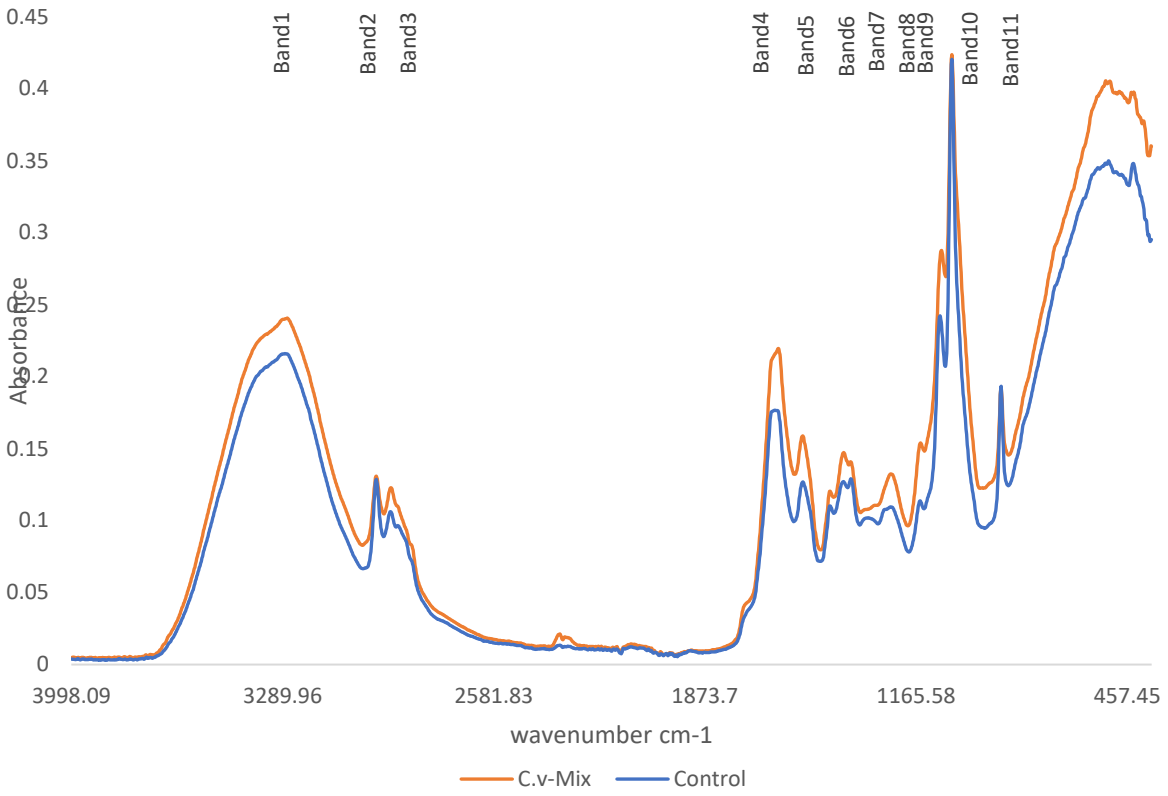

Figure 5.34. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)*, the control, and *Chlorella vulgaris* phenotype obtained from exposure to heavy metals mix (C.v-Mix). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Table 10 illustrates the assignment of the spectra accomplished from FTIR scans to *Chlorella vulgaris* phenotype that exposed to a mixture of heavy metals and the control. bands count of 11 have been included with the associated position of each band. The significance of difference is also listed in the table.

There was a significant difference between the control and the phenotype of *Chlorella vulgaris* that exposed to the metals mixture in band 11 (p <0.05). Likewise, bands 3 and 4 displayed a significant difference in their absorbance (p <0.01). Other bands: 1, 2, 5, 6, 7, 8, 9, and 10 did not show any difference from the control(p >0.05).

Table 5.10 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. vulgaris* before	*C. vulgaris* After	N	Position	Significancy
1	0.176	0.240	30	3270	n
2	0.105	0.131	30	2974	n
3	0.075	0.111	30	2907	**
4	0.074	0.108	30	2897	**
5	0.124	0.124	30	1645	n
6	0.073	0.107	30	1552	n
7	0.093	0.177	30	1380	n
8	0.074	0.126	30	1242	n
9	0.071	0.135	30	1148	*
10	0.281	0.359	30	1045	**
11	0.126	0.148	30	883	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

During the adaptation process, nickel introduction to microalgae strain generated a phenotype that differs from the original strain in its molecular structure. This change is shown in Figure 5.35. The control strain of *Chlorella vulgaris* and the obtained phenotype were scanned with FTIR to monitor any alteration in the cell functional groups.

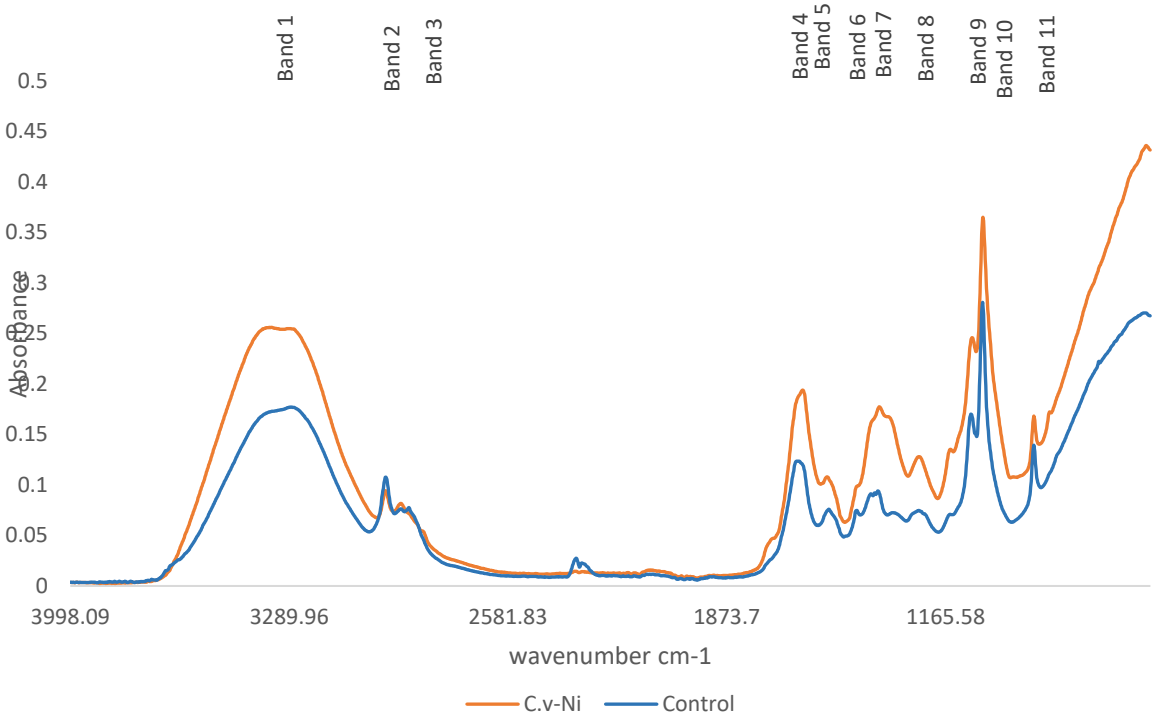

Figure 5.35. FTIR spectra of *Chlorella vulgaris* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlorella vulgaris)*, the control, and *Chlorella vulgaris* phenotype obtained from exposure to nickel (C.v-Ni). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Analysis of the spectra produced from FTIR for both the control and the treated strain was made to find the sign of alteration in cell structure. The results are revealed in Table 11.

Table 5.11 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. vulgaris* before	*C. vulgaris* After	N	Position	Significancy
1	0.176	0.253	30	3270	*
2	0.105	0.107	30	2974	n
3	0.075	0.075	30	2907	n
4	0.074	0.108	30	2897	n
5	0.124	0.218	30	1645	n
6	0.073	0.135	30	1552	n
7	0.093	0.161	30	1380	**
8	0.074	0.121	30	1242	n
9	0.071	0.131	30	1148	**
10	0.281	0.382	30	1045	n
11	0.126	0.178	30	883	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Band 1, that related to protein (amide A), Significantly showed much absorbance in the obtained phenotype than in the control. This outcome suggests that the presence of nickel induced the production of that protein group (p <0.05). Bands 2, 3, 4, 5, 6, 8, 10, and 11 did not substantially vary from the control. On the other hand, bands 7 and 9 related to protein and lipids bending of methyl, and carbohydrates, respectively, displayed a very significant variance in their absorption intensities from the control (P <0.01).

5.4.2 Fourier transform infrared (FTIR) spectroscopy of *Chlamydomonas reinhardtii*

Published information obtained from Coates (2006), Bednarz, *et al*. (2004), Andrew, *et al*. (2010), Benning *et al*. (2004), Giordano *et al.* (2001) Keller (1986), Naumann *et al.* (1996), Stuart (1997), Wong *et al.* (1991), Dean *et al.* (2007), Sigee *et al.* (2002), Maquelin and Kirchner (2002) was used for general identification of spectral absorption bands of *Chlamydomonas reinhardtii*. In addition, analysis of protein, nucleic acid, soluble carbohydrate, and fatty acids, were made by Sigee *et al*. (2002) using pure biochemical standards, to obtain supportive information on band assignment. These bands were issued FTIR spectra to a specific functional group (Table 11). Band contributions were as follows: residual water (band 1), proteins (bands 1, 4, 5, 6 and 7), lipids (bands 2, 3, 6 and 7), carbohydrate (bands 2, 9, 10, 11), cellulose (band 3), and nucleic acids (bands 8, 10).

Peaks in the scans were assigned a functional group per each. Protein spectra were categorised by a clear feature at 3280 cm-1 (amide A). Three sets of strong vibrations, 2975 cm-1, 2927 cm-1, and 2900 cm-1 were for lipids spectra. Protein also presented at 1641 cm-1 (amide I) and 1546 (amide II). Position 1380 cm-1 represent protein and lipids bending of methyl. Band of position 1271 cm-1 stands for nucleic acids. Due to the C-O-C of polysaccharides, carbohydrate absorption bands were found at 1148 cm-1, 1045 cm-1 up to 878 cm-1.

Table 5.12 General band assignment of FTIR spectrum of *Chlamydomonas reinhardtii*

Band	Main peak (cm^{-1})	Typical band assignment from the literature	Wavenumber range (cm^{-1})
1	3327	Water v(O-H) stretching Protein v(N-H) stretching (amide A)	3200-3400
2	2960	Lipid – carbohydrate Mainly vas(CH2) and vs(CH2) stretching	2809-3012
3	1745	Cellulose–Fatty Acids v(C=O) stretching of esters	1763-1712
4	1655	Protein amide I band Mainly v(C=O) stretching	1583-1709
5	1545	Protein amide II band mainly δ(N-H) bending and v(C-N) stretching	1481-1585
6	1455	Protein δas(CH2) and δas(CH3) bending of methyl, Lipid δas(CH2) bending of methyl	1425-1477
7	1390	Protein δs(CH2) and δs(CH3) bending of methyl Carboxylic Acid vs(C-O) of COOgroups of carboxylates Lipid δs(N(CH3)3) bending of methyl	1357-1423
8	1240	Nucleic Acid (other phosphate-containing compounds) vas(>P=O) stretching of phosphodiesters	1191-1356
9	1150	Carbohydrate v(C-O-C) of Polysaccharides	1134-1174
10	1082	Carbohydrate v(C-O-C) of polysaccharides Nucleic Acid (and other phosphate-containing compounds) vs(>P=O) stretching of phosphodiesters	1072-1099
11	1075	Carbohydrate v(C-O-C) of polysaccharides	800-1075

Band assignment based on Coates (2006), Bednarz, et al. (2004), Andrew, et al. (2010), Benning et al. (2004), Giordano et al. (2001), Keller (1986), Naumann et al. (1996), Stuart (1997), Wong et al. (1991), Dean et al. (2007), Sigee et al. (2002), Maquelin and Kirchner (2002). FTIR, Fourier transform infrared.

Biomarkers of *Chlamydomonas reinhardtii* strain have been observed using FTIR before and after contact to each metal used in the study to evaluate absorbance intensity changes upon exposure to specific stress as an essential mean to obtain valuable structural and functional information about the studied system.

Figure 5.36 demonstrates the patterns of FTIR spectra for both control and phenotype treated with cadmium throughout the acclimation process. Eleven bands were selected to compare the two strains to find the impact that might have occurred on the produced strain due to the stress applied by the added metal. Both strains did not vary substantially at most of the bands.

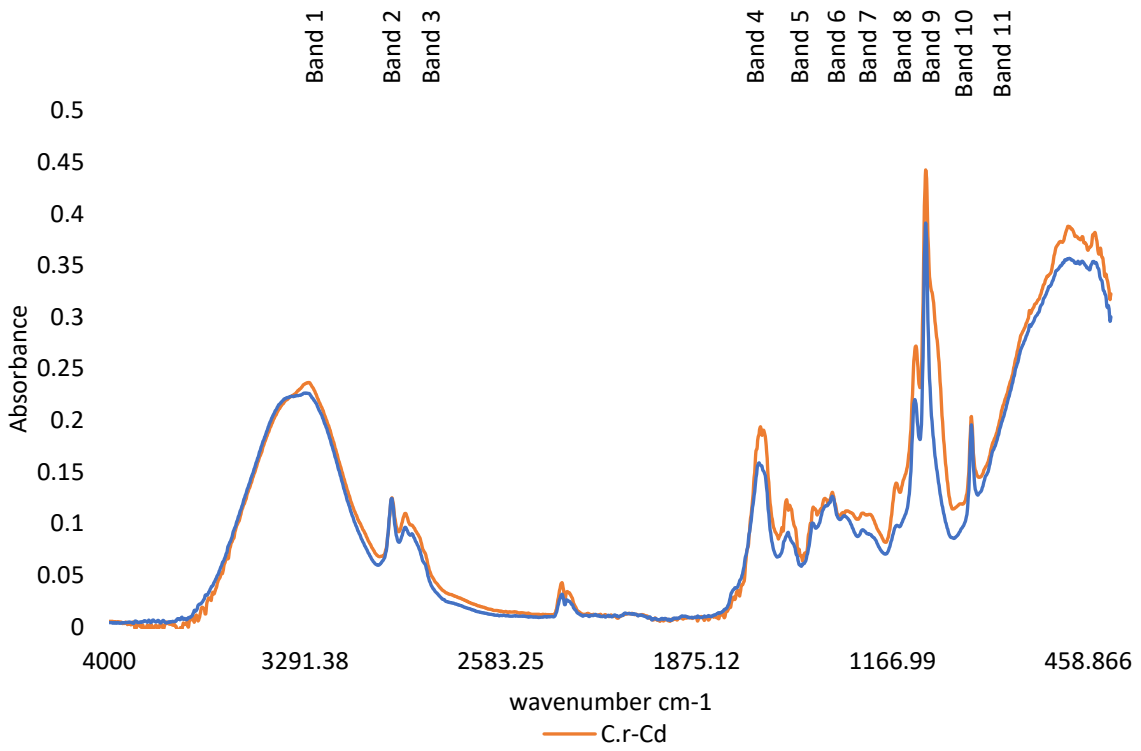

Figure 5.36. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to cadmium (C.r-Cd). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Table 13 lists the absorbance values of *C. reinhardtii* before and after acclimation for each peak and shows the test of significance on how these bands differ. Only band 4 and 10 showed a significant variation from the control (p <0.05). Bands 1, 2, 3, 5, 6, 7, 8, 9, and 11 did not display any difference compared to the original strain.

Table 5.13 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. reinhardtii* before	*C. reinhardtii* After	n	Position	Significancy
1	0.226	0.237	30	3280	n
2	0.123	0.125	30	2975	n
3	0.096	0.110	30	2927	n
4	0.089	0.098	30	2900	*
5	0.156	0.193	30	1641	n
6	0.089	0.122	30	1546	n
7	0.126	0.129	30	1380	n
8	0.093	0.110	30	1271	n
9	0.098	0.138	30	1148	n
10	0.391	0.443	30	1045	*
11	0.192	0.200	30	878	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Similarly, treatment of *C. reinhardtii* with copper did not show a remarkable structural alteration in our experiment when Fourier Transform Infrared (FTIR) spectroscopy was used to monitor the cell construction of the produced phenotype (Figure 5.37).

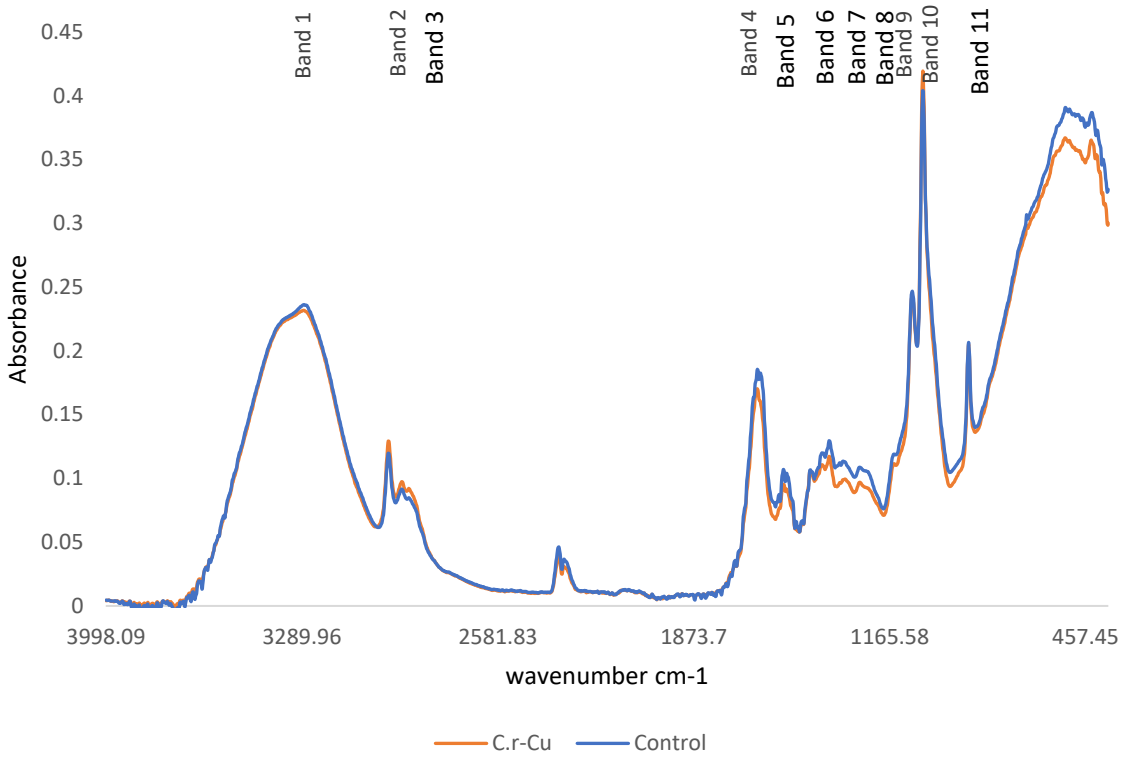

Figure 5.37. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to copper (C.r-Cu). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Table 14 illustrates the absorbance values of *C. reinhardtii* before and after acclimation for each peak and shows the test of significance on how these bands differ. Bands 1, 2, 3, 4, 5, 6, 7, 8, 9, and 11 did not display any difference from the control. The only band that displayed a significant variation from the control (p <0.05) was band 10.

Table 5.14 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. reinhardtii* before	*C. reinhardtii* After	n	Position	Significancy
1	0.226	0.232	30	3280	n
2	0.123	0.129	30	2975	n
3	0.096	0.097	30	2927	n
4	0.089	0.092	30	2900	n
5	0.156	0.170	30	1641	n
6	0.089	0.094	30	1546	n
7	0.126	0.170	30	1380	n
8	0.093	0.097	30	1271	n
9	0.098	0.112	30	1148	n
10	0.391	0.415	30	1045	*
11	0.192	0.205	30	878	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Treatment in which mercury was introduced to *C. reinhardtii* have shown that Hg has a limited impact on the cell structure of the strain used in the study. Figure 5.38 demonstrate that most of the band did not vary between the two compared strains.

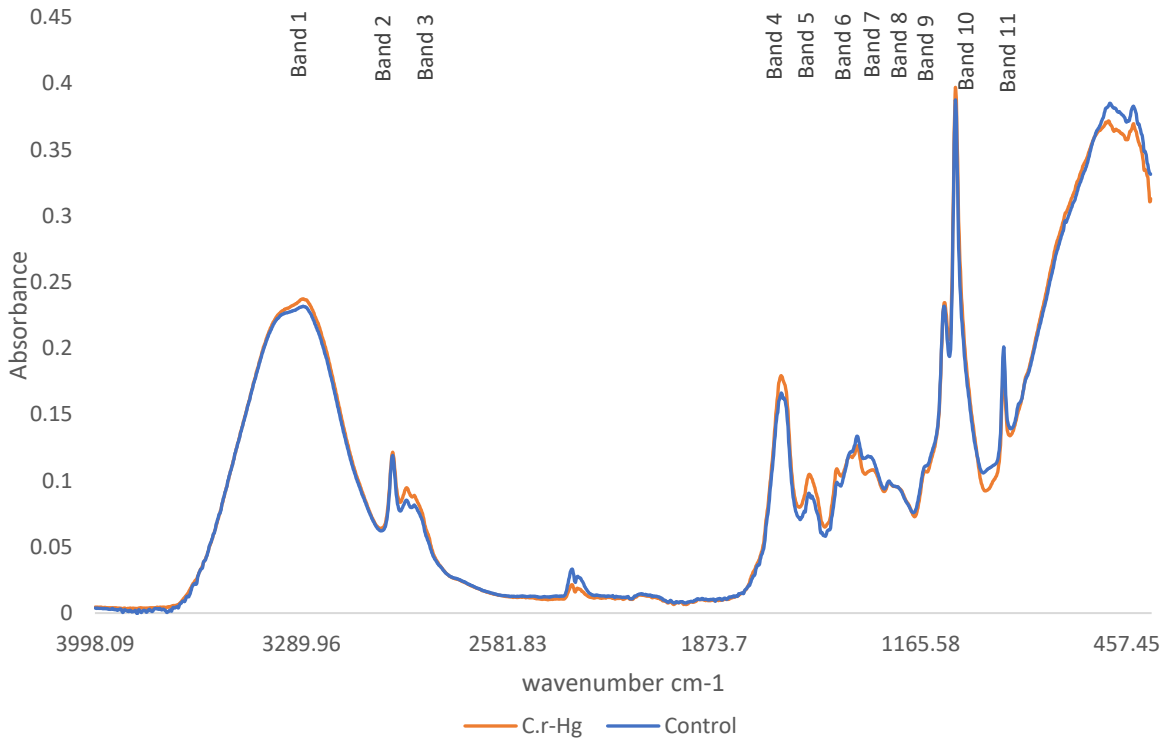

Figure 5.38. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii)*, the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to mercury (C.r-Hg). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Further analysis to test the impact of Hg on the produced *C. reinhardtii* phenotype was made and revealed in Table 15. Band's comparison showed that only band 1 and 10 had varied significantly from the control (p <0.05). Bands 2, 3, 4, 5, 6, 7, 8, 9, and 11 did not show a subsequential variance.

Table 5.15 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. reinhardtii* before	*C. reinhardtii* After	n	Position	Significancy
1	0.234	0.214	30	3280	*
2	0.120	0.118	30	2975	n
3	0.080	0.106	30	2927	n
4	0.083	0.091	30	2900	n
5	0.177	0.191	30	1641	n
6	0.099	0.122	30	1546	n
7	0.123	0.133	30	1380	n
8	0.112	0.113	30	1271	n
9	0.108	0.138	30	1148	n
10	0.396	0.415	30	1045	*
11	0.198	0.201	30	878	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

The structure of *C. reinhardtii* formed after adaptation to heavy metals mix comprising of Cd, Co, Cu, Zn, Ni, and Hg at an equal concentration of each, is presented in Figure 5.39 as a function of FTIR spectra. The collected spectra were then compared to the molecular content of the original strain to find the difference in the cell structure associated with the adaptation process.

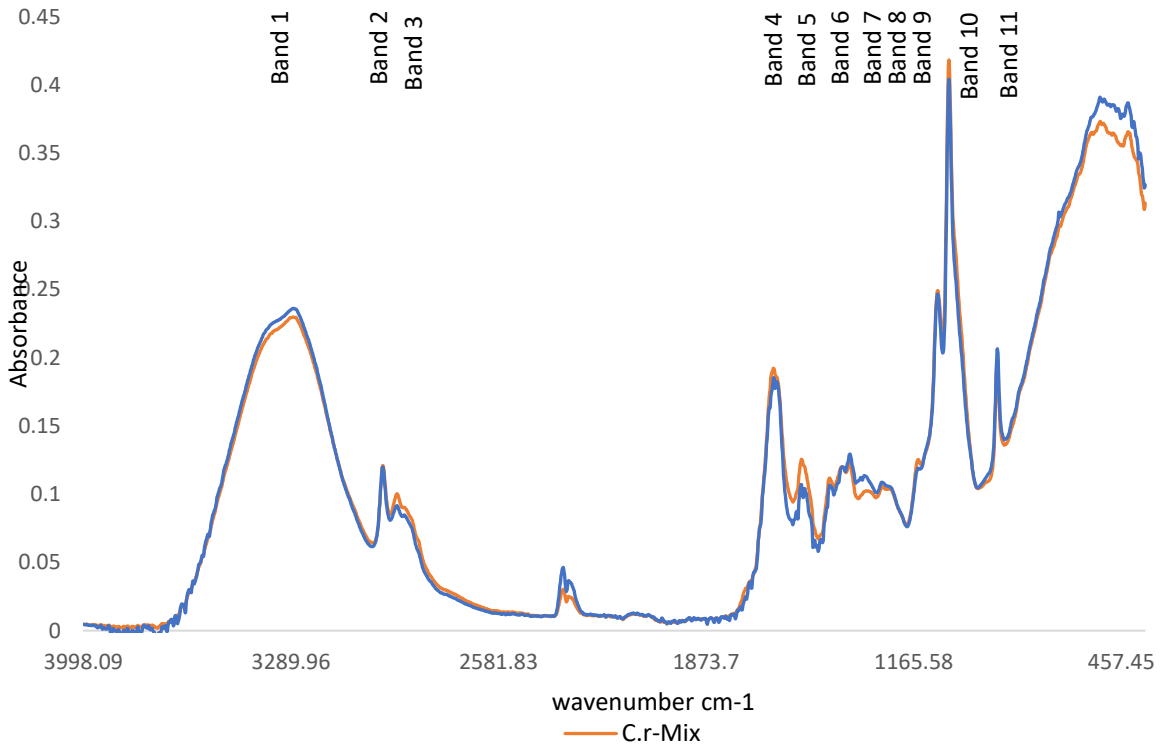

Figure 5.39. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii)*, the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to heavy metals mix (C.r-Mix). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Band assignment is presented in Table 16; the bands 1 to 11 have been listed with the absorbance data for *C. reinhardtii* before adaptation (control) and the phenotype obtained after adaptation at the accompanying position. The significance of the difference is also included in the table at both 99% and 95% probabilities. None of the bands presented any significant variance in the case of heavy metals mix.

Table 5.16 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. reinhardtii* before	*C. reinhardtii* After	n	Position	Significancy
1	0.210	0.229	30	3280	n
2	0.110	0.121	30	2975	n
3	0.084	0.100	30	2927	n
4	0.079	0.090	30	2900	n
5	0.161	0.192	30	1641	n
6	0.096	0.125	30	1546	n
7	0.115	0.127	30	1380	n
8	0.080	0.105	30	1271	n
9	0.099	0.125	30	1148	n
10	0.355	0.414	30	1045	n
11	0.176	0.199	30	878	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Figure 5.40 demonstrates the cell's functional groups of *C. reinhardtii*, a phenotype obtained from exposure to nickel during adaptation effort, compared to the control. The absorbance of some bands shows an effect on the amount of some functional groups in the cell, indicating the changes occurred due to the stress applied on microalgae by nickel.

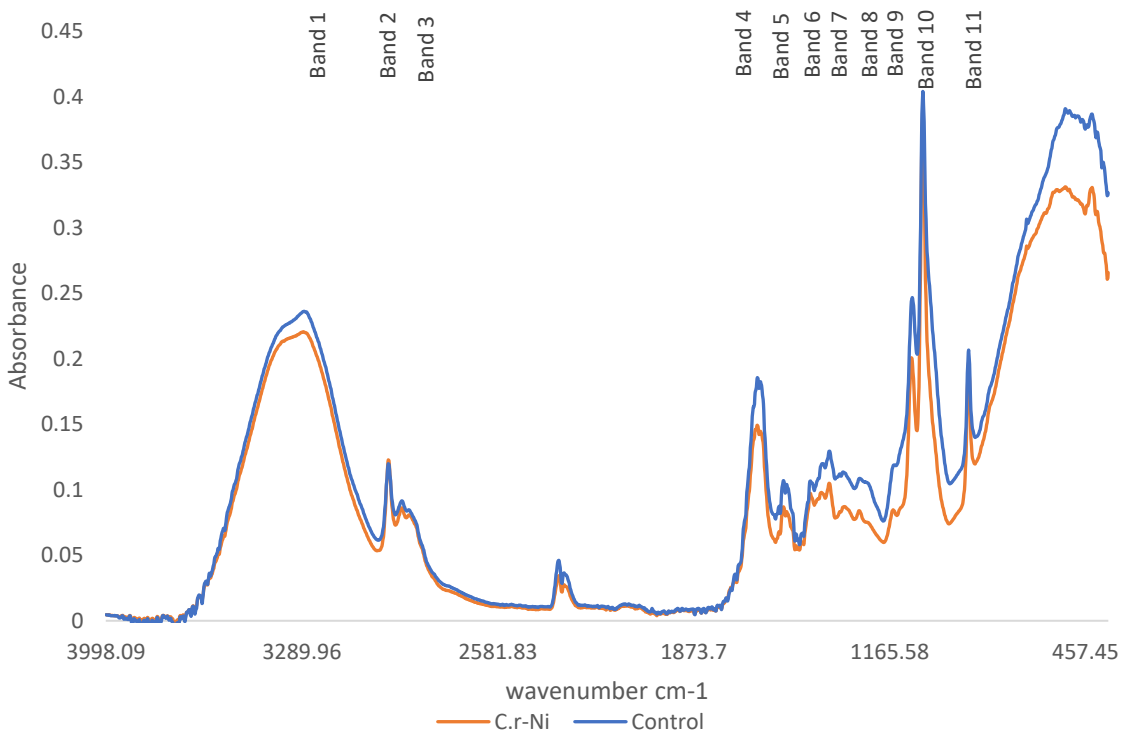

Figure 5.40. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to nickel (C.r-Ni). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Table 17 shows the assignment of the spectra achieved from FTIR scans to *C. reinhardtii* -Ni phenotype and the control (original strain). bands 1 to 11 have been listed with the associated position of each band. The significance of difference is also covered in the table at both 99% and 95% probabilities.

Table 5.17 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. reinhardtii* before	*C. reinhardtii* After	n	Position	Significancy
1	0.230	0.234	30	3280	*
2	0.119	0.118	30	2975	n
3	0.085	0.086	30	2927	*
4	0.082	0.082	30	2900	n
5	0.165	0.181	30	1641	*
6	0.090	0.107	30	1546	n
7	0.123	0.115	30	1380	*
8	0.097	0.093	30	1271	*
9	0.109	0.104	30	1148	*
10	0.382	0.397	30	1045	n
11	0.193	0.187	30	878	*

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Bands 2, 4, 6, and 10 did not display any difference from the control. Bands 1, 3, 5,7, 8, 9, and 11 had significantly lower absorbance compared to the control (p <0.05). This outcome suggests that nickel stress led to a reduction in the production of some functional groups in the cell of microalgae (*Chlamydomonas reinhardtii*).

Generation of a phenotype from the association of zinc and *C. reinhardtii* microalgae through adaptation, resulted in cell alteration in some of the bands that represent a shift in the production of functional groups that these bands represent. This change is shown in Figure 5.41. The control strain of *C. reinhardtii* and the obtained phenotype were scanned with FTIR to monitor any alteration in the cell molecular structure.

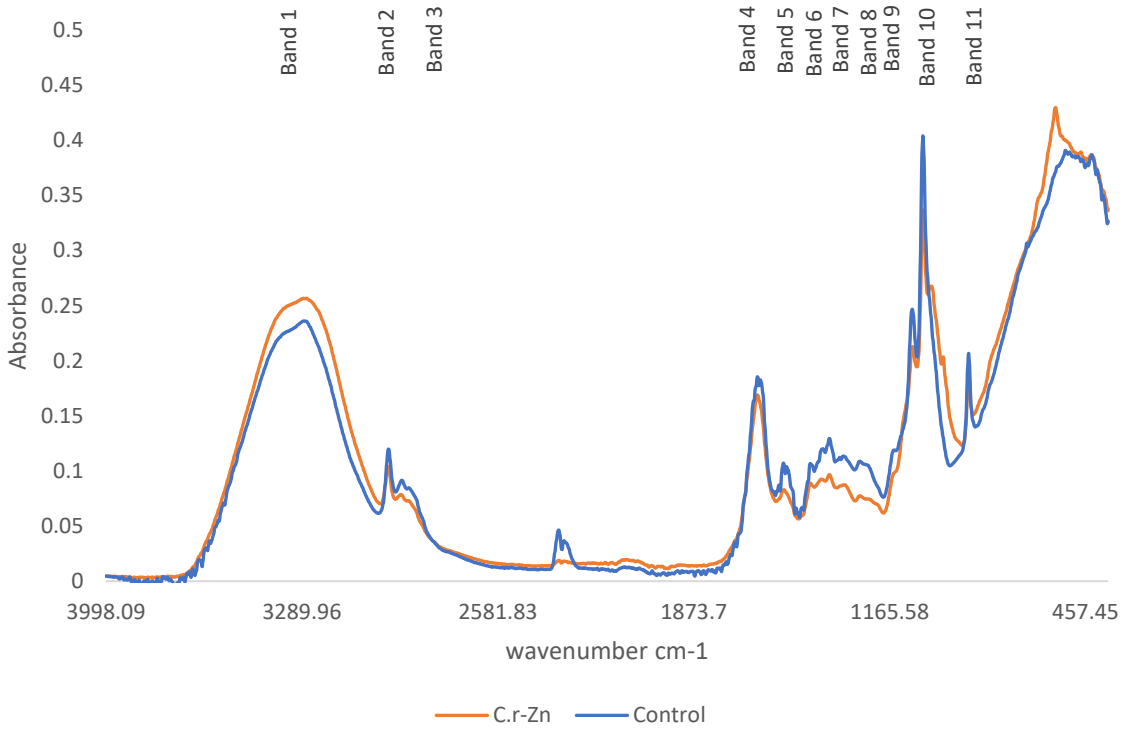

Figure 5.41. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to zinc (C.r-Zn). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 µm; a.u., arbitrary units.

Table 18 illustrates the assignment of the spectra accomplished from FTIR scans to *C. reinhardtii* phenotype that exposed to zinc and the control. bands count of 11 have been comprised with the linked position of each band. The significance of difference is also registered in the table.

Table 5.18 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. reinhardtii* before	*C. reinhardtii* After	n	Position	Significancy
1	0.236	0.256	30	3280	*
2	0.129	0.104	30	2975	n
3	0.092	0.078	30	2927	*
4	0.085	0.073	30	2900	n
5	0.185	0.169	30	1641	n
6	0.107	0.083	30	1546	n
7	0.120	0.097	30	1380	*
8	0.109	0.077	30	1271	*
9	0.119	0.099	30	1148	n
10	0.404	0.336	30	1045	n
11	0.207	0.187	30	878	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Bands 2, 4, 5, 6, 9, 10 and 11 did not show any difference from the control. Bands 1, 3, 7, and 8 had significantly different absorbance values compared to the control (p <0.05). Band 1 has a higher absorbance value which means higher production of the linked functional group (protein amide A); however, bands 3, 7, and 8 had lower absorption suggesting that zinc stress caused a reduction in the production of the associated functional.

Introducing cobalt to *C. reinhardtii* during the adaptation period had an impact on the cell structure of microalgae as shown in Figure 5.42. The produced phenotype showed differences in some bands in comparison to the control.

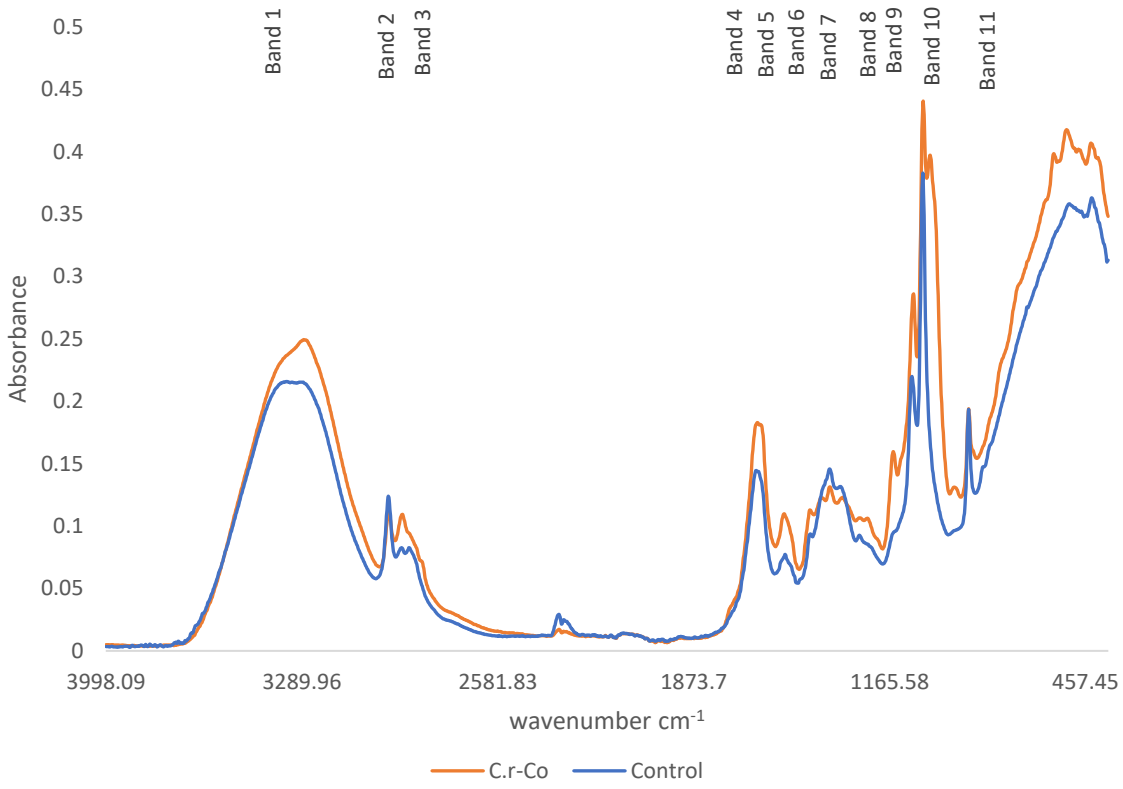

Figure 5.42. FTIR spectra of *Chlamydomonas reinhardtii* showing biomolecular peak assignments from 4000–500 cm^{-1}. The spectrum is a transmission-type micro-spectrum from algae (*Chlamydomonas reinhardtii),* the control, and *Chlamydomonas reinhardtii* phenotype obtained from exposure to cobalt (C.r-Co). The sample was placed on the diamond field directly and covered with pure aluminium slide, and then pressed with the equipment's clip. Equipment: Bruker IR scope II, circular diameter of aperture ~60 μm; a.u., arbitrary units.

Assessment of the spectra produced from Fourier Transform Infrared spectroscopy for both the control and the treated strain was made to find the sign of alteration in cell structure. The results are listed in Table 19.

Table 5.19 Comparison of band intensities at each band position, Mann-Whitney Test.

Band	*C. reinhardtii* before	*C. reinhardtii* After	n	Position	Significancy
1	0.182	0.223	30	3280	*
2	0.111	0.144	30	2975	*
3	0.077	0.117	30	2927	*
4	0.077	0.114	30	2900	*
5	0.123	0.180	30	1641	n
6	0.74	0.109	30	1546	n
7	0.095	0.174	30	1380	*
8	0.072	0.114	30	1271	n
9	0.072	0.135	30	1148	*
10	0.297	0.470	30	1045	*
11	0.149	0.199	30	878	n

N number of spectra, n= not significant, * = significant at 95% probability, **= significant at both 95%, and 99% probabilities.

Band 1, 2, 3, 4, 7, 9, and 10 significantly showed much absorbance in the obtained phenotype than in the control (p <0.05). This result suggests that the presence of cobalt encouraged the production of the functional group assigned to each band. On the other hand, bands 5, 6, 8, and 11 did not substantially vary from the control.

5.5 Results Discussion

The results present data from an experiment in which *C. vulgaris* 211/111 and *C. reinhardtii* 11/45 were cultivated in 3NBBM for a prolonged time with a defined concentration of heavy metal/s over ten generations (stage) and increasing metal/s concentration for the subsequent stages by 10%. This was the most logical increase ratio since LC_{50} for each metal-algae spices were highly distinctive. The metals were introduced as individual heavy metal additions or as a mixture of metals to enhance microalgal resistance to

them. The initial concentration of heavy metals used in stage 1 was derived from the toxicity experiments and represents the LC_{50} for each metal. The adaptation process consisted of five stages (the stages division was used for ease of data presentation) over 315 days (Chapter 5).

The findings from this experiment showed a successful selection and adaptation strategies on enhancing *C. vulgaris* 211/111 and *C. reinhardtii* 11/45 to tolerance of single and mixtures of heavy metals. There was an improvement of metals resistance by 10% to 50% between first stage and third or fifth stage of adaptation of the studied strains (chapter 5). Various studies investigating heavy metal pollution of water bodies have revealed that microalgal diversity may be reduced due to a selection process to the most tolerant species (Fahmi *et al*. 1982; Foster 1982; Gupta and Chandra 1994; Harding and Whitton 1976; Takamura *et al*. 1989). It has also been suggested that exposure to heavy metals leads to adverse impacts on growth, photosynthesis, respiration, cell division, and degeneration of cell components, causing the reduction of microalgal diversity (Wang and Chen 2009). The results corroborate with previous results where a successful adaptation was also reported by Shehata and Whitton (1982), Kuwabara and Leland (1986). De Fillippis and Pallghay (1976) used *Chlorella sp*. to monitor growth parameters impacted by zinc and mercury and reported that long-term exposure to zinc (1 mM ZnCl for 50 d) had developed resistance to zinc in the studied strain. Volskey (1990) compared studies that used different microalgae strains from seven classes. A hindered and eighteen microalgae strains were isolated from polluted and unpolluted sites exposed to elevated concentrations of heavy metals. They found that algae from Chlorellaceae and Chlorophyceae were more adaptable to the increasing concentration of heavy metals than strains belonging to blue-green algae that were more sensitive to the studied metals Cu, Cd, and Zn. Bates *et al.* (1982) studied zinc absorption and transport by *Chlamydomonas variabilis* and *Scenedesmus subspecies* (Chlorophyceae) growing in simi

continuous culture. They reported that both strains accumulated zinc very fast and that the accumulation on cell walls took place within minutes. De Fillippis and Pallghay (1976) used *Chlorella* sp in a study about zinc and mercury bioremediation by living cells. They monitored growth parameters during exposure to these heavy metals; reporting that only 14% of the accumulated mercury was situated in the cell wall. The in-cell mercury concentration was very close to the concentration of sulfhydryl in the cell, suggesting that sulfhydryl is the primary binding site that *Chlorella* uses to accumulate mercury.

Fourier Transform Infrared (FTIR) spectroscopy system is used to attain an infrared spectrum of absorption or emission of a solid, liquid, or gas. High-spectral-resolution data over broad spectral range are simultaneously collected via a FTIR spectrometer; this deliberates a significant advantage over a dispersive spectrometer, which measures intensity over a narrow range of wavelengths at a time. FTIR has been widely used as a rapid and accurate tool for detecting and analysing organic materials for decades since it was initially used to study the cell structure of algae since FTIR can distinguish cell components at the molecular level (Li *et al.,* 2021). The infrared spectrum is a method for identifying molecular structure based on atom vibration and rotation. Atoms of organic molecules that form chemical bonds and functional groups are in a constant state of vibration. When a bundle of IR (infrared radiations) beam with a continuous wavelength passes through the sample, the light of a particular wavenumber is absorbed, which causes the absorbance spectrum. The equivalent of light energy and the differences between two energy levels is the indispensable condition of absorption, thus determining the position of the absorption in the spectrum. Finally, the spectrum is further processed by the Fourier transformation technique to make it more efficiently analysed.

Fourier Transform Infrared (FTIR) spectroscopy was used to scan algal cells for the strains used in this research before and after adaptation to determine the structural changes in the cell after incubation with the heavy metals. Comparison through detecting the marker bands was made in addition to determining the position of each band for both strains *C. vulgaris* and *C. reinhardtii*.

The result showed a significant change in the microalgal cell structure upon exposure to heavy metals in both individual metals and the mixture additions for both *C. vulgaris* 211/111 *and C. reinhardtii* 11/45. Microalgae respond differently to heavy metal toxicity, such as the synthesis of phytochelatins, upregulation of antioxidants, accumulation of compatible solutes, accumulation of low-molecular-weight metabolites, and changes in phytohormone levels (Bajguz and Hayat 2009) and metallothionine (Pérez-Rama *et al.*, 2001). Production of any of the previous cell components depends on the cell mechanism used by microalgae to tolerate heavy metals stress (Bajguz, 2011; Shamim, 2018). The results showed that the changes in cell structure were more significant in *C. vulgaris* 211/111 than in *C. reinhardtii* 11/45, which suggest that *C. vulgaris* 211/111 uses intercellular mechanisms, while *C. reinhardtii* 11/45 uses extracellular mechanisms. Jahan *et al*. (2004) in their study used *C. vulgarise* and *Scenedesmus abundans* to test the impact of Cu and As toxicity on the cell structure, and reported that the changes occurred in *S. abundans* cell was significant and that the main process involved in removing heavy metals was biosorption. The obtained findings have many practical applications apart from heavy metal bioremediation; for example, the use of heavy metals to induce the production of desired cell components such as lipids, proteins, and other economically valuable components. In support of this, Robinson *et al.* (1993) found that an increase of Zn, Cd, Pb, Cu, Hg, and Ag induced the production of phytochelatins in microalgal cells. Other researchers have also reported a

relationship between metals and the increases in protein production in plant, yeast, and microalgal cells (Clements *et al.*1999; Mallick *et al.,* 1994). For example, Mallick *et al.* (1994) found that *Anabaena doliolum* cells were able to produce a low molecular protein that can bind to Cd and reduced its toxicity when introduced in a high concentration. Yang *et al.* (2015) used *Chlorella* sp. for both lipid production and cadmium, copper, manganese, and zinc ions removal under heterotrophic culture conditions; and could efficiently remove them through intracellular accumulation and extracellular immobilization. They reported that algal lipids content increased by 21.07% and 93.90%, respectively, induced by cadmium and copper.

In the last stage of adaptation, the impact of incubating *C. vulgaris and C. reinhardtii* in the heavy metal solutions on removing heavy metals from the solution was tested using ICP-OEC. The concentration of metals was compared before adding microalgal phenotype and then after microalgal growth for one generation (7 days). In general, the presence of the microalgae significantly decreased the concentration of heavy metals in solution. The results further indicated that the biosorption of heavy metals by microalgal biomass is accompanied by an induction of a variety of biochemical changes, some of which directly contribute to the metal tolerance capacity of the cell. Once adapted to higher concentrations of heavy metals, the microalgae quickly removed metal ions from aqueous solutions by biosorption without any toxic effects on the cells. These findings are similar to those reported by Bajguz (2011), who studied the ability of *C. vulgaris* to remove Cd, Pb and Cu from the solution through the production of intracellular components. The author studied the addition of brassinolide to a *C. vulgaris* culture and the impact of this treatment on the cultural response to heavy metals toxicity and the production and levels of intercellular components of *C. vulgaris* such as phytochelatins, upregulation of antioxidants, accumulation of compatible solutes, accumulation of low-molecular-weight metabolites, and changes in phytohormone levels

(ABA, auxins, cytokinins, and gibberellins). Gale (1986) also found that microalgae were able to remove toxic metals from wastewater of up to 99%. Other similar studies reported a 100% lead removal at 1 mg/l concentration within 24 hours at 23 ℃ (Mac and Hardy 1990; Manzoor *et al.*, 2019).

CHAPTER SIX

IMPACT OF MICROALGAL BIOMASS ON THE SURVIVAL OF *DAPHNIA MAGNA* IN THE PRESENCE OF HEAVY METALS

Summary

In this chapter, the impact of microalgal biomass on the survivability of *Daphnia magna* in the presence of heavy metals, was investigated. A concentration of 0.01 mg/l was used for Zn, Ni, Hg, Co, and Cd. For the Cu solution and the mixture of metals, a concentration of 0.001 mg/l was used for each treatment. These concentrations represent the levels at which 50% of the *Daphnia* population was killed by toxicity of the metal/s. An inoculum of 5% v/v of microalgae was added to each flask/ treatment. The data were statistically compared, and Cohen's d values were calculated to see the effect size of algae-pre-treatment on the survival of *Daphnia magna.*

6.1 Survival of *Daphnia magna* in heavy metals solutions pre-treated with adapted *Chlorella vulgaris* 211/111

6.1.1 *Daphnia magna* survival in zinc solution pre-treated with *Chlorella vulgaris* 211/111

Three solutions were used to incubate *D. magna* 0 mg/l of zinc (control), 0.01mg/l of zinc (control+), and 0.01mg/l of zinc that has been treated with *C. vulgaris* phenotype before the incubation of *D. magna.* A one-way analysis of variance (ANOVA) was performed to show

any significant differences from the control (p <0.01). The results showed that there was no significant variation in the survival ratio of *D. magna* grown in zinc solution of 0.01 mg/l that was pre-treated with *C. vulgaris*, from the control (p >0.01). On the other hand, there was a significant decrease in the number of *D. magna* that was incubated in the 0.01mg/l of zinc (control+) compared to the control (p <0.05). The mortality rate of *D. magna* in the presence of zinc, compared to the control, was - 0.40/h (Figure 6.1).

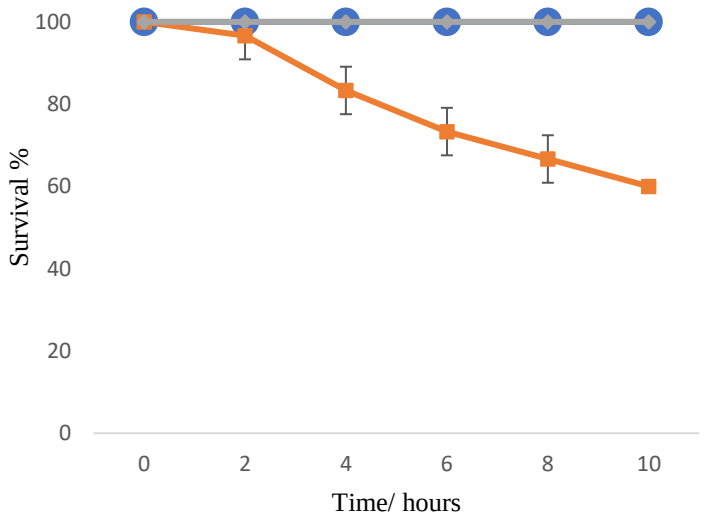

Figure 6.1. *Daphnia magna* survival in 0.01 mg/l zinc solution pre-treated with *Chlorella vulgaris*: the control, 0 mg/l of zinc (●); 0.01mg/l of zinc (■), and 0.01mg/l of zinc that has been treated with *C. vulgaris* phenotype (♦).

To find the practical significance of impact of the pre-treatment with *C. vulgaris* on *Daphnia* survivability, the effect size of the pre-treatment was calculated. The effect size is essential to check the applicability of the findings on a large scale. The effect size of the algal pre-treatment on the survival of *Daphnia* has been assessed by calculating Cohen's d value, 5.77; this suggests that the pre-treatment with *C. vulgaris* has a significant impact on *Daphnia's* survival.

6.1.2 *Daphnia magna* survival in copper solution pretreated with *C. vulgaris* 211/111

The number of survivors is shown in Figure 6.2, where three solutions were used to incubate *D. magna:* 0 mg/l of copper (control), 0.001mg/l of copper (control+), and 0.001mg/l of copper that has been treated with *C. vulgaris* phenotype before the incubation of *D. magna*. In the solution of 0.001mg/l of copper (control+), the mortality rate was -0.48/h in relation to the control (0 mg/l of copper), this differed from the algae pre-treated copper solution and the control (p <0.01). The pre-treated copper solution had 100% survival ratio of *D. magna*, with no significant difference from the control (p >0.01). This outcome suggests that the pre-treatment with *C. vulgaris* phenotype reduced the impact of copper toxicity on *D. magna*. Cohen's d value was calculated to be 9.23, which means a very high impact on the survival chance of *D. magna* when algal pre-treatment was applied on the copper solution before the incubation of *Daphnia*.

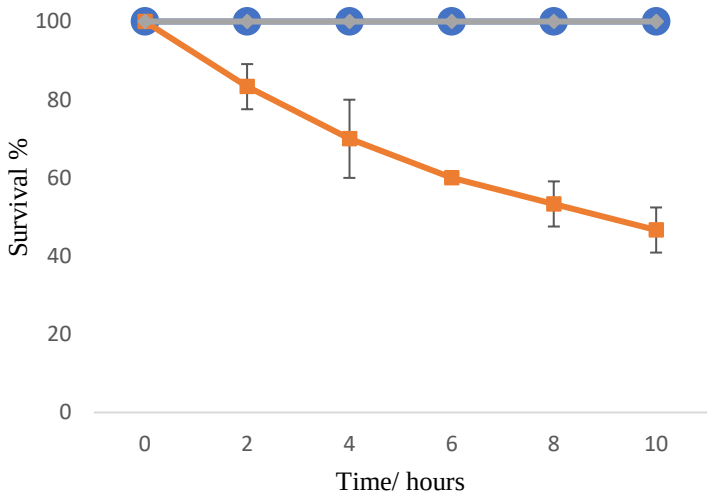

Figure 6.2. *Daphnia magna* survival in 0.001 mg/l copper solution pretreated with *Chlorella vulgaris;* the control, 0 mg/l of copper (●); 0.001mg/l of copper (■), and 0.001mg/l of copper that has been treated with *C. vulgaris* phenotype (♦).

6.1.3 *Daphnia magna* survival in nickel solution pretreated with *C. vulgaris 211/111*

There was a slight difference in *Daphnia's* survival between the control and the algae-treated nickel solution of concentration of 0.01mg/l as shown in Figure 6.3. Statistically, this difference was not significant (p >0.01). The survival ratio of *Daphnia* in the pretreated solution was 96.7% compared to 55% in the control+ (the untreated 0.01 mg/l nickel solution) which is highly significant (p <0.01). In addition, the mortality rate of *D. magna* in the untreated nickel solution in relation to the control was -0.59/h. The effect size of algae pretreatment on the survival of *Daphnia* in the pre-treated nickel solution was assessed by calculating Cohen's d value, 6.93; this suggests that the pre-treatment with *C. vulgaris* has a significant impact on *Daphnia's* survival.

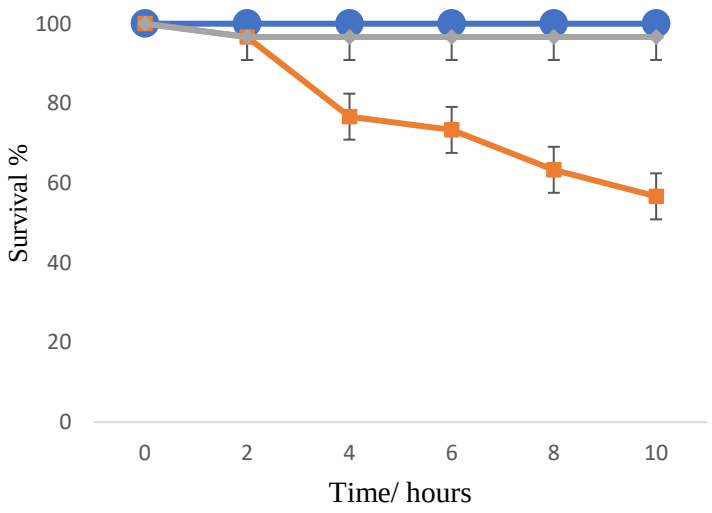

Figure 6.3. *Daphnia magna* survival in 0.01 mg/l nickel solution pretreated with *Chlorella vulgaris; ;* the control, 0 mg/l of Ni (●); 0.01mg/l of Ni (■), and 0.01mg/l of Ni that has been treated with *C. vulgaris* phenotype (♦).

6.1.4 *Daphnia magna* survival in mercury solution pretreated with *C. vulgaris* 211/111

Data in Figure 6.4 illustrates the survival ratio of *D. magna*, where 0.01 mg/l of mercury was added in the control+ treatment, the survival ratio was 48.66%, significantly different from the algae-treated mercury solution (p <0.01). *C. vulgaris* -pretreated mercury solution had a 100% survival ratio, with no significant difference from the control (p >0.01). This finding suggests that the pretreatment successfully reduced mercury toxicity on *D. magna*. The mortality rate of *D. magna* in a concentration of 0.01 mg/l of mercury in relation to the control was found to be -0.47/h. Cohen's d value was found to be 9.24, which means a very high impact on mercury removal and *D. magna* survivability.

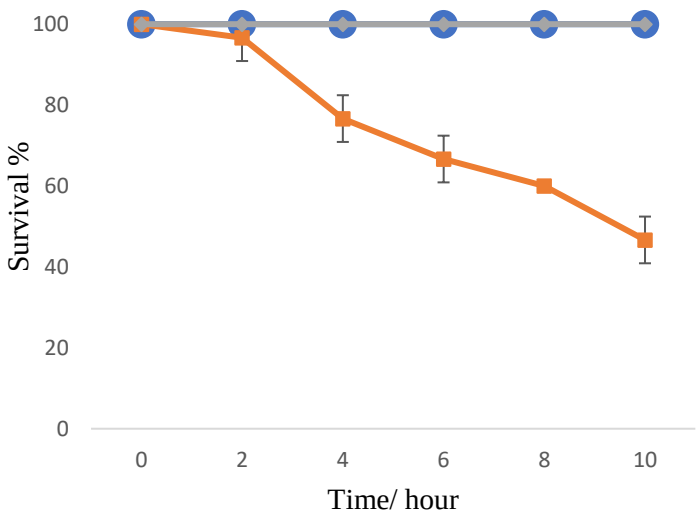

Figure 6.4. *Daphnia magna* survival in 0.01 mg/l mercury solution pretreated with *Chlorella vulgaris ;* the control, 0 mg/l of mercury (●); 0.01mg/l of mercury (■), and 0.01mg/l of mercury that has been treated with *C. vulgaris* phenotype (♦).

6.1.5 *Daphnia magna* survival in cobalt solution pretreated with *C. vulgaris* 211/111

There was a difference in *Daphnia's* survival between the control and the pre-treated cobalt solution of concentration of 0.01mg/l as shown in Figure 6.5 (p <0.01). Which means that the pretreatment with *C. vulgaris* did not entirely eliminate cobalt from the solution or, needs more time remove the metal from the solution. However, the survival ratio of *Daphnia* in the pre-treated solution was 90% compared to 43% in the control+ (the untreated 0.01 mg/l cobalt solution) which was significant (p <0.01). The mortality rate of *D. magna* in a concentration of 0.01 mg/l of cobalt in relation to the control was found to be -0.48/h. The effect size of algae pretreatment on the survival of *Daphnia* in the pre-treated Co solution was assessed by calculating Cohen's d value, 8.08, this means that the pretreatment with *C. vulgaris* has improved *Daphnia's* ratio of survival.

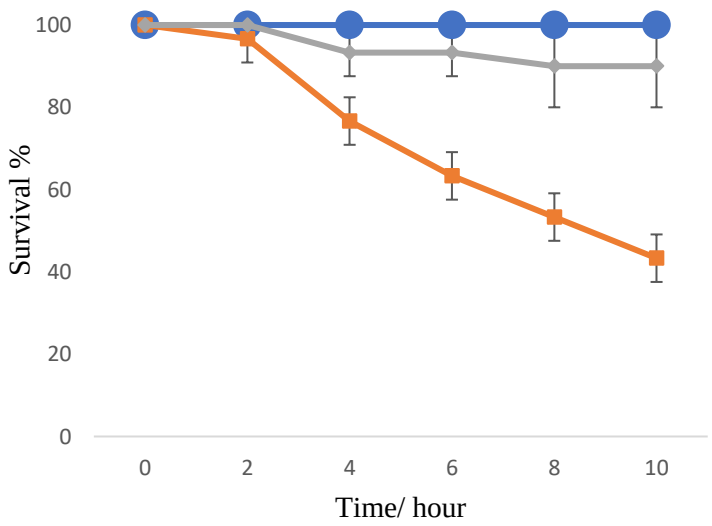

Figure 6.5. *Daphnia magna* survival in 0.01 mg/l cobalt solution pretreated with *Chlorella vulgaris; ;* the control, 0 mg/l of cobalt (●); 0.01mg/l of cobalt (■), and 0.01mg/l of cobalt that has been treated with *C. vulgaris* phenotype (♦).

6.1.6 *Daphnia magna* survival in cadmium solution pretreated with *C. vulgaris* 211/111

The performed statistical analysis showed a significant variation in the survival ratio of *D. magna* grown in a pre-treated solution of 0.01 mg/l cadmium and the control (p <0.01). However, an improved survival of 90% was observed in the pre-treated solution. On the other hand, there was a significant decrease in the population of *D. magna* in the control+, the untreated 0.01 mg/l of cadmium solution, compared to the pre-treated solution of the same concentration and the control (p <0.05). Thus, a reduction of 46% in *Daphnia* population in the control+ has occurred at the end of the incubation (Figure 6.6). The mortality rate of *D. magna* in a concentration of 0.01 mg/l of cadmium in relation to the control was -0.59/h. Cohen's d value has been found to be 6.35, which states a high impact of *C. vulgaris* pre-treatment on cadmium removal and *D. magna* survivability.

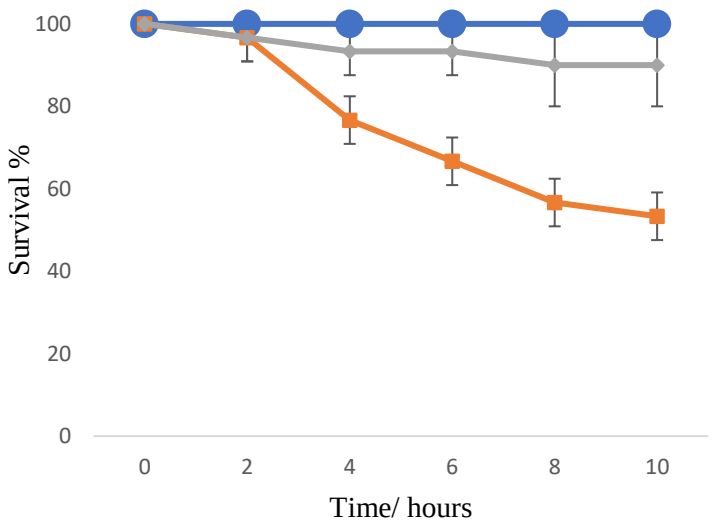

Figure 6.6. *Daphnia magna* survival in 0.01 mg/l cadmium solution pretreated with *Chlorella vulgaris; ;* the control, 0 mg/l of cadmium (●); 0.01mg/l of cadmium (■), and 0.01mg/l of cadmium that has been treated with *C. vulgaris* phenotype (♦).

6.1.7 *Daphnia magna* survival in metals mixture solution pretreated with *C. vulgaris* 211/111

A mixture of the following metals: Zn, Cu, Ni, Hg, Co, and Cd, was prepared to test the combining effect on their removal by *C. vulgaris*. The mixture was prepared by adding equal amounts of each metal in the mixture to prepare the required concentration. For example, to prepare 10 mg/l of the metal mixture, an amount of 1.67 mg/l of each metal (Cu, Zn, Ni, Hg, Co, and Cd) were mixed in one litre of the growth medium to reach a final concentration of 10 mg/l of the metal mixture. The concentration used was 0.001mg/l. The results showed that pre-treatment with *C. vulgaris* significantly reduced the lethal effect of the metals mixture on *Daphnia's* longevity (p <0.01). On the other hand, the survival ratio reduced to 57% in the control+ compared to the microalgae-pretreated solution of the same concentration, where a 90% survival ratio was obtained (Figure 6.7). The mortality rate of *D. magna* in 0.001 mg/l of metals mixture in relation to the control was -0.48/h. Cohen's d value was 9.24, which means that *C. vulgaris* pre-treatment, has a significant impact on metals removal and *Daphnia* survivability.

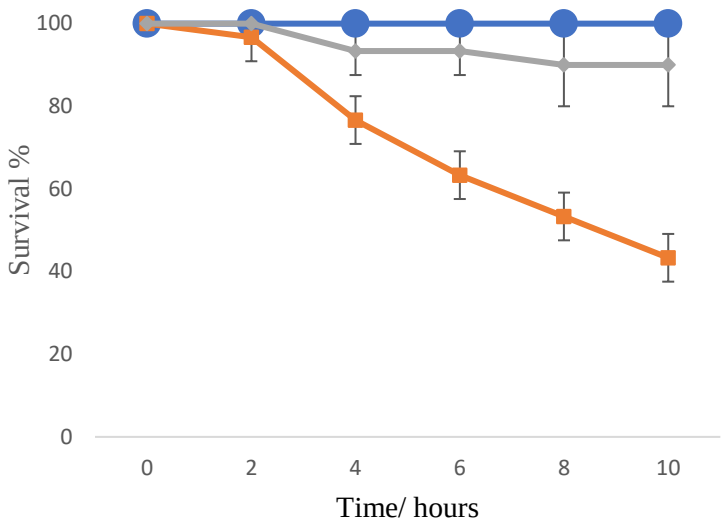

Figure 6.7. *Daphnia magna* survival in 0.001 mg/l mixture of metals solution pretreated with *Chlorella vulgaris;* the control, 0 mg/l of mixture of metals (●); 0.01mg/l of mixture of metals (■), and 0.01mg/l of mixture of metals that has been treated with *C. vulgaris* phenotype (♦).

Table 6.1 shows a summary of the survival parameters of *Daphnia magna* in heavy metals solutions pre-treated with adapted *Chlorella vulgaris* 211/111.

Table 6.1. Survivability parameters of *D. magna* in the metal solution with and without treatment with *C.vulgaris* phenotype

Metal / Parameter	Zn	Cu	Ni	Hg	Co	Cd	Mixture
Survival rate in the pre-treated solution	100%	100%	96.99%	100%	90%	90%	90%
Death rate in the untreated solution	40%	53.67%	43.67%	43.67%	43.67%	43.67%	50.67
Mortality rate per hour	-0.40/h	-0.48/h	-0.47/h	-0.59/h	-0.59/h	-0.48/h	-0.48/h

6.2 Survival of *Daphnia magna* in heavy metals solutions pre-treated with adapted *Chlamydomonas reinhardtii 11/45*

6.2.1 *Daphnia magna* survival in zinc solution pre-treated with *C. reinhardtii 11/45*

Three solutions were used to incubate *D. magna:* 0 mg/l of zinc (control), 0.01mg/l of zinc (control+), and 0.01mg/l of zinc that has been treated with *C. reinhardtii* phenotype before the incubation of *D. magna.* A one-way analysis of variance (ANOVA) was performed to show any significant differences from the control (p <0.01). The results showed that adding *C. reinhardtii* to 0.01mg/l of zinc solution has a positive impact on *D. magna* survival in the metal solution (Figure 6.8). The difference in *Daphnia's* survival ratios between the algae-treated solution and the control+ was significant (p <0.01). A survival ratio of 100% was observed in the pre-treated solution versus 63.33% in the control+. The mortality rate of *D. magna* in a concentration of 0.01 mg/l of zinc in relation to the control was -0.63/h. Cohen's d value was 6.35, which refers to a high impact of the treatment on the survivability of *D. magna*.

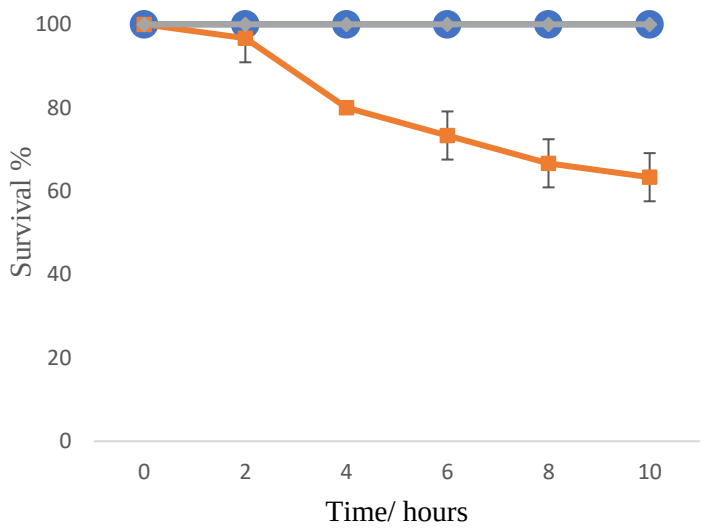

Figure 6.8. *Daphnia magna* survival in 0.01 mg/l zinc solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of zinc (●); 0.01mg/l of zinc (■), and 0.01mg/l of zinc that has been treated with *C. reinhardtii* phenotype (♦).

6.2.2 *Daphnia magna* survival in copper solution pretreated with *C. reinhardtii 11/45*

Adding *C. reinhardtii* to 0.001 mg/l of copper enhanced the survival chance of *D.magna* (Figure 6.9). An improvement was obtained in the survival ratio of 86.67%. In the control+, the survival ratio was 63.3%. Both were significantly different from the control (p <0.01). These results suggest that adding *C. reinhardtii* improved the survivability of *D. magna*, but its presence did not completely remove Cu from the solution. The mortality rate of *D. magna* in a concentration of 0.001 mg/l of Cu in relation to the control was -0.73/h. Cohen's d value was 7.50, suggesting that using *C. reinhardtii* to reduce copper toxicity, highly impacted *Daphnia* survivability.

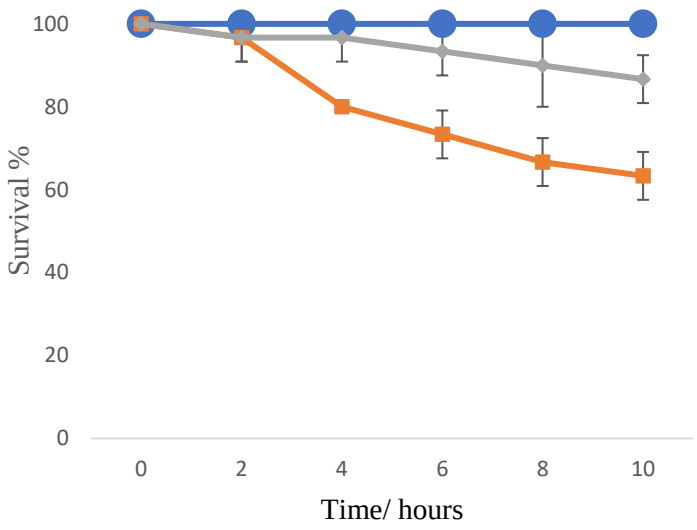

Figure 6.9. *Daphnia magna* survival in 0.001 mg/l copper solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of copper (●); 0.001mg/l of copper (■), and 0.001mg/l of copper that has been treated with *C. reinhardtii* phenotype (♦).

6.2.3 *Daphnia magna* survival in nickel solution pre-treated with *C. reinhardtii 11/45*

Figure 6.10 demonstrates the survival ratio of *D. magna*, where 0.01 mg/l of nickel was added in the control+ treatment. The survival ratio was 53.33%, significantly lower than the solution pre-treated with adapted *C. reinhardtii*, which had a 100% survival ratio, the pre-treated solution showed no significant difference from the control (p >0.01). This outcome suggests that the pre-treatment successfully reduced Ni toxicity on *D. magna*. The mortality rate of *D. magna* in a concentration of 0.01 mg/l of Ni in relation to the control was -0.53/h. Cohen's d value was 8.08, which means this treatment highly impacted *Daphnia* survivability.

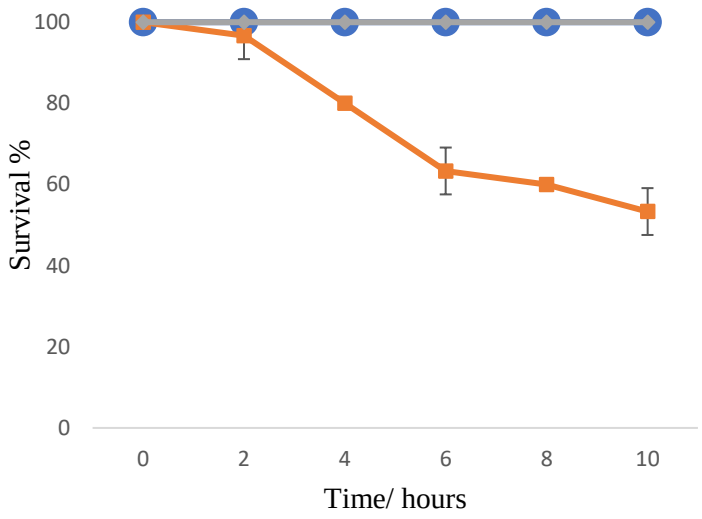

Figure 6.10. *Daphnia magna* survival in 0.01 mg/l nickel solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of nickel (●); 0.01mg/l of nickel (■), and 0.01mg/l of nickel that has been treated with *C. reinhardtii* phenotype (♦).

6.2.4 *Daphnia magna* survival in mercury solution pretreated with *C. reinhardtii 11/45*

The solutions used to incubate *D. magna* were 0 mg/l of mercury (control), 0.01mg/l of mercury (control+), and 0.01mg/l of mercury that has been treated with *C. reinhardtii* phenotype before the incubation of *D. magna.* The analysis of data and comparison with the control showed that there was no variation in the survival ratio of *D. magna* grown in a pre-treated solution of 0.01 mg/l mercury and the control (p >0.01) (both achieved 100% survivors). On the other hand, there was a significant decrease in the number of *D. magna* in the control+ (the untreated 0.01 mg/l of mercury solution) compared to the microalgae-treated solution and the control (p <0.01). A reduction of 56.66% in *D. magna* population in the control+ has occurred at the end of the incubation (Figure 6.11). The mortality rate of *D.*

magna in a concentration of 0.01 mg/l of mercury in relation to the control was found to be -0.43/h. Cohen's d value was found to be 9.81, refers to a very high impact on Hg removal and the survival ratio of *D. magna*.

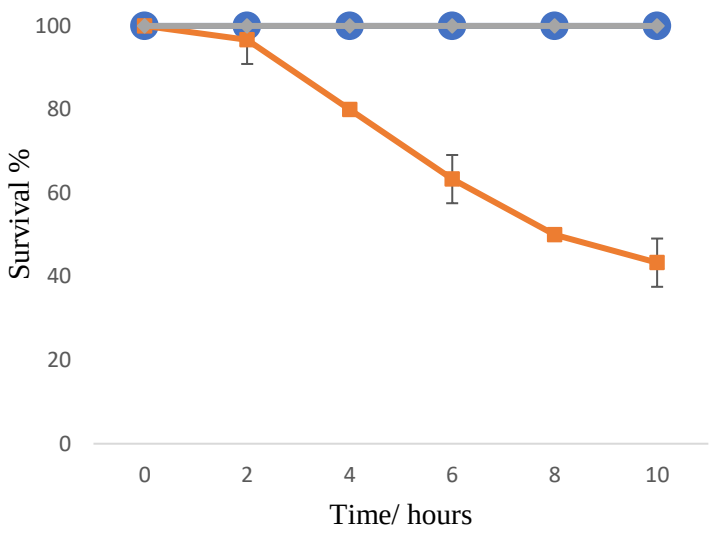

Figure 6.11. *Daphnia magna* survival in 0.01 mg/l mercury solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of mercury (●); 0.01mg/l of mercury (■), and 0.01mg/l of mercury that has been treated with *C. reinhardtii* phenotype (♦).

6.2.5 *Daphnia magna* survival in cobalt solution pre-treated with *C. reinhardtii* 11/45

There was a minor difference in *Daphnia's* survival between the control and the algae-treated cobalt solution of concentration of 0.01mg/l as shown in Figure 6.12. Statistically, this difference was not significant (p >0.01). The survival ratio of *Daphnia* in the pre-treated solution was 96.67% compared to 43.33% in the control+ (the untreated 0.01 mg/l cobalt solution) which is highly significant (p <0.01). The mortality rate of *D. magna* in a concentration of 0.01 mg/l of cobalt in relation to the control was found to be -0.45/h. The

effect size of algae pre-treatment on the survival of *Daphnia* in the pre-treated cobalt solution has been assessed by calculating Cohen's d value, 9.24; this suggests that the pre-treatment with *C. reinhardtii* has a high impact on *Daphnia's* survivability.

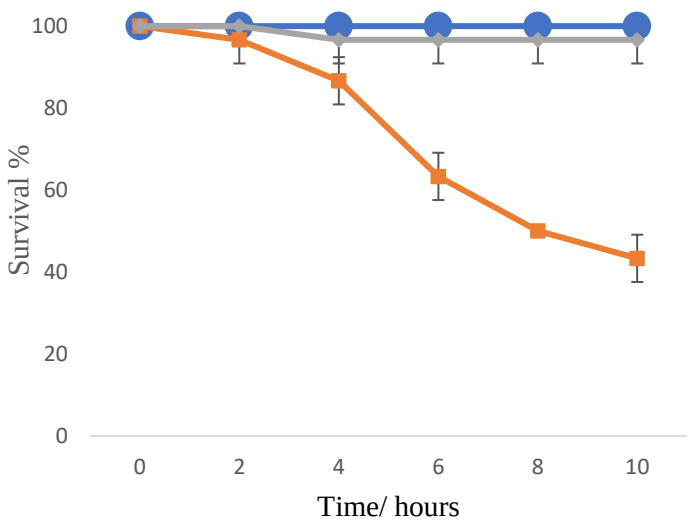

Figure 6.12. *Daphnia magna* survival in 0.01 mg/l cobalt solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of cobalt (●); 0.01mg/l of cobalt (■), and 0.01mg/l of cobalt that has been treated with *C. reinhardtii* phenotype (♦).

6.2.6 *Daphnia magna* survival in cadmium solution pretreated with *C. reinhardtii 11/45*

An insignificant difference in the survival of *Daphnia* between the control and the algae-treated cadmium solution of concentration of 0.01mg/l was observed (Figure 6.5) (p >0.01). This indicates that the pre-treatment with *C. reinhardtii* did not fully remove Cd from the solution. However, the survival rate of *Daphnia* in the pre-treated solution was 90% compared to 46.67% in the control+ (the untreated 0.01 mg/l cadmium solution) which was very significant (p <0.01). The mortality rate of *D. magna* in a concentration of 0.01 mg/l of

Cd in relation to the control was -0.52/h. Cohen's d value was 7.50, which means that the pre-treatment has improved *Daphnia's* survivability.

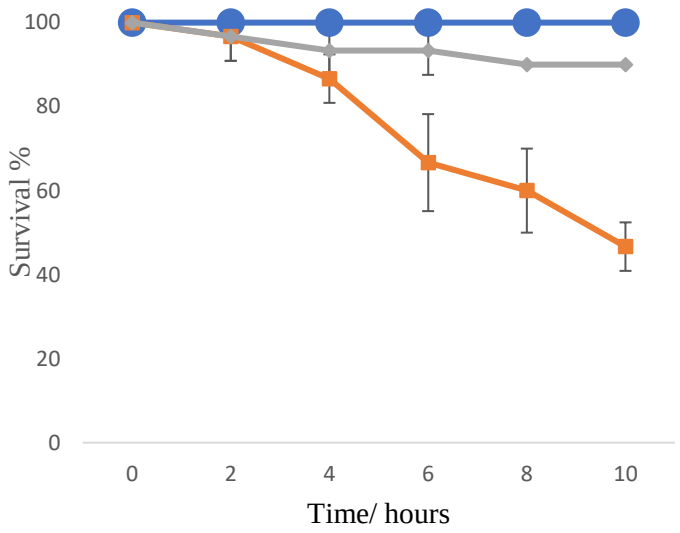

Figure 6.13. *Daphnia magna* survival in 0.01 mg/l cadmium solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of cadmium (●); 0.01mg/l of cadmium (■), and 0.01mg/l of cadmium that has been treated with *C. reinhardtii* phenotype (♦).

6.2.7 *Daphnia magna* survival in metals mixture solution pretreated with *C. reinhardtii 11/45*

Zinc, copper, nickel, mercury, cobalt, and cadmium were used to prepare metal mixture to evaluate their impact altogether on the efficiency of their removal by *C. reinhardtii*. The concentration used was 0.001mg/l. Analysed data showed that pre-treatment with *C. reinhardtii* significantly reduced the lethal effect of the metals mixture on *Daphnia's* population (p <0.01) (Figure 6.14).

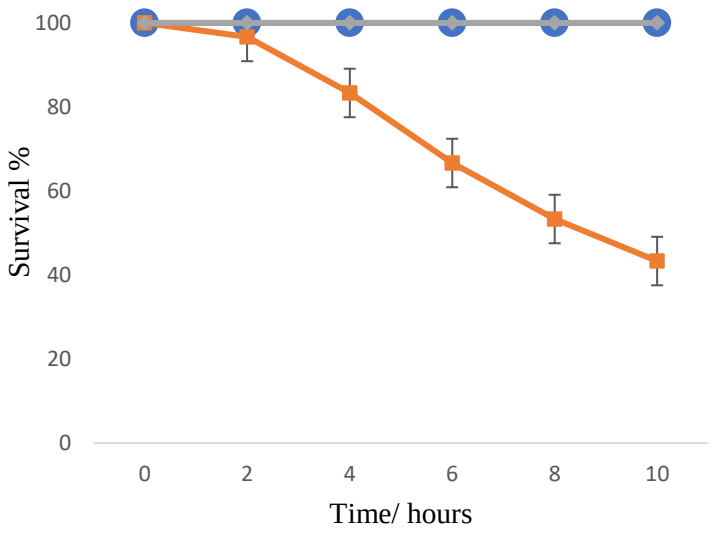

Figure 6.14. *Daphnia magna* survival in 0.001 mg/l metals mixture solution pretreated with *Chlamydomonas reinhardtii;* the control, 0 mg/l of metals mixture (●); 0.001mg/l of metals mixture (■), and 0.001mg/l of metals mixture that has been treated with *C. reinhardtii* phenotype (♦).

The control+ survival rate was 43.33% compared to the algae-treated solution of the metal mixture at a concentration of 0.001mg/l, where a 100% survival ratio was attained. The mortality rate of *D. magna* in the metals mixture in relation to the control was -0.43/h. Cohen's d value was 9.81, which means that *C. reinhardtii* pre-treatment has considerably improved the metals removal and survival ratios of *Daphnia magna*.

Table 6.2 represents a summary of the survival parameters of *Daphnia magna* in heavy metals solutions pre-treated with adapted *C. reinhardtii*.

Table 6.2. Survivability parameters of *D. magna* in the metal solution with and without treatment with *C.reinhardtii* phenotype

Metal / Parameter	Zn	Cu	Ni	Hg	Co	Cd	Mixture
Survival rate in the pre-treated solution	100%	86.67%	100%	100%	96.66%	90%	100%
Death rate in the untreated solution	46.67%	36.67%	46.67%	56.67%	56.67%	53.33%	56.67
Mortality rate per hour	-0.63/h	-0.73/h	-0.53/h	-0.43/h	-0.45/h	-0.52/h	-0.43/h

6.3 A comparison between the survival rate of *Daphnia magna* in the metal/s solution treated with *Chlorella vulgaris* and *Chlamydomonas reinhardtii* phenotypes produced from the adaptation to heavy metals

The incubation of *Chlorella vulgaris* phenotype in 0.01 zinc solution led to an increasing of *Daphnia Magna* survival rate from 50% to 100% compared to the untreated solution. Similarly, the use of the adapted *C. vulgaris* phenotype in the treatment of copper and mercury solutions led to 100% survival of *D. Magna* compared to the control. On the other hand, the use of the adapted phenotype reduced the toxicity of Ni, Co, Cd, and metal mixture to rise the survivability to 96.99%, 90%, 90% and 90% in the treated solutions, respectively (Table 6.3).

The use of *C. reinhardtii* in the treatment of metal solutions resulted in the increase of survivability of *D. magna* in the solution as follows: 100% survival rate in the case of zinc, cobalt, cadmium, and metal mixture. while the survival rates of mercury was 86.67%, 96.66% for copper and 90% for nickel.

Table 6.3. A comparison between the survival rate of *Daphnia magna* in the metal/s solution treated with *Chlorella vulgaris* and *Chlamydomonas reinhardtii* phenotypes produced from the adaptation to heavy metals

Metal	**The survivability of *Daphnia magna* in the solution treated with *Chlorella vulgaris* phenotype**	**The survivability of *Daphnia magna* in the solution treated with *Chlamydomonas reinhardtii* phenotype**
Zn	100%	100%
Hg	100%	86.67%
Co	96.99%	100%
Cd	100%	100%
Cu	90%	96.66%
Ni	90%	90%
Mixture	90%	100%

6.4 Results discussion

The survivability of *Daphnia magna* in heavy metal solutions was measured following incubation with microalgal biomass. Defined concentrations of Zn, Ni, Hg, Co, and Cd, Cu and metals mixture solution were used in individual and mixture treatments. The data were statistically compared, and Cohen's d values were calculated to elucidate the impact of algal-pretreatment on the survival of *Daphnia magna*. The results for the survivability of *Daphnia magna* demonstrates that both *C. vulgaris* 211/111 and *C. reinhardtii* 11/45 successfully reduced the impact of heavy metals on *Daphnia*. *C. vulgaris* was incubated in Zn, Cu, and Hg solutions for three days before incubating *D. magna* in the metal solutions; 100% of *D. magna* survived, which suggest that *C. vulgaris* entirely removed the metals from the solutions. In the case of nickel, the incubation of *C. vulgaris* reduced the toxicity of Ni on *D. magna*, and 96.66% survivability was observed. The survivability of *D. magna* in cobalt, cadmium, and metal mixture solutions previously incubated with *C. vulgaris* for three days, was 90%. When *C. reinhardtii* was incubated with copper, cadmium, and cobalt solutions for three days, survivability of 86.66%, 90%, and 96.66, respectively, for *D. magna* was obtained. 100% survivability of *D. magna* was attained when *C. reinhardtii* was incubated in

Zn, Ni, Hg, and metal mixture solutions. The similar survivability ratio found of *D. magna* for cadmium solution for *C. vulgaris* and *C. reinhardtii* suggests that some heavy metals may require a longer treatment time for a total removal via bioaccumulation (Jahan *et al.,* 2004). Jahan *et al.* (2004) indicted that bioaccumulation of heavy metals in microalgae, *C. vulgaris* and *Scenedesmus abundans* took longer time than biosorption process in addition to the difference of bioaccumulation efficiency between the two microalgal species. As discussed earlier, intercellular and extracellular mechanisms may be used in microalgal tolerance to heavy metals stress. Microalgae successfully eliminated the heavy metals' hazard impact on *Daphnia magna*. The results from biosorption tests also showed the importance of bioactivity of microalgae in cleaning polluted water bodies (Bajguz 2011; Gale,1986; Mac and Hardy, 1990; Manzoor *et al.,* 2019); these studies frequently reported the removal of heavy metals from the solution that reaches up to 100% removal in some of these studies.

CHAPTER SEVEN

CONCLUSION, FUTURE WORK, AND RECOMMENDATIONS

7.1 Conclusion

The results of investigations in this research established the success of adaptation strategies in improving the resistance of heavy metals in green microalgae *Chlorella vulgaris* and *Chlamydomonas reinhardtii*; this is because of their high content of functional groups that play a significant role in heavy metals tolerance. In addition, they are able to alter cell structure to increase the content of these functional groups to adapt to the new conditions. The gradual adaptation to the new conditions for an extended time helped microalgae to improve tolerance to heavy metals toxicity, making them a preferable biological candidate for bioremediation. The importance of these results comes from overcoming a major challenge that faces bioaccumulation use, where living cells are used and are impacted by metals toxicity. Adapting to high levels of heavy metals and improving microalgal resistance to heavy metals toxicity promotes the bioaccumulation process. In addition, the results obtained from enhanced metals bioaccumulation shows its role in improving the mitigation of heavy metals impact on the next organism in the food chain. This was observed from the *Daphnia magna* survivability experiment. The presence of eukaryotic microalgae, *Chlorella vulgaris* and *Chlamydomonas reinhardtii* successfully reduced heavy metals toxicity in the solution used to incubate *D. magna*. The results reveal the possibilities for further exploiting adaptation strategy with eukaryotic algae and designing sustainable bioremediation strategies

that will help mitigate environmental pollution. Therefore, this thesis presents the impact of in vitro strategies and key mechanisms to promote and improve microalgal bioaccumulation and biodegradation of toxic heavy metals in the aquatic environment.

7.2 Future work and recommendations

The application of adaptation strategies significantly improves microalgae resistance to heavy metals toxicity, emphasising this research's role in paving the way for the obtained phenotype to be scaled-up to be applicable for bioremediation in the environment. Furthermore, the findings from the adaptation experiment showed a successful impact of the selection and adaptation strategy on enhancing *C. vulgaris* 211/111 and *C. reinhardtii* 11/45 to the tolerance of single and mixtures of heavy metals. Therefore, it is recommended that the researchers increase knowledge of adaptation strategy with other algal species that might act differently to different metals or pollutants.

The result showed a significant change in cell structure upon exposure to heavy metals in both individual and mixture addition for both *C. vulgaris* 211/111 *and C. reinhardtii* 11/45. This could be a main objective for future studies by using long term exposure to metal ions to produce valuable cell components, this is because adaptation is a safe and natural strategy for promoting molecules production. Microalgae respond differently to heavy metal toxicity, such as the synthesis of phytochelatins, upregulation of antioxidants, accumulation of compatible solutes, accumulation of low-molecular-weight metabolites, and changes in phytohormone levels (Bajguz and Hayat 2009). Production of any of the previous cell

components depends on the mechanism used by microalgae to tolerate heavy metals stress (Bajguz, 2011). Therefore, further investigations on increase knowledge of adaptation strategy with other algal species which might contain different valuable cell molecules that are usable in industries and biotechnological applications.

Bioaccumulation of heavy metals by microalgae successfully promote the survivability of *Daphnia magna* in the solution indicating that this biological strategy creates a better environment for the next organism in the food chain following incubation with microalgal biomass. The results for the survivability of *D. magna* demonstrate that both *C. vulgaris* 211/111 and *C. reinhardtii* 11/45 successfully removed heavy metals danger from the aqueous solution, thereby reducing the impact of heavy metals on *D. magna*. It is recommended that further comparisons are made on the impact of the use of microalgal phenotypes with the use of other biological, chemical, and physical means on the organisms in the food chain and the long-term remediation.

In general, microalgae significantly decreased the concentration of heavy metals in solution. The results further indicated that the biosorption of heavy metals by microalgal biomass is accompanied by an induction of a variety of biochemical changes, some of which directly contribute to the metal tolerance capacity of the cell. Once adapted to higher concentrations of heavy metals, the microalgal biomass quickly eliminated metal ions from aqueous solutions by biosorption without any toxic impacts. Therefore, research is recommended into the broader production of adapted phenotypes to fulfil the environmental demands. In addition, further studies are required to:

- Investigate the effects of algal progeny on metal tolerance, optimise the sorption capacity of algae and study cell function and structure after acclimation.

- Test the interchangeability of acquired tolerance of algal phenotype to a particular heavy metal with another.

- Understand metals recovery from adapted microalgae to use them as a hunting tool, especially for valuable metals.

- Understand the use of adapted phenotypes with different environmental conditions and investigate their effect

- Optimise the adaptation- bioremediation process to reach the final production point of nontoxic outcomes like water and biomass.

- Optimise the process of using adapted phenotypes for bioremediation by adding supporting steps to the process to reach the ultimate goal of a clean environment.

APPENDICES

Appendix 1: 3N-BBM+V (Bold Basal Medium with 3-fold Nitrogen and Vitamins; modified)*

Stocks		**per litre**
1	NaNO3	75 g
2	CaCl2.2H2O	2.5 g
3	MgSO4.7H2O	7.5 g
4	K2HPO4.3H2O	7.5 g
5	KH2PO4	2.5 g
6	NaCl	17.5 g
7	Trace Elements (PIV)	
	Ensure elements are added in the following sequence:	
	Na2EDTA	0.75 g
	FeCl3.6H2O	0.097 g
	MnCl2.4H2O	0.041 g
	ZnCl2.6H2O	0.005 g
	CoCl2.6H2O	0.002 g
	Na2MoO4.2H2O	0.004 g
	Once elements are dissolved autoclave at 15 psi for 15 minutes.	
		Per 100 ml
8	Vitamin B1 (Thiamine hydrochloride)	0.12 g
	Filter sterile	
9	Vitamin B12 (Cyanocobalamin)	0.1 g
	Take 1 ml of this solution and add 99 ml Deionised water. Filter sterile.	

Medium	**per litre**
Stock solution 1 - 6	10 ml each
Stock solution 7 (Trace element)	6 ml
Stock solutions 8 - 9	1 ml each

Make up to 1 litre with distilled water. For agar add 15 g per litre Bacterial Agar. Autoclave at 15 psi for 15 minutes.

*CCAP (Culture Collection of Algae and Protozoa), SAMS Ltd, Scottish Marine Institute, Oban, Argyll, PA37 1QA, UK.

Appendix 2: f/2 Medium*

Stocks		**per litre**
1	NaNO3	15 g
2	NaH2PO4.2H2O	1.13 g
3	**Trace Elements (x10 concentration)**	**Per 200 ml**
	Na2EDTA	8.32 g
	FeCl3.6H2O	6.30 g
	MnCl2.4H2O	0.36 g
	ZnSO4.6H2O	0.044 g
	CoCl2.6H2O	0.02 g
	Na2MoO4.2H2O	0.012 g
	CuSO4.5H2O	0.02 g
4	**Vitamin mix: First make primary stocks of Cyanocobalamin and Biotin.**	
		Per 100 ml
	Vitamin B1 (Thiamine hydrochloride)	0.12 g
	Filter sterile	
	Biotin	0.1 g
	Dispense any excess primary stocks into 1 ml aliquots and freeze	
	For final vitamin mix stock solution:	**Per 200 ml**
	Vitamin B12 (Cyanocobalamin)	0.02 g
	Thiamine HCl (Vitamin B1)	1 ml
	Biotin	1 ml
Medium		**per litre**
	Stock solution 1	1.0 ml
	Stock solution 2	1.0 ml
	Stock solution 3 (Trace element)	0.1 ml
	Stock solutions 4 (Vits)	1.0 ml

Make up to 1 litre with filtered natural seawater. Adjust pH to 8.0 with 1M NaOH or 1 M HCl prior to autoclaving. For agar add 15g per litre Bacteriological Agar. Autoclave at 15 psi for 15 minutes.

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*Guillard RRL & Ryther JH (1962) Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervaceae (Cleve) Gran. Can. J. Microbiol. 8: 229-239.

Appendix 3: pH and temperature averages during adaptation experiments of microalgae

pH and temperature averages during adaptation experiments of *Chlorella vulgaris* to different heavy metals (values are mean ± standard error (n = 3))

Stages	1		2		3		4		5	
Treatment/perimeter	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**
Nickel	7±0.25	25±2	7±0.35	25±2	7±0.35	25±2	7±0.4	25±2	7±0.5	25±2
Cadmium	7±0.75	25±2	7±0.75	25±2	6±0.75	25±2	6±0.75	25±2	6±0.75	25±2
Copper	7±0.5	25±2	7±0.5	25±2	7±0.75	25±2	6±0.25	25±2	6±0.5	25±2
Zink	6±0.25	25±2	6±0.4	25±2	6±0.5	25±2	6±0.5	25±2	6±0.5	25±2
Cobalt	7±0.5	25±2	7±0.5	25±2	7±0.5	25±2	7±0.6	25±2	7±0.8	25±2
Mercury	7±0.5	25±2	7±0.5	25±2	7±0.5	25±2	7±0.6	25±2	7±0.75	25±2
Metal's mixture	7±0.75	25±2	7±0.75	25±2	6±0.25	25±2	6±0.3	25±2	6±0.5	25±2

pH averages during adaptation experiments of *Chlamydomonas reinhardtii* to different heavy metals

Stages	1		2		3		4		5	
Treatment/perimeter	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**	**pH**	**Temperature/ °C**
Nickel	7±0.25	25±2	7±0.35	25±2	7±0.35	25±2	7±0.4	25±2	7±0.5	25±2
Cadmium	7±0.5	25±2	7±0.5	25±2	7±0.5	25±2	7±0.75	25±2	7±0.75	25±2
Copper	7±0.5	25±2	7±0.5	25±2	7±0.5	25±2	7±0.75	25±2	7±0.75	25±2
Zink	6±0.3	25±2	6±0.5	25±2	6±0.5	25±2	6±0.5	25±2	6±0.5	25±2
Cobalt	7±0.5	25±2	7±0.5	25±2	7±0.5	25±2	7±0.75	25±2	7±0.75	25±2
Mercury	7±0.5	25±2	7±0.5	25±2	7±0.5	25±2	7±0.75	25±2	7±0.75	25±2
Metal's mixture	7±0.5	25±2	7±0.7	25±2	6±0.25	25±2	6±0.5	25±2	6±0.5	25±2

Appendix 4: Transformation table from percentage to Probits

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	**0.0**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
99	7.33	7.37	7.41	7.46	7.58	7.65	7.75	7.75	7.88	8.09

Appendix 5: pH, dissolved oxygen, and temperature averages of survival experiments for *Daphnia magna*

Treatment/perimeter	pH	Temperature/ °C	Dissolved oxygen mg/ l
Nickel	7±0.25	25±2	6±0.25
Cadmium	7±0.75	25±2	6±0.25
Copper	7±0.1	25±2	6±0.25
Zink	7±0.25	25±2	6±0.25
Cobalt	7±0.5	25±2	6±0.25
Mercury	7±0.5	25±2	6±0.25
Metal's mixture	7±0.2	25±2	6±0.25

Values are mean ± standard error (n = 3)

Reference

Abbasi S. A. and Soni R. (1989) Relative toxicity to seven heavy metals and their safe concentrations intern. J. envern studies (UK) 35 121-22.

Abbasi SA, Nipaney PC, Soni R (1985) Environmental consequences of the inhibition in the hatching of pupae of Aedes aegypti by mercury, zinc and chromium—the abnormal toxicity of zinc. Int J Environ Stud 24(2):107–114.

Abbasi, S.A., Abbasi, N., Soni, R., 1998. Heavy Metals in the Environment. Mittal Publications, New Delhi.

Abdel-Atty MMFarag NAKassab SESerya RATAbouzid KAM (2014) Design, synthesis, 3D pharmacophore, QSAR, and docking studies of carboxylic acid derivatives as histone deacetylase inhibitors and cytotoxic agents Bioorganic Chemistry 57:65–82.

Abdelhaleim AF, Abdo Soliman JS, Amer AY, Abdo Soliman JS. Association of Zinc Deficiency with Iron Deficiency Anemia and its Symptoms: Results from a Case-control Study. Cureus. 2019 Jan 2;11(1):e3811. doi: 10.7759/cureus.3811. Erratum in: Cureus. 2019 Apr 1;11(4):c20. Abdo Soliman JS [corrected to Abdelhaleim AF]. PMID: 30868025; PMCID: PMC6402732.

Abdel-Ghani, Nour T and Ghadir El-Chaghaby. (2014) "BIOSORPTION FOR METAL IONS REMOVAL FROM AQUEOUS SOLUTIONS: A REVIEW OF RECENT STUDIES".

Abomohra, Abd El-Fatah. Wenbiao Jin, Renjie Tu, Song-Fang Han, Mohammed Eid, Hamed Eladel,(2016).Microalgal biomass production as a sustainable feedstock for biodiesel: Current status and perspectives, Renewable and Sustainable Energy Reviews,Volume 64,Pages 596-606,ISSN 1364-0321.

Abu-Rezq AN, Tolba AS, Khuwaja GA, Foda SG. Best parameters selection for wavelet packet-based compression of magnetic resonance images. Comput Biomed Res. 1999 Oct;32(5):449-69. doi: 10.1006/cbmr.1999.1523. PMID: 10529302.

Adeniyi, Oladapo Martins. Ulugbek Azimov, Alexey Burluka,(2018). Algae biofuel: Current status and future applications,Renewable and Sustainable Energy Reviews,Volume 90,Pages 316-335,ISSN 1364-0321.

Adriano D. C. (1980) Trace elements in the aquatic environment springer Verlag. New York.

Ahluwalia, S. S. & Goyal, D. 2007. Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioresource technology, 98, 2243-2257.

Ahner BA, Kong S, Morel FMM (1995a) Phytochelatin production in marine algae. 1. An interspecies comparison. Limnol Oceanogr 40: 649–657.

Ahner BA, Kong S, Morel FMM (1995b) Phytochelatin production in marine algae. 2. Induction by various metals. Limnol Oceanogr 40: 658–665.

Aksu Z, Açikel Ü. (2002) Modelling of a single-staged bio separation process for simultaneous removal of iron (III) and chromium (VI) by using Chlorella vulgaris. Biochem Eng J. 4(3): 229-38.

Alloway, B.J and Ayres,D.C. (1993). Chemical Principles of Environmental Pollution, 1st ed. Blackie Academic and Professional, London.

Al-rub, F.A.A., El-Naas, M.H., Ashour, I., Al-Marzouqi, M., 2006. Biosorption of copperon Chlorella vulgaris from single, binary and ternary metal aqueous solutions.Pro. Biochem. 41, 457–464.

Al-Waeli A, Pappas AC, Zoidis E, Georgiou CA, Fegeros K, Zervas G. The role of selenium in cadmium toxicity: interactions with essential and toxic elements. Br Poult Sci. 2012;53(6):817-27. doi: 10.1080/00071668.2012.751523. PMID: 23398427.

Anbalagan, R. and Sivakami, R. 2018. Bioaccumulation of Heavy Metals in Water and Algae of Mukkombu in the River Cauvery System, Tiruchirappalli District, Tamil Nadu, India. Int.J.Curr.Microbiol.App.Sci. 7(1): 1067-1072.

ANZECC & ARMCANZ 2000. Australian and New Zealand Guidelines for Fresh and Marine Water Quality, Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand, Canberra.

Arceneaux JE, Boutwell ME, Byers BR (1984) Enhancement of copper toxicity by siderophores in Bacillus megaterium. Antimicrob Agents Chemother 25: 650–652.

Arunakumara, K.K.I.U., Zhang, X. (2008) Heavy metal bioaccumulation and toxicity with special reference to microalgae. J. Ocean Univ. China 7, 60–64.

Asthana RK, Singh AL, Singh SP (1993) Comparison of Ni–sensitive and N–resistant strains of Nostoc muscorum. World J Microbiol Biotechnol 9: 323–327.

Azimi A., Azari A., Rezakazemi M., Ansarpour M. (2017) Removal of heavy metals from industrial wastewaters: A review ChemBioEng. Rev., 4 , pp. 37-59, 10.1002/cben.201600010.

Azov, Y., G. Shelef, and R. Moraine. 1982. “Carbon limitation of biomass production in high-rate oxidation ponds.” Biotechnology and Bioengineering 24 (3): 579-594. Accessed 6 2, 2019. https://ncbi.nlm.nih.gov/pubmed/18546349.

Azevedo, N. F., Bragança, S. M., Simes, L. C., Cerqueira, L., Almeida, C., Keevil, C. W., & Vieira, M. J. (2012). Proposal for a method to estimate nutrient shock effects in bacteria. BMC Research Notes, 5. https://doi.org/10.1186/1756-0500-5-422.

Bajguz A. (2011). Suppression of Chlorella vulgaris growth by cadmium, lead, and copper stress and its restoration by endogenous brassinolide. Archives of environmental contamination and toxicology, 60(3), 406–416.

Bajguz A, Hayat S. Effects of brassinosteroids on the plant responses to environmental stresses. Plant Physiol Biochem. 2009;47:1–8. doi: 10.1016/j.plaphy.2008.10.002.

Baran, A., Bıcak, E., Baysal, Ş. H. & Önal, S. 2007. Comparative Studies On The Adsorption Of Cr(Vi) ions on to various sorbents. Bioresource Technology, 98, 661-665.

Bariaud A, Bury M, Mestre JC (1985) Mechanism of Cd2+ resistance in Euglena gracilis. Physiol Plant 63: 382–386.

Basci, Nurgul. Erdem Kocadagistan, Beyhan Kocadagistan,(2004). Biosorption of copper (II) from.

Bates, S.S., Tessier. A., Campbell, P. G. C., and Buffle, J., (1982) Zinc absorption and transport by Chlamydomonas variabilis Scenedesmus subspecious (Chlorophyceae) growing in Simi continuous culture, J. phycol. 18, 521.

Bednarz, T., A. Losi, W. Gärtner, P. Hegemann, and J. Heberle. 2004. "Functional variations among LOV domains as revealed by FT-IR difference spectroscopy." photochem. photobiol. Sci 3 (6): 575-579.

Ben-Bassat, D. and Mayor, A. M. (1977)reduction of HgCl2 by Chlorella, plant 40-157.

Benoit, J., J. Guilhot, A. Mathe, M.-T. Nourissier-Sobesky, Y. Pélicier and P. Sivadon. "The Psychotherapeutic Process: 10th Congress, Paris, July 1976." (1978).

Berman E. (1980) toxic metals on their analysis Heyden and sons Limited, London.

Bilal M, Rasheed T, Sosa-Hernández JE, et al. Biosorption: An Interplay between Marine Algae and Potentially Toxic Elements-A Review. Marine Drugs. 2018 Feb;16(2). DOI: 10.3390/md16020065. PMID: 29463058; PMCID: PMC5852493.

Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S., Pangilinan, J. & Polle, J. 2010. The Chlorella variabilis NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. The Plant Cell, 22, 2943-2955.

Bockris, J. and Reddy, A. (1977) Modern Electrochemistry: An Introduction to an Interdisciplinary Area, Volume 2. 3th Edition, Plenum Rosetta Edition, New York.

Bowen H. J. M. (1966) Trace elements in biochemistry. Academic press London. New York. 196~. 241 p.

Briffa, J., Sinagra, E., Blundell, R., 2020. Heavy metal pollution in the environment and their toxicological effects on humans. Heliyon 6 (9), e04691.

Brinza, L., Dring, M. J. & Gavrilescu1, M. 2007. MARINE MICRO AND MACRO ALGAL SPECIES AS BIOSORBENTS FOR HEAVY METALS. Environmental Engineering and Management Journal, 6.

Brune, D. (1980). Carbon and light limitation in mass algal culture. Retrieved 6 3, 2019, from http://osti.gov/scitech/biblio/7058423.

Brynjólfsson, S. 2013. Enhancement of carotenoid biosynthesis in the green microalga.

Bulgariu D, Bulgariu L (2016) Potential use of alkaline treated algae waste biomass as sustainable biosorbent for clean recovery of cadmium (II) from aqueous media: batch and column studies. J Clean Prod 112:4525–4533.

Burnison G, Wong, PTS, Chan, Y. Silverbury (1975) Toxicity of cadmium to fresh water algae. Proceeding of canadian fedral biological society, 182.46.

Casey, C.E. and Hambidge, K.M. (1980) Epidemiological Aspects of Human Zinc Deficiency. In: J. O. Nriagu, Ed., Zinc in the Environment, Part II: Health Effects, John Wiley, New York.

Cawse, P. A. (1987) trace and major elements in the atmosphere at rural locations in Great Britain 1972 -1981 in Coughtrey Martin and also earth pollutant transport on fate in ecosystems black hole scientific publication Oxford 89-112.

Chatteijee, A. and S. Schiewer, B io so rp tio n o f C adm ium (II) Io n s b y C itrus P eels in a P a cked B ed C olum n: E ffect o f P rocess P aram eters an d C o m parison o f D ifferen t B reakthrough C urve M odels. Clean-Soil Air Water, 2011. 39(9): p. 874 881.

Chaudhry, G. R. 1994. Biological degradation and bioremediation of toxic chemicals, Dioscorides Press.

Chen, LI, Y., Y.-F., CHEN, P., MIN, M., ZHOU, W., MARTINEZ, B., ZHU, J. & RUAN, R. 2011. Characterization of a microalga Chlorella sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresource technology, 102, 5138-5144.

Chia-Chay, T., Redzwan, G., Hong-Hooi, L., Soon-Kong, Y., Surif, S., & Abdul-Talib, S. (2010, December). Copper (II) biosorption characteristic of Pleurotus spent mushroom compost. In *2010 international conference on science and social research (CSSR 2010)* (pp. 6-10). IEEE.

Chiu, S.-Y., Kao, C.-Y., Chen, C.-H., Kuan, T.-C., Ong, S.-C. & Lin, C.-S. 2008. Reduction of CO 2 by a Chlamydomonas reinhardtii by adaptive laboratory evolution. Bioresource technology, 147, 499-507.

Clarke SE, Stuart J, Sanders–Loehr J (1987) Induction of siderophore activity in Anabaena spp. and its moderation of copper toxicity. Appi Environ Microbiol 53: 917–922.

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 18: 3325–3333.

Coates, John. 2006. “Interpretation of Infrared Spectra, A Practical Approach.” Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation.

Commission of the European Communities. Study D8369. (1979). Inter-laboratory Test Programme concerning the study of the ecotoxicity of a chemical substance with respect to

Cooper, G. M. and Goodman, T. M. (1975) rren Hair. N. England Journal of Medicine 292, 483-484.

Cooper, Helen. 2015. Use of naturally acidified environments to determine tolerance and acclimation potential of marine organisms to ocean acidification.

Costas, Eduardo, Emma Huertas, Beatriz Baselga-Cervera, Camino García-Balboa, and Victoria López-Rodas. 2014. “Phytoplankton Ability to Physiological Acclimatization and Genetic Adaptation to Global Warming.” International Journal of Biology 6 (4): 24.

Coughtrey P. (1987), Pollutant transport and fate in ecosystems, Blackwell Scientific Publications (Ed.).

Das, N. & Das, D. 2013. Recovery of rare earth metals through biosorption: An overview. Journal of Rare Earths, 31, 933-943.

De Filippis LF (1978) The effect of sublethal concentrations of mercury and zinc on Chlorella. IV. Characteristics of a general reducing enzyme system for metallic ions. Z Pflanzenphysiol 86: 339–352.

De Filippis LF, Pallaghy CK (1994) Heavy metals: Sources and biological effects. In: Rai LC, Gaur JP, Soeder CJ (eds) Algae and water pollution. E. Schweizerbart’sche Verlagsbuchhandlung, Stuttgart, pp 31–77.

De Filippis, L. F. and Pallaghy, C. K. (1976) The effect of sublethal concentration of mercury and zinc on Chlorella sp.79-323.

Demayo, A., Taylor, M.C., Taylor, K.W. and Hodson, P.V. (1982) Toxic Effects of Lead and Lead Compounds on Human Health, Aquatic Life, Wildlife Plants, and Livestock. Critical Reviews in Environmental Control, 12, 257-305.

Diep P, Mahadevan R, Yakunin AF. Heavy Metal Removal by Bioaccumulation Using Genetically Engineered Microorganisms. Front Bioeng Biotechnol. 2018 Oct 29;6:157. doi: 10.3389/fbioe.2018.00157. PMID: 30420950; PMCID: PMC6215804.

Dragosits, M., Mozhayskiy, V., Quinones-Soto, S., Park, J. & Tagkopoulos, I. 2013. Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in Escherichia coli. Mol Syst Biol, 9.

Ebert D. (2005)Ecology, Epidemiology, and Evolution of Parasitism in Daphnia [Internet]. Bethesda (MD): National Center for Biotechnology Information (US; Chapter 2, Introduction to Daphnia Biology. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2042/.

Edmundson, S. J. 2012. Algae Cultivation on Landfill Leachate: Exploring a Novel Nutrient Supply for Sustainable Bio-Resource Production. University of Florida.

Eisler, R. 1971. Cadmium poisoning in FunduZus heteroclitus and other marine organisms. J, Fish. Res. Bd. Can. 28: 1225-1234.

El Ouaer, M., HALAOUI, M., TRABELSI, I. & HASSEN, A. 2016. Adaptation and use of Chlorella sp. for landfill leachate treatment. International Journal of Innovation and Applied Studies, 14, 807.

Eladel,(2016).Microalgal biomass production as a sustainable feedstock for biodiesel: Current status and perspectives, Renewable and Sustainable Energy Reviews,Volume 64,Pages 596-606,ISSN 1364-0321.

Elshorbagy, walid (2013). water treatment. 10.5772/2883isbn: 978-953-51-0928-0.

Enfrin, M., Dumée, L. F., & Lee, J. (2019). Nano/microplastics in water and wastewater treatment processes–origin, impact and potential solutions. Water research, 161, 621-638.

Engström, N. 2012. Cultivation of seven different species of marine microalgae using simulated flue gas mimicking effluents from paper mills as carbon source.

Environment Canada. (1996) Biological test method. Acute Lethality Test Using Daphnia spp. EPS 1/RM/11. Environment Canada, Ottawa, Ontario, Canada. EPA OPPTS 850.1010. (1996). Ecological Effects Test Guidelines - Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids.

Erbe JL, Adams AC, Taylor KB, Hall LM (1996) Cyanobacteria carrying a smt–lux transcriptional fusion as biosensors for the detection of heavy metal cations. J Ind Microbiol 17: 80–83.

Erdmann, Norbert, and Martin Hagemann. 2001. Salt Acclimation of Algae and Cyanobacteria: A Comparison. Accessed 5 7, 2019. https://link.springer.com/chapter/10.1007/978-3-642-59491-5_11.

Ernest, M. (1984) Introduction on environmental chemistry and global cycles of chromium, nickel, cobalt, barrylium, arsenic, cadmium, and selenium on their derivatives. Toxicological and environmental chemistry 8,9-38.

Ernst WHO (1990) Poly y-glutamylcysteinyl glycines or phytochelatins and their role in Cadmium tolerance of Silene vulgaris. Plant Cell Physiol 13: 913–921.

Ernst WHO, Verkleij JAC, Schat H (1992) Metal tolerance in plants. Acta Bot Neerl 41:229–249.

Esposito, A., Pagnanelli, F., Lodi, A., Solisio, C. & Veglio, F. 2001. Biosorption of heavy metals by Sphaerotilus natans: an equilibrium study at different pH and biomass concentrations. Hydrometallurgy, 60, 129-141.

Expósito N, Carafa R, Kumar V, Sierra J, Schuhmacher M, Papiol GG. Performance of Chlorella Vulgaris Exposed to Heavy Metal Mixtures: Linking Measured Endpoints and Mechanisms. International Journal of Environmental Research and Public Health. 2021; 18(3):1037.

Falk, H. L. (1974) Chemical Carcinogens, mutagens on teratogens. In: WD Mckee (Ed) environmental problems in medicine. Charlies C. Thomas's publisher Springfield, Illinois.

FAO (Food and Agriculture Organization), "Compilation of Legal Limits for Hazardous Substances in Fish and Fishery Products," FAO Fisheries Circular No. 464, 1983, pp. 5-100.

Farhadian M, Vachelard C, Duchez D, et al. (2008) In situ bioremediation of monoaromatic pollutants in groundwater: A review. Bioresource Technology Journal 99: 5296–5308.

Fergusson, J. E. (1990). The heavy elements: chemistry, environmental impact and health effects\Jack E. Fergusson (No. 628.53 F4.).

Finney D.J. (1978). Statistical Methods in Biological Assay. 3rd ed. London. Griffin, Weycombe, UK.

Fisher, A. A., and Shapiro, A. (1956). J. Amer. med. Ass., 161, 717.

Fisher, J and Shapiro, J. 1964. Effect of yellow organic acids on iron and other metals in water. J. Am. Water Works Assoc. 56(8): 1062-1082.

Fisher, Ronald A. (1930) The Genetical Theory of Natural Selection. Oxford: Clarendon Press.

Fishner A. A. and Sharpiro A. (1956) allergic eczematous contact limited dematitis due to metallic nickel journal of the American medicine Medical Association 161,717-727.

Fogg GE, Westlake DF (1955) The importance of extracellular products of algae in freshwater. Proc Int Assoc Theor Appi Limnol 12: 2119–2132.

Fondriest Environmental, Inc. "pH of Water." Fundamentals of Environmental Measurements. 19 Nov. 2013. Web. < http://www.fondriest.com/environmentalmeasurements/parameters/water-quality/ph/ >.https://secure.clixoo.com/purchase/oilgae/wwt/report.html.

Forbes, A. A. and Krimmel, B. A. (2010) Evolution Is Change in the Inherited Traits of a Population through Successive Generations. *Nature Education Knowledge* 3(10):6

Forstenr, U. (1980). Cadmium in polluted sediments. In: J. Nriagu (Ed). Cadmium in the eviironment part !. Wiley. New york.

Foster PL (1977) Copper exclusion as a mechanism of heavy metal tolerance in a green alga. Nature 269: 322–323.

Foster PL (1982) Metal resistance of Chlorophyta from rivers polluted by heavy metals. Freshwater Biol 12: 41–61.

Frac, Magdalena, Stefania Jezierska-Tys and Jerzy Tys. "Microalgae for biofuels production and environmental applications: A review." African Journal of Biotechnology 9 (2010): 9227-9236.

Freyer G . Functional morphology and the adaptive radiation of the Daphniidae (Branchiopoda: Anomopoda). Philos Trans R Soc. 1991;331:1–99.

Fu Z, Xi S. The effects of heavy metals on human metabolism. Toxicol Mech Methods. 2020 Mar;30(3):167-176. doi: 10.1080/15376516.2019.1701594. Epub 2019 Dec 17. PMID: 31818169.

Fu, W., Gudmundsson, O., Feist, A. M., Herjolfsson, G., Brynjolfsson, S. & Palsson, B. Ø. 2012. Maximizing biomass productivity and cell density of Chlorella vulgaris by using light-emitting diode-based photobioreactor. Journal of biotechnology, 161, 242-249.

Fu, W., Guðmundsson, Ó., Paglia, G., Herjólfsson, G., Andresson, Ó. S., Palsson, B. Ø. & Brynjólfsson, S. 2013. Enhancement of carotenoid biosynthesis in the green microalga Dunaliella salina with light-emitting diodes and adaptive laboratory evolution. Applied microbiology and biotechnology, 97, 2395-2403.

Fu, W., Guðmundsson, Ó., Paglia, G., Herjólfsson, G., Andresson, Ó. S., Palsson, B. Ø. & future applications, Renewable and Sustainable Energy Reviews, Volume 90,Pages 316-335,ISSN 364-0321.

Gao, Yang, Yunluan Cui, Wei Xiong, Xiaobo Li, and Qingyu Wu. 2009. "Effect of UV-C on algal evolution and differences in growth rate, pigmentation and photosynthesis between prokaryotic and eukaryotic algae." Photochemistry and Photobiology 85 (3): 774-782.

Gate, H. V. and Dodakundi, G. B. (1979) Effect of dye factory effluent on the developing emperyos of Microhyla orate Indian journal of environmental health 20,239.

Gale N.L. The role of algae and other microorganisms in metal detoxification and environmental clean-up. Biotechnol. Bioeng. Symp. 1986;16:171–180.

Gatten, R. E., Echternacht, A. C., & Wilson, M. A. (1988). Acclimatizationversus acclimation of activity metabolism in a lizard.PhysiologicalZoology,61(4), 322–329.

Gaur, V.K., Sharma, P., Gaur, P., Varjani, S., Ngo, H.H., Guo, W., Chaturvedi, P., Singhania, R.R., 2021. Sustainable mitigation of heavy metals from effluents: Toxicity and fate with recent technological advancements. Bioengineered (Doi: 10.1080/21655979.2021.1978616).

Gaur, J. P., & Rai, L. C. (2001). Heavy Metal Tolerance in Algae. Retrieved 6 19, 2019, from https://link.springer.com/chapter/10.1007/978-3-642-59491-5_12.

Genter RB (1996) Ecotoxicology of inorganic chemical stress on algae. In: Stevenson RJ, Bothwell ML, Lowe RL (eds) Algal ecology — freshwater benthic ecosystems. Academic Press, California, pp 403–468.

Genter RB, Cherry DS, Smith EP, Cairns J Jr. 1987. Algal-periphyton population and community changes from zinc stress in stream mesocosms. Hydrobiologia 153:261–275.

Gesamp. IMO/FAO/UNESCO/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution: Report of the seventeenth session. Geneva, Switzerland: World Health Organization; 1987. (Reports and Studies No. 31)

Gimmler H, Weis U (1992) *Dunaliella acidophila* - life at pH 1.0. In: Avron M, Ben Amotz A (eds) Dunaliella. physiology, biochemistry and biotechnology. CRC Press, Boca Raton, Florida, pp 99–133.

Gledhill M, Nimmo M, Hill SJ, Brown MT (1999) The release of copper-compexing ligands by the brown alga Fucus vesiculosus (Phaeophyceae) in response to increasing total copper levels. J Phycol 35: 501–509.

Gorelova, O. A., O. I. Baulina, T. T. Ismagulova, Kamilya Kokabi, E. S. Lobakova, Irina Selyakh, Larisa Semenova, et al. 2019. "Stress-induced changes in the ultrastructure of the photosynthetic apparatus of green microalgae." Protoplasma 256 (1): 1-17.

Greene B, Darnall DW (1990) Microbial oxygenic photoautotrophs (cyanobacteria and algae) for metal-ion binding. In: Ehrlich HL, Brierley CL (eds) Microbial mineral recovery. McGraw-Hill, New York, pp 227–302.

Grill E, Winnacker E-L, Zenk MH (1987) Phytochelatins: a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc Natl Acad Sci USA 84: 439–443.

Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environmental Health and Safety Publication. Series on Testing and Assessment. No. 23. Paris 2000.

Gulnaz, O.; Saygideger, S. & Kusvuran, E.J. (2005). Studyof Cu (II) biosorption by dried activated sludge: Effectof physico-chemical environment and kinetics study.Journal of Hazardous Materials, 120(1-3):193-200.

Gupta, C.K. and Krishnamurthy, N. (2004) Extractive Metallurgy of Rare Earths. CRC Press, Boca Raton, Florida.

Gupta, M.P. Sharma, N. B. Sarin, and A. K. Sinha, "Differential response of arsenic stress in two varieties of Brassica juncea L," Chemosphere, vol. 74, no. 9, pp. 1201–1208, 2009.

Haines DW, Rogers KH, Rogers FEJ. 1987. Loose and firmly attached epiphyton: their relative contributions to algal and bacterial carbon productivity in a Phragmites marsh. Aquatic Botany 29:169-176.

Hájek, Josef, Jana Kvíderová, and Michael Roger Worland. 2010. The ice nucleation activity of green algae isolated from low-temperature environments.

Hall A (1980) Heavy metal cotolerance in a copper tolerant population of the marine fouling alga Ectocarpus siliculosus (Dillw.) Lyngbye. New Phytol 85: 73–78.

Hall, J.L. Cellular mechanisms for heavy metal detoxification and tolerance, Journal of Experimental Botany, Volume 53, Issue 366, 1 January 2002, Pages 1–11.

Hall, K. J, Yesaki, I. and Chan, J. (1976) trace metals and chlorinated hydrocarbons in the sediments of metropolitan watershed technical reports number 10 wastewater research centre university of British Columbia.

Haney, J.F., And C. Buchanan. 1987. Distribution and Biogeography on Daphnia in the Arctic . Mem. Ist. Ital. Idrobiol. **45**: 77-105.

Harris EH. Introduction to Chlamydomonas and its laboratory use. In: Harris EH, Stern DB, Witman GB, editors. The Chlamydomonas Sourcebook. Oxford: Academic Press; 2009.

Hart, B. T. (1982): A water quality criteria for heavy metals. Australian Governmental Publishing Services, Canberra, Australia.

Hassett, J. M., Janet, J. C., Smith, J. E. (1979) control of heavy metals and the environment using algae in management on control of heavy metal and heavy metals environment, conference England -Scotland CEP consultants Ltd 210.

Hirooka,S., Hirose, Y.,Yu Kanesaki, Sumio Higuchi, Takayuki Fujiwara, Ryo Onuma, Atsuk o Era, Ryudo Ohbayashi, Akihiro Uzuka, Hisayoshi Nozaki, Hirofumi Yoshikawa, Sh in-ya Miyagishima. Acidophilic green algal genome provides insights into adaptation to an acidic environment. Proceedings of the National Academy of Sciences Sep 2017, 114 (39) E8304-E8313; DOI: 10.1073/pnas.1707072114.

Hobscheman J. R. (1967) Effect of copper on the cry fish or connects rustalus Girard mode of toxic action crustaceana 12, 141-110.

Hodson, R. D. 1979. "Labor in the Monopoly, Competitive and the State Sectors of Production." Politics and Society 8 (3/4) : 429-480.

Hoffmann, J.P. (1998), WASTEWATER TREATMENT WITH SUSPENDED AND NONSUSPENDED ALGAE. Journal of Phycology, 34: 757-763.

Horvath, D. I. (1976) trace element and health. In: Neubern P.M. trace substances and health miracle Decker incorporation New York and basil 319 357.

Horváth, Z., Palla, L. (1976) Dyons in classical SU(3) gauge theory and a new topologically conserved quantity. Physical Review D, 14 (6). 1711-1714 doi:10.1103/physrevd.14.1711.

Hosono, H., Uemura, I., Takumi, T., Nagamune, T., Yasuda, T., Kishimoto, M., Nagashima, H., Shimomura, N., Natori, M. & Endo, I. 1994. Effect of culture temperature shift on the cellular sugar accumulation of Chlorella vulgaris SO-26. Journal of fermentation and bioengineering, 78, 235-240.

Howe, G., and S. Merchant, 1992 The biosynthesis of membrane and soluble plastidic c-type cytochromes of Chlamydomonas reinhardtii is dependent on multiple common gene products. EMBO J. 11: 2789–2801.

Hudson P. V., Blunt, B. R., Jensen. D. and Morgan, S. (1979) Effect of fish egg on a predicted and observed to ronak toxicity of lead train boat or root and Lake Ontario waters journal of Great Lakes research 5 84-89.

Hughes, Adam D., and Andréa G. Grottoli. 2013. "Heterotrophic Compensation: A Possible Mechanism for Resilience of Coral Reefs to Global Warming or a Sign of Prolonged Stress?" PLOS ONE 8 (11).

Hutchinson T.C. (1973) comparative study studies of the toxic of the toxicity of heavy metals to phytoplankton on their synergetic synergistic interactions water pollution research in Canada 8, 68-90.

Hutchinson T.C. and stocks P. M. (1975) Heavy metals toxicity and although bioassays water quality parameters am SoC test master space tech Paul be 573PP 320-343.

Hutchinson, G.E., 1969. Eutrophication, past and present. Eutrophication: Causes, Consequences, Correctives. National Academy of Sciences, Washington, D.C, pp. 17±26.

Ismail, I., and Moustafa, T. (2016). Biosorption of heavy metals by bacteria isolatedfrom activated sludge.Heavy Met. Sources, Toxic. Remediat. Tech.91, 131–174.

ISO 6341. (1996). Water quality - Determination of the inhibition of the mobility D. magna Straus (Cladocera, Crustacea) - Acute toxicity test. Third edition, 1996.

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary toxicology, 7(2), 60–72. https://doi.org/10.2478/intox-2014-0009.

Javanbakht V, Alavi SA, Zilouei H. Mechanisms of heavy metal removal using microorganisms as biosorbent. Water Sci Technol. 2014;69(9):1775-87. doi: 10.2166/wst.2013.718. PMID: 24804650.

Ji, Yongcheng, Sherrell, Robert M., (2008), Differential effects of phosphorus limitation on cellular metals in Chlorella and Microcystis, Limnology and Oceanography, 53, doi: 10.4319/lo.2008.53.5.1790.

Jin X, Kushner DJ, Nalewajko C (1996) Nickel uptake and release in nickel-resistant and -sensitive strains of Scenedesmus acutus f. alternans (Chlorophyceae). Environ Exp Bot 36: 401–411.

JinShui Yang, Jing Cao, GuanLan Xing, HongLi Yuan (2015) Lipid production combined with biosorption and bioaccumulation of cadmium, copper, manganese, and zinc by oleaginous microalgae Chlorella minutissima UTEX2341.

Jodłowska, Sabina, and Adam Latała. 2012. Mechanisms of Photoacclimation on Photosynthesis Level in Cyanobacteria.

Jokel, M. (2017) Regulation of photosynthesis under dynamic light conditions in Chlamydomonas reinhardtii: Impact on hydrogen production. Researchgate.

Jones, D., Williamson, K. & Owen, A. 2006. Phytoremediation of landfill leachate. Waste Management, 26, 825-837.

Kalbasi, Roozbeh, and Neda Mosaddeg. 2012. "Application of Atomic Absorption for Determination of Metal Nanoparticles in Organic-Inorganic Nanocomposites.".

Kästner. 1993. Lehrbuch der speziellen Zoologie, Band 1, Teil 4. Heidelberg, Germany, Elsevier GmbH, Spektrum Akademischer Verlag.

Kessler E (1985) An extremely cadmium sensitive strain of Chlorella. Experientia 41: 1621.

King P, Anuradha K, Lahari SB, Kumarb YP, Prasad VSRK (2008) Biosorption of zinc from aqueous solution using Azadirachta indica bark: equilibrium and kinetic studies. J Hazard Mater 152:324–329.

Kirchgessner M., Roth, P., Spoerl R., Schnegg, Welgand (1977) A comparative view of trace elements on growth nutrition metabolism 21,119-143. Kirchgessner, M., R. J. Kellner, F. X. Roth and K. Ranfft. 1977. Zur schatzung des futterwertes mittels rohfaser und der zellwandfraktionen der detergentien-analyse. Landwirtsch. Forsch. 30:245-250.

Klimmek, S., Stan, H., Wilke, A.L., Bunke, G., & Buchholz, R. (2001). Comparative analysis of the biosorption of cadmium, lead, nickel, and zinc by algae. *Environmental science & technology*, *35 21*, 4283-8.

Korkaric, Muris, Muris Korkaric, Mao Xiao, Mao Xiao, Renata Behra, Rik I.L. Eggen, and Rik I.L. Eggen. 2015. "Acclimation of Chlamydomonas reinhardtii to ultraviolet radiation and its impact on chemical toxicity." Aquatic Toxicology 167: 209-219.

Kumar, V., & Nanda, M. (2018). Microalgae: A Promising Tool for Remediation of Heavy Metals. In A. Rathoure (Ed.), *Biostimulation Remediation Technologies for Groundwater Contaminants* (pp. 141-153). IGI Global.

Kratochvil, D.B. Volesky (1998) Advances in the biosorption of heavy metals, Trends in Biotechnology, 16 (7) (1998), pp. 291-300.

Krom, M. D., N. Kress,S.Brenne, And L. I. Gordon. 1991.Phosphorus limitation of primary productivity in the EasternMediterranean Sea. Limnol. Oceanogr. 36: 424–432.

Lagerwerf, J. V. (1972). Lead,mercury, and cadmium as environmental contaminats. In: Micronutrients in agriculture soil science society of america incorporation, madison, wisconsin.

Lauwerys R, Buchet JP, Roels H, Hubermont G. Placental transfer of lead, mercury, cadmium, and carbon monoxide in women. I. Comparison of the frequency distributions of the biological indices in maternal and umbilical cord blood. Environ Res. 1978 Apr;15(2):278-89. doi: 10.1016/0013-9351(78)90104-4. PMID: 668658.

Lee, Y.-K. 2007. Algal Nutrition – Heterotrophic Carbon Nutrition. Handbook of Microalgal Culture. Blackwell Publishing Ltd.

Li, Y., Chen, Y.-F., Chen, P., Min, M., Zhou, W., Martinez, B., ZHU, J. & RUAN, R. 2011. Characterization of a microalga Chlorella sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresource technology, 102, 5138-5144.

Li, L., Wu, J., Yang, L., Wang, H., Xu, Y., & Shen, K. (2021). Fourier Transform Infrared Spectroscopy: An Innovative Method for the Diagnosis of Ovarian Cancer. *Cancer management and research*, *13*, 2389–2399. https://doi.org/10.2147/CMAR.S291906.

Lo, Y.-C., Cheng, C.-L., Han, Y.-L., Chen, B.-Y. & Chang, J.-S. 2014. Recovery of high-value metals from geothermal sites by biosorption and bioaccumulation. Bioresource Technology, 160, 182-190.

Lucas, S., Pangilinan, J. & Polle, J. 2010. The Chlorella variabilis NC64A genome reveals

Maeda, S. M. Mizoguchi, A. Ohki, T. Takeshita (1990) Bioaccumulation of zinc and cadmium in freshwater alga, Chlorella vulgaris. Part I. Toxicity and accumulation Chemosphere, 21 , pp. 953-963.

McHardy B.M., George J.J. Bioaccumulation and toxicity of zinc in green alga *Cladophora glomerata*. Environ. Pollut. 1990;66:55–66.

Mahipal Singh Sankhla, Mayuri Kumari, Manisha Nandan, Rajeev Kumar, Prashant Agrawal. 2016. Heavy Metals Contamination in Water and their Hazardous Effect on Human Health-A Review. Int.J.Curr.Micl.App.Sci. 5(10): 759-766.

Malakootian M, Yousefi Z, Khodashenas Limoni Z. (2016) Removal of zinc from industrial wastewaters using microscopic green algae Chlorella vulgaris. Sci J Ilam Univ Med Sci; 23(6): 40-50.

Malcata, F.X. (2011) Microalgae and Biofuels: A Promising Partnership? Trends in Biotechnology, 29, 542-549. https://doi.org/10.1016/j.tibtech.2011.05.005.

Malik, S., Washington, C., & Purewal, T. S. (1999). Solution and adsorption behaviour of lecithin surfactants in CFC suspensions: a light scattering study in aerosol propellants. International journal of pharmaceutics, 186(1), 63-69.

Manisalidis I, Stavropoulou E, Stavropoulos A, Bezirtzoglou E. Environmental and Health Impacts of Air Pollution: A Review. Front Public Health. 2020 Feb 20;8:14. doi: 10.3389/fpubh.2020.00014. PMID: 32154200; PMCID: PMC7044178.

Manzoor Faezeh , Karbassi Abdolreza *, Golzary Abooali , Removal of Heavy Metal Contaminants from Wastewater by Using Chlorella vulgaris Beijerinck: A Review, Current Environmental Management (Discontinued) 2019; 6(3) . https://dx.doi.org/10.2174/2212717806666190716160536.

Maret, W., & Moulis, J.-M. (2013). The Bioinorganic Chemistry of Cadmium in the Context of Its Toxicity. In A. Sigel, H. Sigel, & R. K. Sigel, Cadmium: From Toxicity to Essentiality (pp. 10.1007/978-94-007-5179-8_1). Netherlands: Springer .

Marquardt, Ronny, Hendrik Schubert, Daniel A. Varela, Pirjo Huovinen, Luis Henríquez, and Alejandro H. Buschmann. 2010. “Light acclimation strategies of three commercially important red algal species.” Aquaculture 299: 140-148.

Masironi, R. 1969 trace elements and cardiovascular diseases. bulletin of World Health Organisation, 40. 305-312.

Maxwell, D. P., Falk, S., Trick, C. G. & Huner, N. P. 1994. Growth at low temperature mimics high-light acclimation in Chlorella vulgaris. Plant Physiology, 105, 535-543.

McKnight, D. M., and F. M. M. Morel. 1979. Release of weak and strong copper-complexing agents by algae. Limnol. Oceano gr. 24:823-837.

Meagher, R. B. 2000. Phytoremediation of toxic elemental and organic pollutants. Current opinion in plant biology, 3, 153-162.

Mehta SK, Gaur JP (1999) Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in Chlorella vulgaris. New Phytol 143: 253–259.

Mehta, R., Shukla, H., & Bisen, P. (1992). Physiological and genetical characterization of a novel Synechococcus sp. isolate, showing resistance against cyanide, heavy metals and drugs. Environmental Technology, 13(3), 253-258.

Meikle, A.J. Gadd G.M. , R.H. 1990. Reed Manipulation of yeast for transport studies: critical assessment of cultural and experimental procedure.

Melis Anastasios, Thomas Happe, Hydrogen Production. Green Algae as a Source of Energy, Plant Physiology, Volume 127, Issue 3, November 2001, Pages 740–748.

midheaven I why (1975) histochemical observation on pure emission of heavy metals into tests buds of goldfish Walton of Japanese Society of science and fisheries 41 631.

Mishra, A. and Malik, A. (2012) Simultaneous Bioaccumulation of Multiple Metals from Electroplating Effluent Using Aspergillus lentulus. Water Research, 46, 4991-4998.http://dx.doi.org/10.1016/j.watres.2012.06.035.

Missouri R. (1969) trace elements and cardiovascular diseases bulletin of World Health Organisation 40, 305-312.

Moejes, Fiona Wanjiku, Anna Matuszyńska, Kailash Adhikari, Roberto Bassi, Federica Cariti, Guillaume Cogne, Ioannis Dikaios, et al. 2017. "A systems-wide understanding of photosynthetic acclimation in algae and higher plants." Journal of Experimental Botany 68 (11): 2667-2681.

Morais, C. A. D., & Ciminelli, V. S. T. (2004). Process development for the recovery of high-grade lanthanum by solvent extraction. *Hydrometallurgy*, *73*(3-4), 237-244.

Morelli E, Scarano G, Ganni M, Nannicini L, Serriti A (1989) Copper binding ability of the extracellular organic material released by Skeletonema costatum. Chem Spec Bioavail 1:71–76.

Moua, Bethany K. 2016. Acclimation to Nutrient Limitation in Microalgae. Accessed 5 6, 2019. https://ir.library.oregonstate.edu/concern/honors_college_theses/s7526f38q.

Mukhopadhyay, Mausumi & Noronha, S. & Suraishkumar, G K. (2008). Copper Biosorption in a Column of Pretreated Aspergillus niger Biomass. Chemical Engineering Journal - CHEM ENG J. 144. 386-390. 10.1016/j.cej.2008.02.007.

NAS (1974) Water quality criteria Environmental Protection Agency Washington DC ecological research series EPA -R3-73-033.

Nas-NRC (National Academy of Sciences-National Research Council), "Division of Medical Sciences, Medical and Environmental Effects of Pollutants Nickel," National Academic Press, Washington DC, 1975.

Nas-NRC, "National Academy of Sciences-National Research Council, Food and Nutrition Board, Recommended Dietary Allowances," 8th Edition, National Academy Press, Washington DC,1974.

Nausch, M., And N. G. Wasmund. 2004. Phosphorus dynamicsduring the transition from nitrogen to phosphate limitation inthe central Baltic Sea. Mar. Ecol. Prog. Ser. 266: 15–25.

Nielsen FH, Ollerich DA. Proceedings: Nickel, 1974: a new essential trace element. Federation Proceedings. Jun;33(6):1767-1772. PMID: 4827528.

Nielsen, E. S., Hansen, V. K. & Jørgensen, E. G. 1962. The adaptation to different light intensities in Chlorella vulgaris and the time dependenee on transfer to a new light intensity. Physiologia Plantarum, 15, 505-517.

NRCC (National Research of Council Canada). 1979. Effects of mercury in the Canadian environment. Publ. No. 16739. Associate Committee on Scientific Criteria for Environmental Quality, Subcommittee on Heavy Metals and Certain Other Elements, Ottawa. NTP (National Toxicology Program). 1993. OECD (Organisation for Economic Co-operation and Development). 1993. Co-operative risk production

activities for certain dangerous chemicals: Mercury. Draft Status Report. Environment Directorate.

Nriagu, J. Global inventory of natural and anthropogenic emissions of trace metals to the atmosphere. Nature 279, 409–411 (1979).

O'Dell B. L. (1976) Biochemistry of copper medical Clinical Nutrition America 60, 687-703. doi: 10.1016/S0025-7125(16)31853-3.

OECD (2011), Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris,

OECD Guidelines for the Testing of Chemicals. Guideline 211 Reproduction Test adopted September 1998.

Oilgae Guide to Algae-based Wastewater Treatment, (2017). Web source accessed on septemper 2018 :Wastewater_Treatment_Using_Algae_Report_Preview.pdf (oilgae.com).

Olguin EJ (2003) Phycoremediation: key issues for cost effective nutrient removal Processes. Biotechnol Adv 22(1-2):81- 90.

Olguín EJ, Galicia S, Mercado G, Pérez T (2003) Annual productivity of Spirulina (Arthrospira) and nutrient removal in a pig wastewater recycling process under tropical conditions. J Appl Phycol 15:249–257.

Oliveira, R. C., Jouannin, C., Guibal, E. & Garcia, O. 2011. Samarium (III) and praseodymium (III) biosorption on Sargassum sp.: batch study. Process Biochemistry, 46, 736-744.

Oswald W, Gootas HB (1957) Photosynthesis in sewage treatment Trans Am Soc Civ Eng 122: 73-105.

Pal, R., & Rai, J.P. (2010). Phytochelatins: Peptides Involved in Heavy Metal Detoxification. *Applied Biochemistry and Biotechnology, 160*, 945-963.

Pamukoglu MY, Kargi F (2007) Effects of operating parameterson kinetics of copper (II) ion non pre-treated powered waste sludge (PWS). Enzym Microb Technol 2:76–82.

Pandey, V. D. (2017). Cyanobacteria-Mediated Heavy Metal Remediation. Retrieved 6 19, 2019, from https://link.springer.com/chapter/10.1007/978-3-319-49727-3_6.

Park S-H, Kyndt J, Chougule K, Park J-J, BrownJK (2018) Low-phosphate-selected Auxenochlorella protothecoides redirects phosphate to essential pathways while producing more biomass.PLoS ONE 13(6): e0198953.

Patterson, G., Whitton, B. A. (1981) chemistry of water sedimented and Algal filaments in groundwater draining on old lead zinc mind in: water heavy metals in northern England environmental and biological aspect Durham University 65-71.

Peng, G., Tisch, U., Adams, O., Hakim, M and N. Shehada. 2009. Diagnosing lung cancer in exhaled breath using gold nanoparticles. Nat. Nanotechnol., 4: 669-673.

Perelo, L.W. (2010) Review: In situ and bioremediation of organic pollutants in aquatic sediments. J. Hazard. Mater. 177:81-89.

Pérez-Rama M, Herrero López C, Abalde Alonso J, Torres Vaamonde E. Class III metallothioneins in response to cadmium toxicity in the marine microalga Tetraselmis suecica (Kylin) Butch. Environ Toxicol Chem. 2001 Sep;20(9):2061-6. PMID: 11521835.

Perrin, D. D. and Agarwal, R. P. (1976). Metal-induced toxicity and chelation therapy. In: Williams, H (1980). Fate of chromium in a tributary of the Iraja River, Rio De Janeiro. Environmental pollution, 1.117-126.

Perrineau, M. , Zelzion, E. , Gross, J. , Price, D. C., Boyd, J. and Bhattacharya, D. (2014), Evolution of salt tolerance in Chlamydomonas. Environ Microbiol, 16: 1755-1766. doi:10.1111/1462-2920.12372.

Pettersson A, Kunst L, Bergman B, Roomans GM (1985) Accumulation of aluminium by Anabaena cylindrica into polyphosphate granules and cell walls: an X-ray energy-dispersive microanalysis. J Gen Microbiol 131: 2545–2548.

Pianka, Eric. R. (2000)Evolutionary Ecology, 6th ed. San Francisco: Addison-WesleyLongman.

Pick U (1992) ATPases and ion transport in *Dunaliella*. In: Avron M, Ben Amotz A (eds) *Dunaliella* physiology, biochemistry and biotechnology. CRC Press, Boca Raton, Rorida, pp 99–133.

Pick U, Ben Amotz A, Karni L, Seebergts CJ, Avron M (1986) Partial characterization of K and Ca uptake systems in the halotolerant alga Dunaliella salina. Plant Physiol 81: 875–881PubMed.

Pietilainan. K. (1976). Synergitic and antagonistic effects of lead and cadmium on aquatic primary production. In: Proceedings of Accumulation of dieldrin in an algae (Scendesmus obliquus), Daphnia magna and the guppy (Poecillia reticulata). Journal of fisheries research board of canada. 29. 1413.

Pinto, T.S., Malcata, F.X., Arrabaça, J.D. et al. Rubisco mutants of Chlamydomonas reinhardtii enhance photosynthetic hydrogen production. Appl Microbiol Biotechnol 97, 5635–5643 (2013).

Plá, Celis, and Paula Soledad María. 2015. Vulnerability and acclimation of Mediterranean Sea macroalgae, to environmental stress related to climate change: use of indicators physiological state = Vulnerabilidad y aclimatación de macroalgas del Mar Mediterráneo, frente al estrés ambiental derivado del Cambio Climático: uso de indicadores del estado fisiológico.

.

Polukhina, Iryna, Rikard Fristedt, Emine Dinc, Pierre Cardol, and Roberta Croce. 2016. "Carbon Supply and Photoacclimation Cross Talk in the Green Alga Chlamydomonas reinhardtii." Plant Physiology 172 (3): 1494-1505.

Prasad H. H, and Oberleas J. 1976 effect of growth hormone on nonhypophysectomised zinc deficient rats and zinc on hypophysectomised J lab clin med 63;486-494 (1976).

Prasad MNV (1995) Cadmium toxicity and tolerance in vascular plants. Environ Exp Bot 35: 525–540.

Raharinirina, N. Alexia, Gunnar Brandt, and Agostino Merico. 2017. "A Trait-Based Model for Describing the Adaptive Dynamics of Coral-Algae Symbiosis." Frontiers in Ecology and Evolution 5.

Rai LC, Gaur JP, Kumar HD (1981a) Phycology and heavy metal pollution. Biol Rev 56:99–151.

Rai LC, Gaur JP, Kumar HD (1981b) Protective effects of certain environmental factors on the toxicity of zinc, mercury and methylmercury to Chlorella vulgaris Beij. Environ Res 25: 250–259.

Rai LC, Mallick N, Singh JB, Kumar HD (1991) Physiological and biochemical characteristics of a copper tolerant and a wild-type strain of Anabaena doliolum. J Plant Physiol 138: 68–74.

Rai LC. Gaur JP, Kumar HD (1981) Protective effects of certain environmental factors onthe toxicity of zine, mercury and methylmercury to Chlorella vulgaris Beij. Environ Res.25 250-259.

Rathnakumar S., R. Y. Sheeja, & T. Murugesan. (2009). Removal of Copper (II) from Aqueous Solutions Using Teak (Tectona grandis L.f) Leaves. https://doi.org/10.5281/zenodo.1073164.

Raven, John A., Mario Giordano, John Beardall, and Stephen C. Maberly. 2011. "Algal and aquatic plant carbon concentrating mechanisms in relation to environmental change." Photosynthesis Research 109: 281-296.

Rebelo FM, Caldas ED. Arsenic, lead, mercury and cadmium: Toxicity, levels in breast milk and the risks for breastfed infants. Environ Res. 2016 Nov;151:671-688. doi: 10.1016/j.envres.2016.08.027. Epub 2016 Sep 10. PMID: 27619212.

Reed RH, Gadd GM (1990) Metal tolerance in eukaryotic and prokaryotic algae. In: Shaw AJ (ed) Heavy metal tolerance in plants: evolutionary aspects. CRC Press, Boca Raton, pp 1105–1118.

Reese RN, Mehra RK, Tarbet EB, Winge DR (1988) Studies on the dipeptidyl transpeptidase (phytochelatin synthase). Proc Natl Acad

Regaldo, L. M., Gervasio, S. G., Troiani, H. E., & Gagneten, A. M. (2013). Bioaccumulation and Toxicity of Copper and Lead in Chlorella vulgaris.

Rehman K, Fatima F, Waheed I, Akash MSH. Prevalence of exposure of heavy metals and their impact on health consequences. J Cell Biochem. 2018 Jan;119(1):157-184. doi: 10.1002/jcb.26234. Epub 2017 Aug 2. PMID: 28643849.

Renou, S., Givaudan, J., Poulain, S., Dirassouyan, F. & Moulin, P. 2008. Landfill leachate treatment: Review and opportunity. Journal of hazardous materials, 150, 468-493.

Rijstenbil JW, Haritonidis S, Malea P, Seferlis M, Wijnholds JA (1998) Thiol pools and glutathione redox ratio as possible indicators of copper toxicity in the green macroalgae Enteromorpha spp. from the Scheldt Estuary (SW) Netherlands, Belgium and Thermaikos Gulf (Greece, N Aegean Sea). Hydrobiologia 385: 171–181.

Robinson NJ (1989) Algal metallothioneins: secondary metabolites and proteins. J Appi Phycol 1: 5–18.

Robinson NJ, Tommey AM, Kuske C, Jackson PJ (1993) Plant metallothioneins. Biochem J 295: 1–10.

Romera, E., Gonzalez, F., Ballester, A., Blazquez, M. L. & Munoz, J. A. 2006. Biosorption with algae: a statistical review. Crit Rev Biotechnol, 26, 223-35.

Rym, Ben Dhiab. 2012. Photosynthetic Behavior of Microalgae in Response to Environmental Factors.

Sabljic, A. (2009). ENVIRONMENTAL AND ECOLOGICAL CHEMISTRY - Volume I Encyclopedia of Life Support Systems; Chemical Sciences, Eng EOLSS Publishers Company Limited, 2009510229, 9781848261860.

Sacasa Castellanos, Claudia (2013)., "Batch and Continuous Studies of Chlorella Vulgaris in Photo-Bioreactors" *Electronic Thesis and Dissertation Repository*. 1113.

Safi, C. Bachar Zebib, Othmane Merah, Pierre-Yves Pontalier, Carlos Vaca-Garcia, Morphology, composition, production, processing and applications of Chlorella vulgaris: A review, Renewable and Sustainable Energy Reviews,Volume 35, 2014, Pages 265-278, ISSN 1364-0321, https://doi.org/10.1016/j.rser.2014.04.007.

Sandstead H. H. (1975) Some trace elements which are essential to human nutrition: zinc, copper, Manganese, and chromium progress in food and nutrition science one 371-391.

Sandstead HH. Zinc in human nutrition. New York, NY, Academic Press. 1981.

Saroussi, Shai, and Sven Beer. 2007. “Acclimations of macroalgae as reflected in photosynthetic parameters derived from PAM fluorometry, and possible implications for abundance patterns.” Marine Ecology 28 (3): 377-383.

Sayed, O. H. & El-Shahed, A. M. 2000. Growth, photosynthesis and circadian patterns in Chlorella vulgaris (Chlorophyta) in response to growth temperature. Cryptogamie Algologie, 21, 283-290.

Schönknecht, Gerald, Andreas P. M. Weber, and Martin J. Lercher. 2014. “Horizontal gene acquisitions by eukaryotes as drivers of adaptive evolution.” BioEssays 36 (1): 9-20.

Schramel P. Determination of eight metals in the international biological standard by flameless atomic-absorption spectrometry. Analytica Chimica Acta. 1973 Nov;67(1):69-77. DOI: 10.1016/s0003-2670(01)80233-6. PMID: 4759896.

Senge, M. & Senger, H. 1990. Functional changes in the photosynthetic apparatus during light adaptation of the green alga Chlorella fusca. Journal of Photochemistry and Photobiology B: Biology, 8, 63-71.

Senge, M. & Senger, H. 1991. Adaptation of the photosynthetic apparatus of Chlorella and Ankistrodesmus to blue and red light. Botanica acta, 104, 139-143.

Sert, Ş., Kutahyali, C., İnan, S., Talip, Z., Çetinkaya, B. & Eral, M. 2008. Biosorption of lanthanum and cerium from aqueous solutions by Platanus orientalis leaf powder. Hydrometallurgy, 90, 13-18.

Shamshad I, Khan S, Waqas M, Asma M, Nawab J, Gul N, Raiz A, Li G. Heavy metal uptake capacity of freshwater algae (Oedogonium westti) from aqueous solution: A mesocosm research. Int J Phytoremediation. 2016;18(4):393-8. doi: 10.1080/15226514.2015.1109594. PMID: 26515662.

Shamim, S., 2018, 'Biosorption of Heavy Metals', in J. Derco, B. Vrana (eds.), Biosorption, IntechOpen, London. 10.5772/intechopen.72099.

Sharma A. D. (2013). Low nickel diet in dermatology. *Indian journal of dermatology*, *58*(3), 240. https://doi.org/10.4103/0019-5154.110846.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998 A Look Back at the U.S. Department of Energy’s Aquatic Species Program-Biodiesel from Algae, National Renewable Energy Program.

Shehata FHA, Whitton BA (1982) Zinc tolerance in strains of the blue-green alga Anacystis nidulans. Br Phycol J 17: 5–12.

Shrift, A. 1954. Sulfur-selenium antagonism. I. Antimetabolite action of selenate on the growth of Chlorella vulgaris. American Journal of Botany, 223-230.

Shrift, A., Nevyas, J. & Turndorf, S. 1961. Mass adaptation to selenomethionine in populations of Chlorella vulgaris. Plant physiology, 36, 502.

Silver S, Phung LT (1996) Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol 50: 753–789.

Sittig, M. (1975) Copper in environmental sources and emissions handbook noves data corporation park Ridge New Jersey England 48-51.

Sittig, M. Toxic metals, 1976: pollution control and worker protection. United States: N. p., 1976. Web.

Sivalingam, P. M. (1981) Toxicity of trace metals on Chlorella vulgaris isolated from palm oil mill sludge Jpn. phycol. Sci., 28 -162.

Spencer DF & Greene RW 1981. Effects of nickel on seven species of freshwater algae. Environmental Pollution (Series A) 25, 241-247.

Starodub ME, Wong PTS, Mayfield CI, Chau YK (1987) Influence of complexation and pH on individual and combined heavy metal toxicities to a freshwater green alga. Can J Fish Aquat Sci 44: 1173–1180.

Stephan C.E. (1977). Methods for calculating an LC50. In Aquatic Toxicology and Hazard Evaluation (edited by F.I. Mayer and J.L. Hamelink). ASTM STP 634 - American Society for Testing and Materials. Pp65-84

Stevenson, K., A. McVey, I Clark, and K Stevenson. 2016. "General calibration of microbial growth in microplate readers."

Stockinger, S.E. (1963) Nickel In: Fasest D.W. and Irish industrial hygiene on toxicology volume 2 interscience New York 1118- 1122.

Stokes PM, Maler T, Riordan JR (1977) A low molecular weight copper-binding protein in a copper tolerant strain of Scenedesmus acutiformis. In: Hemphil DD (ed) Trace substances in environmental health. University of Missouri Press, Columbia, pp 146–154.

Stokes, P., Hutchinson, T., & Krauter, K. (1973). Heavy-metal tolerance in algae isolated from contaminated lakes near Sudbury, Ontario. Botany, 51(11), 2155-2168.

Stokinger, H. E. (1963). Effects of air pollutants on wildlife. Conn. Med. 27:487–92.

Suppl. 2012;101:133–164. doi:10.1007/978-3-7643-8340-4_6 The American Heritage® Science Dictionary Copyright © 2011. Published by Houghton Mifflin Harcourt Publishing Company. All rights reserved.

Šupraha, L., Gerecht, A., Probert, I., & Henderiks, J. (2015). Eco-physiological adaptation shapes the response of calcifying algae to nutrient limitation. Scientific Reports, 5(1), 16499-16499.

Synechococcus sp. isolate, showing resistance against cyanide, heavy metals and drugs. Environmental Technology, 13(3), 253-258.

Takahashi H, Buchner P, Yoshimoto N, Hawkesford MJ, Shiu SH. 2012. Evolutionary relationships and functional diversity of plant sulfate transporters. Frontiers in Plant Science 2, 119.

Taylor S. R. 1964 abundance off chemical element in the continental crust: Anew table *Geochemica cosmochemica acta*, 28 1273-1285.

Taylor, M. C, Demayo (1981) Copper. In guidelines for surface water quality volume one inorganic chemistry substances inland water directory Environment Canada.

Tchounwou P.B., Yedjou C.G., Patlolla A.K., Sutton D.J. (2012), Molecular, clinical and environmental toxicicology volume 3: Environmental toxicology, molecular Clin. Environ. Toxicol. 10.1007/978-3-7643-8340-4.

Tipton, I.H. and M. Cook. 1963. Trace elements in human tissue. II. Adult subjects from the United States. Health Phys. 9: 103-145.

Tittel, J., Bissinger, V., Gaedke, U., & Kamjunke, N. (2005). Inorganic carbon limitation and mixotrophic growth in Chlamydomonas from an acidic mining lake. Protist, 156(1), 63-75.

Twiss MR, Nalewajko C (1992) Influence of phosphorus nutrition on copper toxicity to three strains of Scenedesmus acutus (Chlorophyceae). J Phycol 28: 291–298.

Umino, Y., Satoh, A. & Shiraiwa, Y. 1991. Factors Controlling Induction of External Carbonic Anhydrase and Change in K½ (CO2) of Photosynthesis in Chlorella regularis. Plant and cell physiology, 32, 379-384.

Underwood, E.J. 1971. Trace Elements in human and animal nutrition, New York. Academic Press 461 – 477.

Vanoverbeke, J., De Gelas, K. & De Meester, L. (2007). Habitat size and the genetic structure of a cyclical parthenogen, Daphnia magna. Heredity 98, 419–426 https://doi.org/10.1038/sj.hdy.6800958.

Velmurugan, N., Sung, M., Yim, S. S., Park, M. S., Yang, J. W. & Jeong, K. J. 2013. Evaluation of intracellular lipid bodies in Chlamydomonas reinhardtii strains by flow cytometry. Bioresource Technology, 138, 30-37.

Verma SK, Singh HN (1991) Evidence for energy–dependent copper efflux as a mechanism of Cu2+ resistance in the cyanobacterium Nostoc caldcóla. FEMS Microbiol Lett 84: 291–294.

Vijayaraghavan K, Yun YS. Bacterial biosorbents and biosorption. Biotechnol Adv. 2008 May-Jun;26(3):266-91. doi: 10.1016/j.biotechadv.2008.02.002. Epub 2008 Feb 15. PMID: 18353595.

Vijayaraghavan, K., & Joshi, U.M. (2014). Application of Ulva sp. biomass for single and binary biosorption of chromium(III) and manganese(II) ions: Equilibrium modeling. *Environmental Progress, 33*, 147-153.

Vishwakarma, Gajendra Singh, Gargi Bhattacharjee, Nisarg Gohil, and Vijai Singh. 2020. “bioremediation of pollutants.” In Current status, challenges and future of bioremediation, by Vijai Singh, Vimal Chandra Pandey. Elsevier.

Vogeli-Lange, R. and Wagner, G.J., 1990. Subcellular localization of a transport function for Cd-binding peptides. Plant Physiology, 92, pp.1086-1093.Järup L. Hazards of heavy metal contamination. Br Med Bull. 2003;68:167-82. doi: 10.1093/bmb/ldg032. PMID: 14757716.

Volesky, B. (1990) Biosorption of Heavy Metals. CRC Press, Inc., pp. 99- 174.

Volesky, B. 2003. Sorption and biosorption, BV Sorbex.

Wagatsuma T, Akiba R (1989) Low surface negativity of root protoplasts from aluminum-tolerant species. Soil Sci Plant Nutrition 35: 443–452.

Walker, C. H., Sibly, R. M., & Peakall, D. B. (2005). Principles of ecotoxicology. CRC press.

Wang, J. & Chen, C. 2009. Biosorbents for heavy metals removal and their future. Biotechnol Adv, 27, 195-226.

Wang, L., Xue, C., Wang, L., Zhao, Q., Wei, W. & Sun, Y. 2016. Strain improvement of Chlorella sp. for phenol biodegradation by adaptive laboratory evolution. Bioresource Technology, 205, 264-268.

Wang, Lu, Hualin Wang, Xiurong Chen, Youjun Zhuang, Zeya Yu, and Tianjun Zhou. 2018. "Acclimation process of cultivating Chlorella vulgaris in toxic excess sludge extract and its response mechanism." Science of The Total Environment 858-869.

Wang, W. (1986) Acclimation and response of algal communities from different sources to zinc toxicity. water air soil pollution 28-335.

Wang, W., Pranolo, Y. & Cheng, C. Y. 2011. Metallurgical processes for scandium recovery from various resources: A review. Hydrometallurgy, 108, 100-108.

Wang, X., Zhao, C., Zhao, P., Dou, P., Ding, Y. & XU, P. 2009. Gellan gel beads containing magnetic nanoparticles: an effective biosorbent for the removal of heavy metals from aqueous system. Bioresour Technol, 100, 2301-4.

Wang, Yingjun, and Martin H. Spalding. 2007. CO2 concentrating mechanisms in eukaryotic microalgae.

Wasewar, Kailas, Surinder Singh, and Sushil Kansal. 2020. "Process intensification of treatment of inorganic water pollutants." In inorganic Pollutants in Water, by Pardeep Singh, Sushil Kansal, Pooja Devi, 245-271. Elsevier.

WHO, "Environmental Health Criteria 108: Nickel. International Programme on Chemical Safety," World Health Organization, 1989.

WHO. Guidelines for drinking-water quality. Sixty-first meeting; 10–19 June 2003; Rome. 2004. Joint FAO/WHO Expert Committee on Food Additives, Avaible from http://ftp.fao.org/es/esn/jecfa/jecfa61sc.pdf).

Wilhelm, C. & Wild, A. 1984. The variability of the photosynthetic unit in Chlorella II. The effect of light intensity and cell development on photosynthesis, P-700 and cytochrome f in homocontinuous and synchronous cultures of Chlorella. Journal of plant physiology, 115, 125-135.

Wilkie AC, Mulbry WW. (2002) Recovery of dairy manure nutrients by benthic freshwater algae. Bioresour Technol. Aug;84(1):81-91. doi: 10.1016/s0960-8524(02)00003-2. PMID: 12137274.

William Falk Adverse Drug Reactions 1974o

Wiszniowski, J., Robert, D., Surmacz-Gorska, J., Miksch, K. & Weber, J. 2006. Landfill leachate treatment methods: A review. Environmental Chemistry Letters, 4, 51-61.

Wojciechowska, E., Gajewska, M. & Obarska-Pempkowiak, H. 2010. Treatment of landfill leachate by constructed wetlands: three case studies. Polish J. of Environ. Stud, 19, 643-650.

Wong, Y, Ho Y H, Ho K C, and Yung KKL Leung H M. 2017. "Growth Medium Screening for Chlorella vulgaris Growth and Lipid Production." (aquac mar biol) 6 (1).

Woolhouse HW (1983) Toxicity and tolerance in the response of plants to metals. In: Lange O, Nobel PS, Osmond CB, Ziegler H (eds) Physiological plant ecology. Ill Responses to the chemical and biological environment. Springer, Berlin Heidelberg New York, pp 125–300.

Xiong, C., JIA, Q., CHEN, X., WANG, G. & YAO, C. 2013. Optimization of polyacrylonitrile-2-aminothiazole resin synthesis, characterization, and its adsorption performance and mechanism for removal of Hg (II) from aqueous solutions. Industrial & Engineering Chemistry Research, 52, 4978-4986.

Xue H-B, Sigg L (1990) Binding Of Cu (II) To Algae In A Metal Buffer. Water Res 24: 1129–1136.

Yang J, Cao J, Xing G, Yuan H. Lipid production combined with biosorption and bioaccumulation of cadmium, copper, manganese and zinc by oleaginous microalgae Chlorella minutissima UTEX2341. Biore sour Technol 2015; 175: 537-44.

Yang, Wenqiang, Claudia Catalanotti, Matthew C. Posewitz, Jean Alric, and Arthur R. Grossman. 2014. Insights into Algal Fermentation.

Yokota, A. & Canvin, D. T. 1986. Changes of ribulose bisphosphate carboxylase/oxygenase content, ribulose bisphosphate concentration, and photosynthetic activity during adaptation of high-CO2 grown cells to low-CO2 conditions in Chlorella pyrenoidosa. Plant physiology, 80, 341-345.

Yoshimura E, Nagasaka S, Sato Y, Satake K, Mori S (1999) Extraordinary high aluminium tolerance of the acidophilic alga, Cyanidium caldarium. Soil Sci Plant Nutr 45: 721–724.

Yu, S., Zhao, Q., Miao, X. & Shi, J. 2013. Enhancement of lipid production in low-starch mutants Chlamydomonas reinhardtii by adaptive laboratory evolution. Bioresource technology, 147, 499-507.

Yuan, Y.W., Liu, C., Marx, H.E., Olmstead, R.G., 2009. The pentatricopeptide repeat (PPR) gene family, a tremendous resource for plant phylogenetic studies. New Phytol. 182, 272–283.

Yun, Y.-S., Park, J. M. & Yang, J.-W. 1996. Enhancement of CO2 tolerance of Chlorella vulgaris by gradual increase of CO2 concentration. Biotechnology techniques, 10, 713-716.

Yuvraj, Vidyarthi, A.S. & Singh, J. Enhancement of Chlorella vulgaris cell density: Shake flask and bench-top photobioreactor studies to identify and control limiting factors. Korean J. Chem. Eng. 33, 2396–2405 (2016).

Zeng, X., GUO, X., SU, G., DANQUAH, M. K., ZHANG, S., LU, Y., SUN, Y. & LIN, L. 2015. Bioprocess considerations for microalgal-based wastewater treatment and biomass production. Renewable and Sustainable Energy Reviews, 42, 1385-1392.

Zhao, X., QU, J., Liu, H., Wang, C., Xiao, S., Liu, R., Liu, P., Lan, H. & Hu, C. 2010. Photoelectrochemical treatment of landfill leachate in a continuous flow reactor. Bioresource technology, 101, 865-869.

Zwolak I. 2020 The Role of Selenium in Arsenic and Cadmium Toxicity: an Updated Review of Scientific Literature. Biol Trace Elem Res. Jan;193(1):44-63. doi: 10.1007/s12011-019-01691-w. Epub 2019 Mar 15. PMID: 30877523; PMCID: PMC6914719.

Gupta M, Chandra P. Lead accumulation and toxicity in *Vallisneria spiralis* and *Hydrilla verticillata* (L.) Royle. J Environ Sci Health A. 1994;29:503–516. doi: 10.1080/10934529409376051.

Fahmi H, Shehata A, Whitton BA. Zinc tolerance in strains of the blue-green alga *Anacystis nidulans*. Br Phycol J. 1982;17:5–12. doi: 10.1080/00071618200650021.

Harding JPC, Whitton BA. Resistance to zinc of *Stigeoclonium tenue* in the field and the laboratory. Br Phycol J. 1976;11:417–426. doi: 10.1080/00071617600650471.

Takamura N, Kusai F, Watanabe M. Effects of Cu, Cd, Zn on photosynthesis of freshwater benthic algae. J Appl Phycol. 1989;1:39–52. doi: 10.1007/BF00003534.

Järup L. Hazards of heavy metal contamination. Br Med Bull. 2003;68:167-82. doi: 10.1093/bmb/ldg032. PMID: 14757716.

Mallick N, Pandey S, Rai LC. 1994 Involvement of a Cdinduced low molecular weight protein in regulating Cd toxicity in the diazotrophic cyanobacterium Anabaena doliolum. Biometals 7, 299–304.

Jahan, K., Mosto, P., Mattson, C., Frey, E., & Derchak, L. (2004). Metal uptake by algae. *WIT Transactions on Ecology and the Environment*, *78*.

Benning LG, Phoenix VR, Yee N, Tobin MJ (2004). Molecular characterization of Cyanobacterial silification using synchrotron infrared micro-spectroscopy. Geocimica et Cosmochimica Acta. 68: 729-741.

Giordano M, Kansiz M, Heraud P, Beardall J, Wood B, McNaughton D (2001). Fourier transform infrared spectroscopy as a novel tool to 3824 Afr. J. Biotechnol. investigate changes in intracellular macromolecular pools in the marine alga Chaetoceros muellerii (Bacillariphyceae). J. Phycol. 37: 271-279.

Keller RJ (1986). Sigma library of FTIR spectra. Sigma Chemical Company: St Louis.

Naumann D, Schultz CP, Helm D (1996). What can infrared spectroscopy tell us about the structure and composition of intact bacterial cells? In: Mantsch HH and Chapman D (ed.) Infrared Spectroscopy of Biomolecules, Wiley: Chichester. pp. 279-310.

Sigee DC, Dean A, Levado E, Tobin MJ (2002). Fourier-transform infrared spectroscopy of Pediastrum duplex: characterization of a micro-population isolated from a eutrophic lake. Eur. J. Phycol. 37: 19-26.

Stuart B (1997). Biological Applications of Infrared Spectroscopy. Wiley: Chichester. pp. 25-180.

Wong PTT, Wong RK, Caputo TA, Godwin TA, Rigas B (1991). Infrared spectroscopy of exfoliated human cervical cells: evidence of extensive structural changes during carcinogenesis. Proc. Natl. Acad. Sci. 88: 10988-10992.

Maquelin K, Kirchner C (2002). Identification of medically relevant microorganisms by vibrational spectroscopy. J. Microbiol. Method, 51: 255-271.

Dean AP, Martin MC, Sigee DC (2007). Resolution of codominant phytoplankton species in a eutrophic lake using synchrotron-based Fourier transform infrared spectroscopy. Phycologia, 46(2): 151-159.