UNIVERSITÉ DU QUÉBEC À MONTRÉAL

Determination of thiol compounds (Glutathione and Phytochelatins) in alga *Chlamydomonas reinhardtii* exposed to Zn^{2+} and zinc nanoparticles

THESIS

PRESENTED

AS A REQUIREMENT

FOR THE MASTER IN CHEMISTRY

BY

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August 2019

UNIVERSITÉ DU QUÉBEC À MONTRÉAL

Détermination des composés thiols (glutathion et phytochélatines) chez l'algue *Chlamydomonas reinhardtii* exposée au Zn^{2+} et aux nanoparticules de zinc

MÉMOIRE

PRÉSENTÉ

COMME EXIGENCE PARTIELLE

DE LA MAÎTRISE EN CHIMIE

PAR

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Août 2019

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to my supervisor Professor David Dewez, for his guidance, great support, kind advice throughout this research, and constructive suggestions which were determinant for the accomplishment of this research. It was an honor for me, not only for his scientific knowledge, but also for his extraordinary human qualities.

I would also like to thank Mahshid Samadani thank you for her great support and significant guidance throughout this research.

I would also like to show gratitude to department of chemistry and biochemistry at UQAM who Professors UQAM's helped me during the course of the master's program and the technicians of the chemistry department, I am grateful to Mrs. Sonia Lachance for her help and consideration.

I would like to express my grateful to my colleague Venus Esmaeili who always was beside me and helped me throughout this research.

I am using this opportunity to express my gratitude to my colleague in our lab who supported me throughout this research.

Last but not the least, I tend to say a special thanks to my mother and my love, who offered her encouragement through phone calls without you I could not carry out these studies. This thesis stands as a testament to your absolute love.

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LIST OF ABREVIATIONS

AAS	Atomic Adsorption Spectrometry
ABS	Absorption by chlorophyll
ACN	Acetonitrile
ATP	Adenosine tri-phosphate
Chl a	Chlorophyll *a*
Chl b	Chlorophyll *b*
Chl	Chlorophyll
Cys	Cysteine
EDTA	Ethylene diamine tetra acetic acid
ET	Electron transport
FM	Maximum fluorescence (at approx. T = 1 sec)
Fo	Initial fluorescence
Fs	Stationary Chl fluorescence
Fv	Variable Chl fluorescence
Glu	Glutamic acid
Gly	Glycine
GSH	Glutathione
HEPES	4 - (2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HNO_3	Nitric acid
HPLC-FL	High performance liquid chromatography coupled with a fluorescence detector

ICP-MS	Mass Spectrometry inductively coupled plasma source
LED	Light Emitting Diode
mBrB	Monobromobimane
Me-PC	Metal-Phytochelatin complexes
MeOH	Methanol
MSA	Metasulfonic acid
NADPH	Nicotinamide adenosine diphosphate (reduced)
PCn	Phytochelatins with n = 2, 3, 4 ...
PI	Photosynthetic index
PSI	Photosystem I
PSII	Photosystem II
RC	Reaction center
SH	Thiol functional group
TCEP	Tris (2-carboxyethyl) phosphine
Zn	Zinc
Zn-NP	Zinc Nanoparticle

RÉSUMÉ

Les nanoparticules de zinc (Zn-NP) sont un groupe de contaminant émergent qui représentent un risque de toxicité pour le milieu aquatique. Les Zn^{2+} sont libérés des Zn-NP, induisant des effets toxiques sur le métabolisme des microalgues. Les cellules algales peuvent synthétiser des composés thiols pour contrôler la concentration intracellulaire de zinc. Les phytochélatines (PC_n) sont en particulier des ligands pouvant lier les métaux bioaccumulés comme le zinc. L'objectif de cette étude est d'étudier l'effet des Zn-NP et des Zn^{2+} sur la physiologie et l'induction des composés thiols (glutathion et phytochelatins) chez l'algue *Chlamydomonas reinhardtii*. Les cellules d'algues ont été exposées pendant 24 à 72 heures à 30 et 60 µM de Zn-NP et de chlorure de zinc. L'effet des Zn-NP et des Zn^{2+} sur la croissance des algues pendant 72 h a montré une inhibition dépendante à la concentration intracellulaire de zinc total et à la durée d'exposition. En outre, le niveau d'accumulation maximale de zinc intracellulaire a été obtenu après 48 h pour les cellules d'algues exposées à 60 µM de chlorure de zinc et à 24 h pour les cellules d'algues exposées à 60 µM de Zn-NP. Les Zn-NP et les Zn^{2+} ont induit la synthèse des PC_2 et PC_4, qui était dépendante de la concentration de zinc et de la durée d'exposition. L'induction du glutathion a été détectée seulement sous l'effet des Zn-NP, et le niveau de glutathion maximal se trouvait à 24 h sous l'effet de 30 µM de Zn-NP. Ces résultats indiquent que les Zn-NP et les Zn^{2+} ont affectés différemment l'induction du glutathion et des phytochélatines. Par conséquent, cette étude a démontré que l'effet physiologique des Zn-NP était significativement différente par rapport à celle des Zn^{2+}.

Mots-clés : Zinc, Nanoparticules de ZnO, Algue verte, *Chlamydomonas reinhardtii*, Glutathion, Phytochélatines.

ABSTRACT

Zinc nanoparticle (Zn-NP) is an emergent contaminant that represent a risk of toxicity for the aquatic environment. Zn^{2+} released from Zn-NP induces toxic effects on the metabolism of microalgae. Algal cells can synthesize thiol compounds to control the intracellular concentration of zinc. Especially, phytochelatins (PC_n) are metal-bonding ligands synthesized upon the accumulation of zinc. The goal of this study was to investigate the effect of Zn-NP and Zn^{2+} on the physiology and the induction of phytochelatins of microalga *Chlamydomonas reinhardtii*. Algal cells were exposed during 24-72 h to 30 and 60 µM of Zn-NP and zinc chloride. The effect of Zn-NP and Zn^{2+} on the growth of algal culture during 72 h showed an inhibitory effect related to the total zinc concentration and the time of exposure. Furthermore, the maximum accumulation level of intracellular zinc was reached at 48 h for algal cells exposed to 60 µM of zinc chloride, and at 24 h for algal cells exposed to 60 µM of Zn-NP. Both Zn-NP and Zn^{2+} induced the synthesis of PC_2 and PC_4, which was dependent to the concentration of zinc and the time of exposure. The induction of glutathione was only detected under the effect of Zn-NP, and the maximum glutathione level was found at 24 h under 30 µM of Zn-NP. These results indicate that Zn-NP and Zn^{2+} affect differently the induction of glutathione and phytochelatins. Therefore, this study demonstrates that the physiological effect of Zn-NP was significantly different compared to Zn^{2+}.

GENERAL INTRODUCTION

In the past 15 years, many molecular technologies have been developed and used to study the physiology of alga *Chlamydomonas reinhardtii* (Hanikenne, 2003a). This development has made the green alga *Chlamydomonas reinhardtii* to become an important model for bioanalytical experiments. This Alga can produce thiol compounds (SH groups) that are caled glutathione and phytochelatins, when it is exposed to heavy metals, as a defensive mechanism. Various species of algae have mechanisms for the detoxification of metals in order to survive, based on (Torrecili *et al.*, 2004a) and (Skowronska, 2001). External detoxification is done by uptake of metals into algal cells, and internal detoxification is done through precipitation or immobilization of metals by ligands (Scheidegger *et al.*, 2010a). Various environmental factors like as pH, concentration of free metal ions, and organic or inorganic ligands can affect life of algae by approaching to optimal growth (Torricelli *et al.*, 2006). In the last decade, human activities and industrial developments in al over the world rejected pollutants into the environment. One of the most important pollution problems is the effect of heavy metals on the environment that can affect human and animal health. Some of pollutants stay a longtime in the environment without decay, such as fertilizers, pesticides, insecticides, that can pollute the lands and agricultural soils affecting the food chain. Because of this, in the recent years, scientific research had a wide concern about pollutants, especially heavy metals. Zinc is a heavy metal that can affect the aquatic environment in high concentration, but ionic zinc at low concentration is a required element for algae metabolism. High concentration (more than 1 μM) is toxic for algae by altering the metabolism of algae, according to (Skowronska, 2003a). To control the toxic effect of zinc, the intracellular accumulation will induce the synthesis of phytochelatins in the cellular system of plant, algae, and fungi (Tsuji *et al.*, 2002a). We can also mention that the toxicity effect of zinc on plants was indicated by the reduction of growth of the root and shoot, the deterioration of

metabolic functions, and the degradation of pigments in leaves (Yadav, 2009a). According to Yadav (2009b), the soil can be polluted with zinc from fertilizers, sewage sludge, urban composts or others activities. Moreover, the concentration of zinc that has been measured in the agricultural soil, and it was between 150 to 300 mg/kg. For this reason, we selected to investigate in this research study the effect of zinc on algal cells, since it represents a hazardous metal for the aquatic environment.

In these recent years, metal nanoparticles have been used intensively in the industry representing a risk of toxicity for the aquatic environment (Navaro *et al.*, 2008a). For this reason, we also investigated the effect of zinc nanoparticles on the physiology of alga *Chlamydomonas reinhardtii*, under the same experimental condition for ionic zinc, in order to understand the toxicity effect of zinc nanoparticles in comparison to ionic zinc. Also, we can mention that the inhibitory effect of nanoparticles was previously investigated on algae, based on (Miazek *et al.*, 2015a), showing different mechanisms of action: 1) The generation of reactive oxygen species inside the cell; 2) the release of metal ions from nanoparticles causing toxic effects; and 3) the interaction with nutrients in the growth media. On the other hand, it was showed that the effect of nanoparticles is related to the size of the nanoparticle, especially for the interaction with living cell compared to others parameters (Shang *et al.*, 2014a).

Glutathione (GSH) and phytochelatins play an important role for the detoxification of toxic metals in plants (lower plants and higher plants), algae, and fungus. The compound GSH is a monothiol that play the role of free metals scavenger, and phytochelatins (PCn) are a polymer of GSH (Torriceli *et al.*, 2004b). In others words, GSH is a substrate for phytochelatin synthesis that is catalyzed by the enzyme PC synthase (PCS). The compounds GSH and phytochelatins have the ability to bind toxic metals using their thiol group (SH) that exist in the structure of the cysteine amino acid in glutathione (Torriceli *et al.,* 2004c; Skowronska *et al.*, 2001b). In this reaction, the concentration of hydrogen ions (pH) is very important in the formation of bonding with metals as an electrophile (Millar *et al.*, 2003; Skowronska, 2000). This study reported

that PC-SH could play the role of bioindicator for metal contamination. Also, phytochelatins can regulate metal homeostasis and detoxification in the cell because of their affinity to metal bonding, according to (Le Faucheur *et al.*, 2006a).

Moreover, it was previously showed that zinc ions can be coordinated by two thiol groups of cysteine (Kobayashi *et al.*, 2006) with a bonding stoichiometry between zinc and thiol group of phytochelatins (PC2), as 1:1 at pH of 7.5. In addition, the HNMR spectra of Zn-PC2 and the UV-VIS absorbance of Zn-PC2 at 220 nm demonstrated the bonding stoichiometry of 1:1. Beside of this, according to (Wu *et al.*, 2012a), the synthesis of PCn and the stoichiometry between PCn and metals are related to the species of algae and the nature of the metal. We can mention that several important factors can affect the production of GSH and PCn, such as 1) the species of algae; 2) the toxicity of metal; 3) the concentration of metal; 4) the interaction with the metal. In addition, the following physiological parameters such as the pH and the temperature can affect the production of GSH and PCn. In fact, previous studies reported the bonding between PCn and metals in phytoplankton after metal exposure, such as cadmium, lead, nickel, copper, cobalt (Kawakashi *et al.*, 2005; Le Faucheur *et al.*, 2004, 2006b; Scheidgger *et al.*, 2010b). According to (Hirata *et al.*, 2001), the detoxification of heavy metals by using algae has high efficiency and a low cost in comparison with others methods.

There are many analytical techniques that can help us in the detection of GSH and PCn in biological samples, such as the cathodic stripping voltammetry and the polarography. With this technique, it was possible to detect directly the GSH and PCn without the derivatization step. This technique was sensitive but not specific, and for this reason, it was not useful for the detection of thiol compounds (Hirata *et al.*, 2001b).

The other technique that we can mention is HPLC (High Pressure Liquid Chromatography) coupled with ICP-MS (Plasma-Mass Spectrometry) or ES-MS (Electrospray-Mass Spectrometry). In my research project, we used UHPLC (High Pressure Liquid Chromatography) with a diode array fluorescence detector. UHPLC

has a higher speed in the analysis of samples than HPLC, as a result, the retention time decreases and we can analyze more samples. The LOD (Limit of Detection) of this method is in the range of nM-μM, which is an advantage. It is a very simple method for the extraction step. Moreover, it is a selective and sensitive method by using thiol compounds. For the measurement of ionic zinc and zinc nanoparticles inside of the cell, we used the analytical technique ICP-OES, a sensitive and repeatable technique. We can also mention instrumental advantages for the ICP-OES, as low LOD and a high speed in analysis.

According to the scientific literature on the subject, the effect of zinc nanoparticles has not been investigated on the cellular physiology of alga *Chlamydomonas reinhardtii*. Until now, studies have been focused on the ionic zinc exposure to algal cells of *C. reinhardtii*, but the effect of zinc nanoparticles on the physiological responses and the detoxification mechanism in the cellular system is still unclear. Moreover, Zn-NP possess different chemical properties than Zn^{2+}, and the toxicity effect of Zn-NP (size 35 nm, 99% purity) can be different compared to the toxicity effect of Zn^{2+}.

CHAPTER I

THE STUDIES ON METALIC CONTAMINATION

1. 1 Heavy metals

Some heavy metals exist in al over our environment, they are found in the foods, in the water, and in the air. These metals can represent a risk of toxicity for human health depending of their bioavailability. Toxic metals can enter our body via drinking, eating, eye contact and absorption via the skin. In our body, they create harm at the cellular level by initiating oxidative stress. This harm can make many diseases and health problems. We can mention that heavy metals have been well investigated in previous studies, such as mercury, cadmium, lead, nickel, copper, and cobalt according to (farceur *et al.*, 2004, 2006c), (Scheidgger *et al.*, 2010c). In particular, Zn^{2+} is also a heavy metal that can affect the environment in high concentration, but at low concentration, it is a required element for the metabolism of living organisms. At high concentration (more than 1 μM), it is known to be toxic by altering the metabolism of algae, according to (Pawlik-skowronska, 2003b).

1.1.2 Sources of heavy metals

Heavy metal can come from different sources such as sediments, rocks, weathering and soil, and the rock cycle. Heavy metals are released from the surface and groundwater, the atmosphere, and anthropogenic sources such as industrial production and agriculture activities. During the last decade, human activities and industrial development in al over the world contributed to propagate several pollutants and contaminants into the environment. These metals can be part of fertilizers and pesticides contaminating the lands and agricultural soils. Since heavy metals are

persistent in the environment, scientists had a wide concern about their pollution during the recent years (Tsuji *et al.*, 2002b).

1.1.3 Toxic effects of heavy metals to human health

In the human body, heavy metals can interrupt metabolic processes and biological functions in organs such as the kidneys, bone, liver, heart, and brain (Singh *et al.*, 2011). In the following table 1-1, the toxic effects of different heavy metals on human health are presented.

Table 1-1 Effect of heavy metals in human body (www.IndianPharmacology.org).

Pollutants	*Major sources*	*Effect on human health*	*Permissible level (mg/l)*
Arsenic	Pesticides, fungicides, metal smelters	Bronchitis, dermatitis, poisoning	0.02
Cadmium	Welding, electroplating, pesticide fertilizer, Cd and Ni batteries, nuclear fission plant	Renal dysfunction, Lung disease, Lung cancer, Bone defects (Osteomalacia, Osteoporosis), increased blood pressure , kidney damage, bronchitis, gastrointestinal disorder, bone marrow, cancer	0.06
Lead	Paint, pesticide, smoking, automobile emission, mining, burning of coal	Mental retardation in children, developmental delay, fatal infant encephalopathy, congenital paralysis, sensor neural deafness and, acute or chronic damage to the nervous system, epilepticus, liver, kidney, gastrointestinal damage	0.1
Manganese	Welding, fuel addition, ferromanganese production	Inhalation or contact causes damage to central nervous system	0.26
Mercury	Pesticides, batteries, paper industry	Tremors, gingivitis, minor psychological changes, acrodynia characterized by pink hands and feet, spontaneous abortion, damage to nervous system, protoplasm Poisoning	0.01
Zinc	Refineries, brass manufacture, metal Plating, plumbing	Zinc fumes have corrosive effect on skin, cause damage to nervous membrane	15
Chromium	Mines, mineral sources	Damage to the nervous system, fatigue, irritability	0.05
Copper	Mining, pesticide production, chemical industry, metal piping	Anemia, liver and kidney damage, stomach and intestinal irritation	0.1

1.1.4 Toxic effects of heavy metals on plants

Pollution of agricultural soil with heavy metals is an environmental concern worldwide, due to effects of heavy metals and damages on the soil quality that are

irrecoverable according to (Yalda, 2010a) and (Bell *et al.*, 2001). It was determined that the word agricultural soil was contaminated by toxic metals as Cd, Zn, Cu, Ni, Co, Cr, Pb and As. This contamination was the result of the intensive use of fertilizers, pesticides, sewage sludge application, and industrial waste for a long period (Schwartz *et al.*, 2001).

For instance, the soil can be polluted by the cadmium (Cd^{2+}) according to (Yalda, 2010b), and the regulatory limit for Cd^{2+} is 100 mg/Kg of soil. The effect of high level of Cd^{2+} on plants is indicated by the decrease of the photosynthesis performance. In addition, we can mention others symptoms that high levels of Cd^{2+} can induced on plants as chlorosis and the inhibition of the root growth, which can finally cause death.

As an essential micronutrient for the metabolism, copper (Cu^{2+}) was determined to have a key role for CO_2 assimilation and ATP synthesis. The soil can be polluted by Cu^{2+} via different ways such as mining activities, and smelting in the industry. For the toxicity effects of Cu^{2+} on plants, we can mention chlorosis caused by the generation of oxidative stress.

As a non-essential element, mercury (Hg^{2+}) is considered as a toxic contaminant in the food chain (Han *et al.*, 2006a). Mercury can change into different chemical forms, but, in the soil, we have the ionic form Hg^{2+}. Also, Hg^{2+} can be added to the soil from organic compounds and clay particles (Han *et al.*, 2006b). In fact, Hg^{2+} can induce physiological damage in plants, for example, it can bind to the water channel proteins and block the water flow in the plant cellular system (Yalda, 2009c). Also, maximum levels of Hg^{2+} can induce an oxidative stress through the generation of ROS.

Cobalt (Co^{2+}) is found in the crust of the earth in three forms, cobalite, eryhrite, and smaltite. Co^{2+} can be added to the soil because of the consumption of fossil fuels. Plants can uptake slight amount of Co^{2+} from soil from different mechanisms (Yalda, 2009d), (Kukier *et al.*, 2004). We can mention that the phytotoxicity effect of Co^{2+} on plants such as the growth inhibition of the shoot, and the decrease of the biomass.

Nickel (Ni^{2+}) is a metal found at paucity concentration in natural soil, and the concentration of Ni^{2+} can be increased in soil as a result of human and mining activities, consumption of oil and coal, and the use of phosphate fertilizers (Yalda, 2010c), (Hirata *et al.*, 2005a). The concentration of Ni^{2+} in polluted soil is 20-30-fold higher than natural soil. The symptoms of excess of Ni^{2+} in soil are chlorosis and necrosis.

Lead (Pb^{2+}) is a toxic element that has been discovered in the soil, and the pollution of Pb^{2+} is the result of mining and smelting activities, consumption of papers, gasoline, and the use of paints (Yalda, 2010d). Pb^{2+} has detrimental effect on growth and physiological processes in plants such as the photosynthetic activity, it can induce an inhibitory effect on enzymatic activities by denaturation sulfhydryl groups, and it can induce the oxidative stress by boosting the amount of ROS (Reddy *et al.*, 2005), (Yalda, 2010e).

Chromium (Cr^{2+}) is a contaminant in soil that can have a critical toxic effect on the environment via the tanning industry. This industry produces wastewater with high levels of Cr^{2+}. In fact, Cr^{2+} is one of the most toxic and carcinogen elements that has been reported by the International Agency of Cancer (Yalda, 2009f). We can mention symptoms caused by the excess of Cr^{2+} in plants such as the growth inhibition, chlorosis, tops and root becoming lose and limp, instability in nutrient content, and the change in physiological processes (Yalda, 2010f), (Yalda, 2009j).

Moreover, the soil and land can be polluted by Zn^{2+} (Zn^{2+}), as a result of the intensive use of fertilizers, pesticides, and others industrial activities. In polluted land and soil, the concentration of Zn^{2+} has been calculated between 150 to 300 mg/Kg of soil. Although it is necessary for living organisms at low concentration, Zn^{2+} can be for plants at high dose (Yalda, 2009h): We can mention the toxicity effects of Zn^{2+} on plants, such as the inhibition of the root and shoot growth, chlorosis, genesis purplish-red color in leaves, and the decrease in metabolic functions. Moreover, Zn^{2+} is a heavy metal that can affect aquatic environment. In particular, it can deteriorate the metabolism in algae. Beside that, Zn^{2+} is an inducer of the synthesis of phytochelatins

in algae, which is a molecular defense mechanism. For this reason, the effect of Zn^{2+} on microalgae was investigated in my project.

1.2. The zinc

1.2.1 Physical and chemical properties

The metal zinc has many physical properties as the solidity, a color bluish-white metal, and it is ductile or malleable at room temperature. The atomic number of the zinc element is 30 and the standard atomic weight is 65.38 amu, according to (Toxicity of industrial metals, 1969). The zinc is an abundant element that is part of 0.004 % of the Earth crust. The zinc can be found in different forms: The sphalerite (ZnS) is the most common zinc mineral; The calamine ($ZnCO_3$) is another form produced as a result of the oxidation of sphalerite (Criteria, 2001). For the chemical and physical properties, see the data from the Merck Index (1989).

1.2.2 Zinc in the environment

In 2016, 322,000 tons of zinc was produced from mines in Canada, and 28.4% of this amount was produced in Québec. The zinc has several uses, but the main use of zinc in Canada is for galvanizing iron and steel for steel protection corrosion (www.canada.ca/health-canada/services/publications). The other consumption of zinc is divided between industries: The zinc oxide is used for tires and rubber products, rolled zinc is applied for architectural and building applications, and dry cell batteries. The main pollution sources in the environment that contain zinc are fertilizers, pesticides, sewage and mining residues (Yalda, 2009i). The concentration of Zn^{2+} in natural surface water is more than 0.1 mg/L. The concentration of Zn^{2+} in tap water can be higher due to the use of zinc in plumbing compound, and the concentration of Zn^{2+} in ground water is variable between 10 to 40 µg/L. The increasing concentration of

Zn^{2+} in water up to 5 mg/L can result in having a styptic taste and a greasy film on boiling.

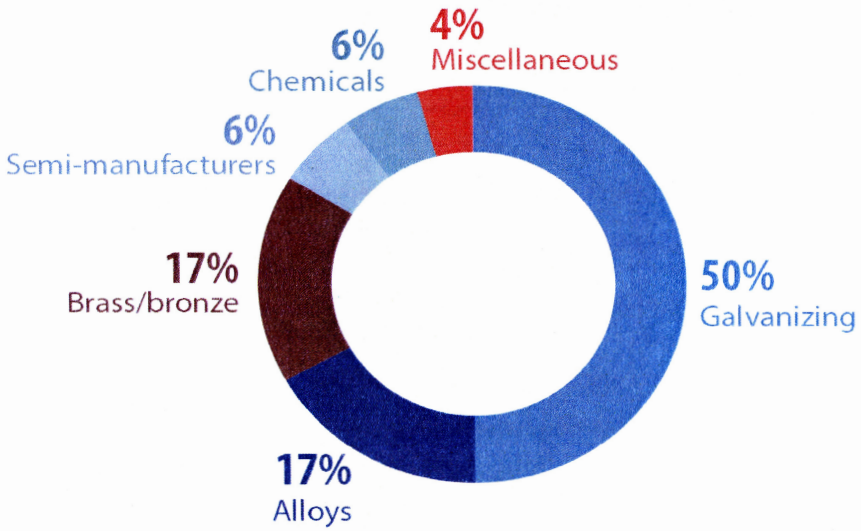


Figure 1.1 Major zinc sources and uses in Canada in 2016 (www.nrcan.gc.ca/mining-materials/facts/Zn^{2+}).

1.2.3 Toxicity of Zn^{2+} for human health

The Zn^{2+} is an essential element for al living organisms, playing an important role for the metabolism, which includes the replication of DNA and the translation of proteins (www.canada.ca/health-canada/services/publications, 1987). The Zn^{2+} can be part for almost 200 enzymes, such as carbonic anhydrase, aspartase, transcarbamylase, and alcohol dehydrogenase. The food sources represent the main uptake of Zn^{2+} for al ages. For Canadian people, the recommended dietary allowance is 2 mg/day for children, 9 mg/day for men and 8 mg/day for women.

Although the Zn^{2+} is not toxic at low concentration, it can be toxic at high concentration. According to Health Canada (1987), the toxicity of Zn^{2+} can cause symptoms as an alteration in the cholesterol metabolism, and the decrease of the

immune response. The Zn^{2+} can enter the human body via three ways (Plum *et al.*, 2010): By inhalation, through the skin, or via the ingestion of contaminated food. Each way can affect particular parts of the body (Figure 1.2).

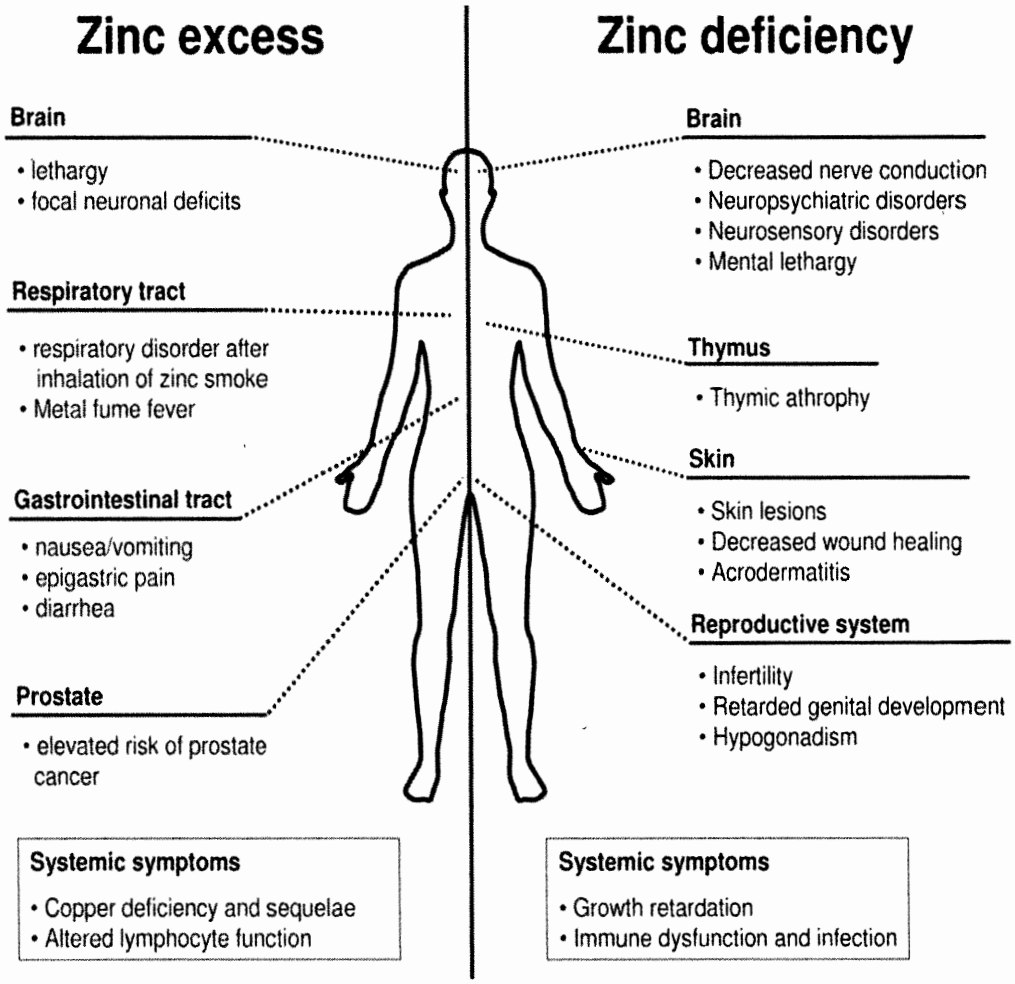


Figure 1.2 The effects of Zn^{2+} excess (left) and deficiency (right), (Laura *et al.*,2010)

Workers can be exposed by inhalation to smoke that emanate from various industrial facilities, which include zinc oxide or zinc chloride. Concerning dermal exposure, the absorption of Zn^{2+} happens via the skin, which can cause an irritation symptom on the

skin. Moreover, toxicity effects of Zn^{2+} can happen from oral exposure when the amount uptake is more than the recommended dietary allowance. From such exposure, symptoms have been reported as vomiting, abdominal pain, and nausea. According to a 1984 national study, the amount of Zn^{2+} intake from drinking water for Canadian adults was between 33.8 to 97.5 µg per day (www.canada.ca/health-canada/services/publications, 1987).

1.2 Nanoparticles

1.3.1 Physical and chemical properties

Size of nanoparticles is between 1-100 nm in at least one dimension (Miazek *et al.*, 2015b), (Zhang *et al.*, 2014). Industrial methods for the production of nanoparticles involved mechanical processes, laser and UV irradiation, hydrothermal process, sol-gel process, or microbial biosynthesis (Miazek *et al.*, 2015c), (Tavakoli *et al.*, 2007). It is well known that engineered or synthesized nanoparticles from different sources can have different physical and chemical properties (Navarro *et al.*, 2008b), (Miazek *et al.*, 2015c). Also, the toxicity of nanoparticles will depend on the size of nanoparticles (surface area) (Shang *et al.*, 2014b), (Navarro *et al.*, 2008c). These days, the increasing use of Zn-NP al over the world may represent a risk for the human health, the quality of the environment, and the life of animals and plants.

1.3.2 Uses of Zn-NP in Canada

Properties of nanoparticles are antifungal, anticancer, antibacterial, and antiviral. Nanoparticles have several uses in our life that can help us to improve our goods. For example, it can be used in the pharmaceutical and cosmetic industries: For components in cream, powders, and dental paste. It can also be used in the electronics and

electrotechnology: For components in photoelectronics, chemical sensors, solar cells, and UV lasers (Schrofel *et al.*, 2014). Moreover, it can be used in the textile industry: As components to absorb UV radiations (Kołodziejczak-Radzimska, 2014; Aruoja *et al.*, 2008a).

1.3.3 Toxicity of Zn-NP

There are three ways that nanoparticles may enter the human body: by inhalation, through the skin, or by ingestion of contaminated food. The nanoparticles can reach the extracellular fluid, in which the nanoparticles are coated by molecules or proteins (Shang *et al.*, 2014c). Then, the internalization into cells can take place by endocytosis, a receptor-mediated active uptake or by passive diffusion across the cell membrane. The cytotoxic effects of Zn-NP were determined on human cell lines, such as the monocytes, the lung epithelial cells, and the lymphoblasts (Shang *et al.*, 2014d). In addition, the cytotoxic effects of Zn-NP were also evaluated on algal and plant cells, and yeast (*S. cerevisia*), causing the generation of reactive oxygen species, the inhibition of growth, the release of toxic metal ions, and the inhibition of the absorption of nutrients (Kasemets *et al.*, 2009). One of the important properties of metal oxide nanoparticles is the increase in solubility, and the released of metal ions is a significant factor for the cytotoxicity of nanoparticles (Shang *et al.*, 2014e), (Navarro *et al.*, 2008d). Previous works on green algae (*P. subcapitata*) demonstrated that Zn-NP caused a strong cytotoxicity, which was related to the solubility of Zn^{2+} (Franklin *et al.*, 2007; Aruoja *et al.*, 2008b).

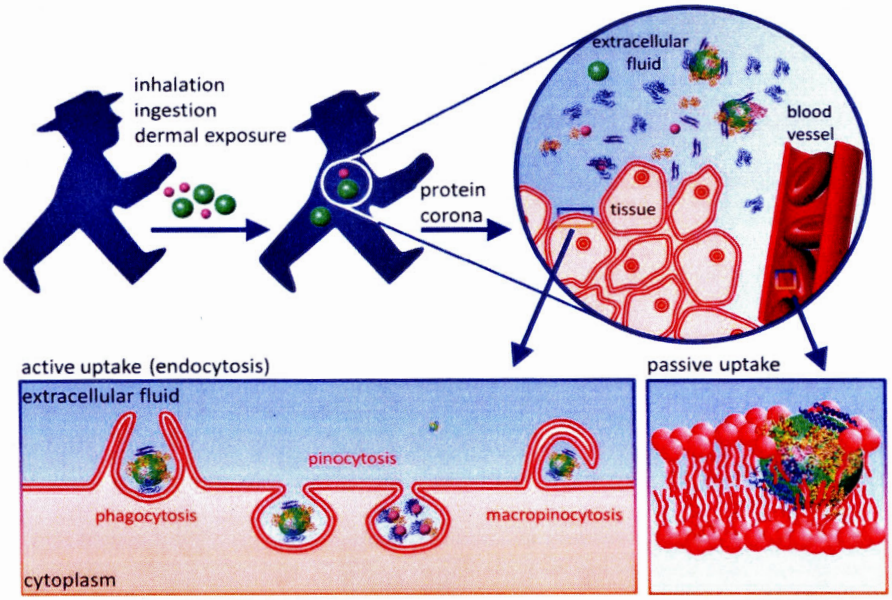


Figure 1.3 The uptake of nanoparticles in human body, tissues and the cellular system (Shang *et al.*, 2014f).

1.3.4 Interaction of nanoparticles with environmental contaminants

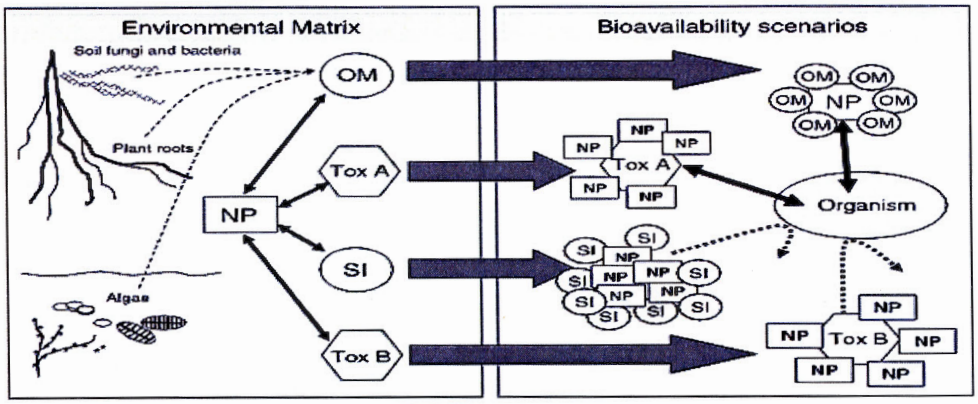


Figure 1.4 The interaction of nanoparticles with environmental contaminants, such as toxicant A and B, organic compounds (OM), salt ions (SI), and their uptake in aquatic organisms (Navarro *et al.*, 2008e).

In the aquatic environment, organic compounds (OM) can increase the stability of nanoparticles, and as a result the complex can enter into the organism. But the presence of salt ions (SI) can decrease the stability of nanoparticles, and as a result the bioavailability of nanoparticles decreases, limiting he entrance of the complex inside the organism (Navarro *et al.*, 2008f). Therefore, the bioavailability of nanoparticles can be increased or decreased, which depends of the interaction of nanoparticles with others compounds. Different pollutants can be adsorbed to nanoparticles depending to their surface area to volume ratio and their complexing capability (Navarro *et al.*, 2008j). As a result of this, their transport, bioavailability, and toxic effect may be changed. Moreover, organic pollutants can interact with hydrophobic nanoparticles like carbon nanotubes and fullerenes (Navarro *et al.*, 2008h), (Wang *et al.*, 2007).

1.3.5 Positive effect of nanoparticles

According to (Navarro *et al.*, 2008i), the effect of TiO_2 nanoparticles was investigated, and they show an increase in the dry weight, the chlorophyll synthesis, and in the metabolism in photosynthesis. These observations could be due to the properties of nanoparticles, or it can be related to the physiological tolerance of plants to metals stress. Also, nanoparticles with high surface area can affect the uptake of nutrients that are essential for the growth of algae (Wang *et al.*, 2007b; Navarro *et al.*, 2008k).

CHAPTER II
THIOL COMPOUNDS IN ALGAE

2.1 Thiol compounds in algae

Glutathione (GSH) is a monothiol that plays the role of free-scavenger (Torriceli *et al.*, 2004d), and phytochelatins (PCn) are polymers of GSH. Also, in plants, GSH plays an important role in cellular processes as defense mechanism against ROS (Yaldav *et al.*, 2010g). In fact, GSH controls the level of H_2O_2. As well as GSH can have two form, such as the reduced form (GSH) and the oxidized form (GSSG) (Hirata *et al.*, 2005b). The ratio of GSH/GSSG is very important for the reduction potential of glutathione, this ratio depends on pH, concentration of GSH, biosynthesis of GSH, and catabolism of GSH (Yaldav *et al.*, 2010h). Glutathione has been founded in cytosol, chloroplast, vacuole, and mitochondria.

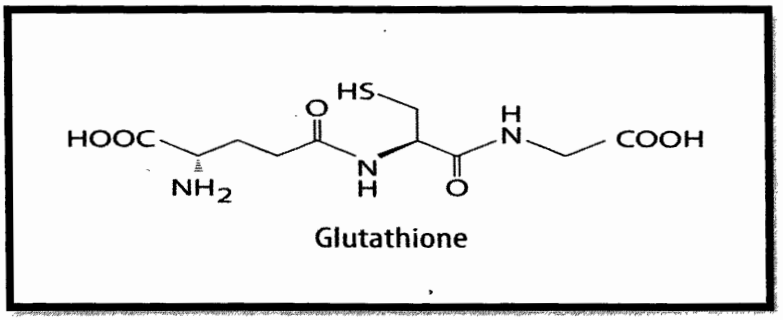


Figure 2.1 The structure of glutathione (GSH).

In other words, GSH is a substrate for phytochelatins synthesis that is catalyzed by the enzyme PC synthase (PCS) (Hirata *et al.*, 2005c). Phytochelatins are metal-bonding polypeptides synthesized by micro algal cells as a result to the exposure to different heavy metals (Le Faucheur *et al.*, 2006c). Phytochelatins play significant role in the

detoxification of heavy metals in the environment, and the homeostasis regulation of metals due to their affinity of metal binding (Le Faucheur *et al.*, 2006b), (Scheidegger *et al.*, 2011a). Therefore, phytochelatins are able to reduce the toxicity of metals by means of decreasing the intracellular concentration of free metals (Le Faucheur *et al.*, 2006d; Scheidegger *et al.*, 2011b).

Micro algal cells can prevent the toxicity of ionic metals *via* two mechanisms: The intracellular metal detoxification and the extracellular exclusion barrier. For the intracellular detoxification, we have metal precipitation or metal immobilization *via* ligands (Scheidegger *et al.*, 2011c). Moreover, for the extracellular mechanisms, the uptake of metals into algal cells is restricted by means of reducing the bioavailability of metals *via* non-specific ligands expulsion, or *via* changing the metal concentration and metal affinity (Scheidegger *et al.*, 2011d; Soldo *et al.*, 2005). The principal property of phytochelatins is their capability for chelating metals, and playing a main role in metal tolerance (Suarez *et al.*, 2010a).

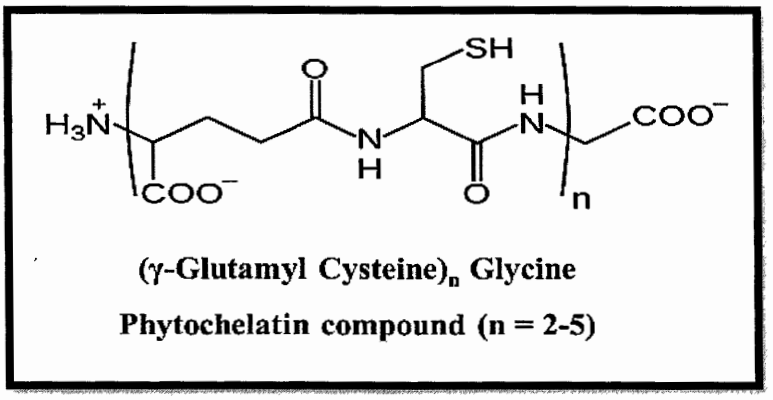


Figure 2.2 The structure of phytochelatins (PCn).

The GSH and phytochelatins play an important function in the detoxification of toxic metals, and we can find these compounds in many species of plants (lower plants and higher plants), algae, and fungus (Torriceli *et al.*, 2004e), (Hirata *et al.*, 2005d). The

thiol compounds such as glutathione and phytochelatins have the ability to bind toxic metals by means of their thiol group (-SH), according to (Torriceli *et al.*, 2004f) and (Skowronska *et al.*, 2001c). The concentration of hydrogen ions (pH) is very important for the bonding between the -SH group and the metals (Millar *et al.*, 2003). Therefore, phytochelatins is known to play a role in metal homeostasis and metal detoxification bbecause of their high affinity for bonding to metals (Le Faucheur *et al.*, 2006f). Several factors can affect the production of GSH and PCn, such as the species of algae, the chemical nature of metal, the concentration of metal, the toxicity level of metal, the environmental pH and temperature, and the growth rate of algae (Yun Wu *et al.*, 2012b).

The effect of several metals such as cadmium, lead, nickel, copper, and cobalt have been investigated in different species of algae in previous studies, according to (Le Faucheur *et al.*, 2004; Le Faucheur *et al.*, 2006e), (Scheidgger *et al.*, 2010d). The induction of intracellular phytochelatins in phytoplankton under metals exposure was reported by (Kawakashi *et al.*, 2005b). As well as, in *C. reinhardtii* exposed to Cd, the synthesis of iso-phytochelatins was investigated (Miazek *et al.*, 2015d). Also, for *C. reinhardtii* exposed to Pb^{2+}, the kinetics of PC synthesis was reported by (Scheidegger *et al.*, 2011e). Although cadmium is known to be the principal inducer of phytochelatins, the synthesis of phytochelatins can be activated in algal cells under the exposure to high concentration of Zn^{2+} (Suarez *et al.*, 2010b). The Figure 2.3 show the interaction of phytochelatins with Zn^{2+}. In previous studies, the surface of electrodes was modified with phytochelatins, and this was used as a biosensor in order to detect heavy metals in different biological, environmental, and pharmaceutical samples (Miazek *et al.*, 2015e), (Adam *et al.*, 2005). Therefore, phytochelatins can be used as a biological or biochemical indicator of the contamination of toxic metals.

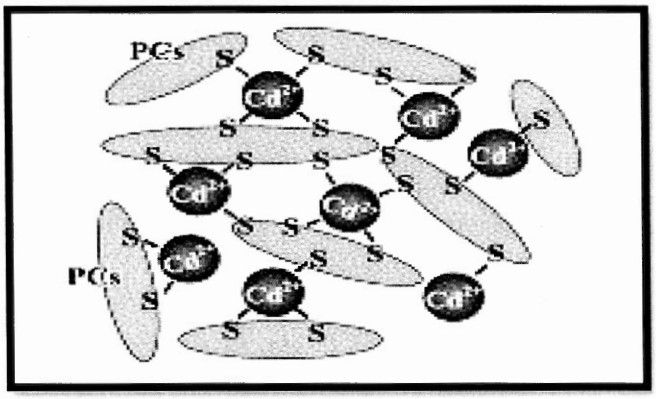


Figure 2.3 The interaction between phytochelatins and Cd^{2+} (Rama et Rai, 2009).

CHAPTER III
ALGAL MODEL *C. REINHARDTII*

3.1 Algal model *C. reinhardtii*

Microalgae are photosynthetic and aquatic organisms that use the light of solar in order to change CO_2 into organic carbon, (Miazek *et al.*, 2015f), (Virgilio *et al.*, 2005a). For growing microalgae, the optimal temperature is between 20 and 30°C. Also, microalgae can synthetize abundant compounds as a result of stress, such as metal stress and high temperature. It was shown that the exposure to heavy metals is an important method for the synthesis of target compounds with algal culture (Virgilio *et al.*, 2005b). Microalgal cells against metals stress have two defensive mechanisms: The first one is the synthesis of chelating agent, such as the glutathione and phytochelatins; The other mechanism is the synthesis of antioxidant compounds (Miazek *et al.*, 2015g; Hanikenne, 2003b).

3.2 Why *C. reinhardtii*

In my project, *C. reinhardtii* (CC-125) was selected as a basic model to investigate the effect of Zn^{2+} and Zn-NP. Al experiments were done in presence and absence of Zn^{2+} and Zn-NP. Beside of that, we investigated the release of metal ions from Zn-NP in comparison with Zn^{2+} for understanding their effect on algal cells of *C. reinhardtii*. This include the effect on the growth rate, on the production of phytochelatins, on the kinetics of PCs, and on the cellular tolerance for these metallic contaminants.

Green alga *C. reinhardtii* as a practical biological model has a lot of advantages such as laboratory cultivation, rapid growth, toxicological studies, analytical experiments, and low cost (Harris, 2001a). For many years, this unicellular has been applied for

several studies and projects for example, photosynthesis, respiration, nitrogen assimilation, and flagella motility (Hanikenne, 2003c). *C. reinhardtii* species are spread al over the world in soil and freshwater. Algal cells can grow in a simple medium of inorganic salts, such as HSM, or in Tris–Acetate–Phosphate (TAP) medium (Harris, 1989).

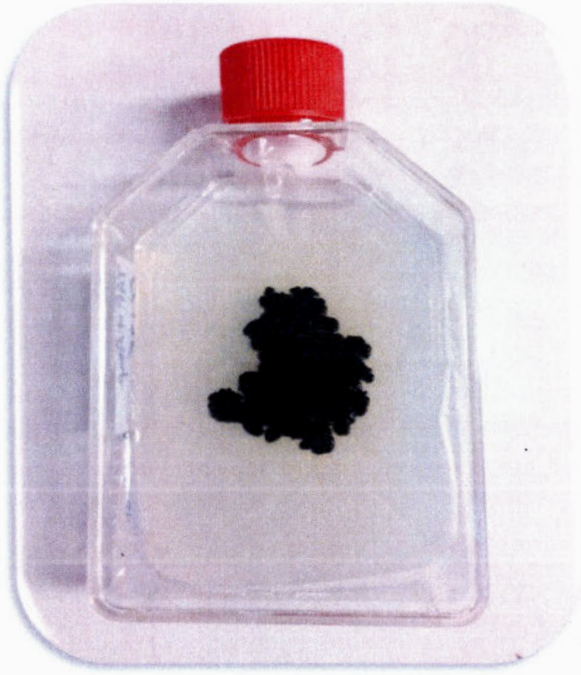


Figure 3.1 Lab stock of algal culture in solid HSM (high salt medium with agar).

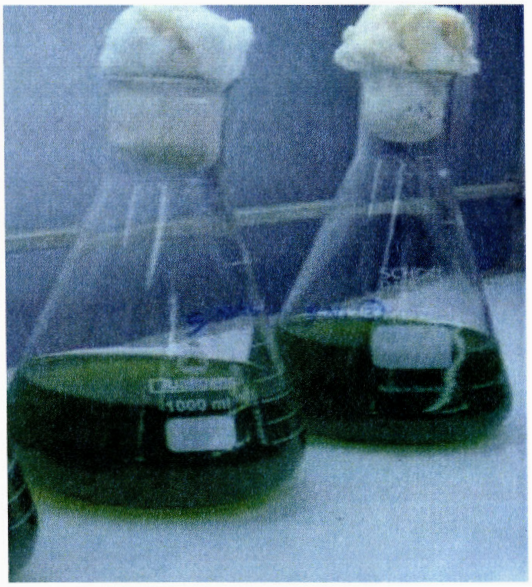


Figure 3.2 Liquid stock of algal culture in HSM (high salt medium).

3.3. Cell architecture

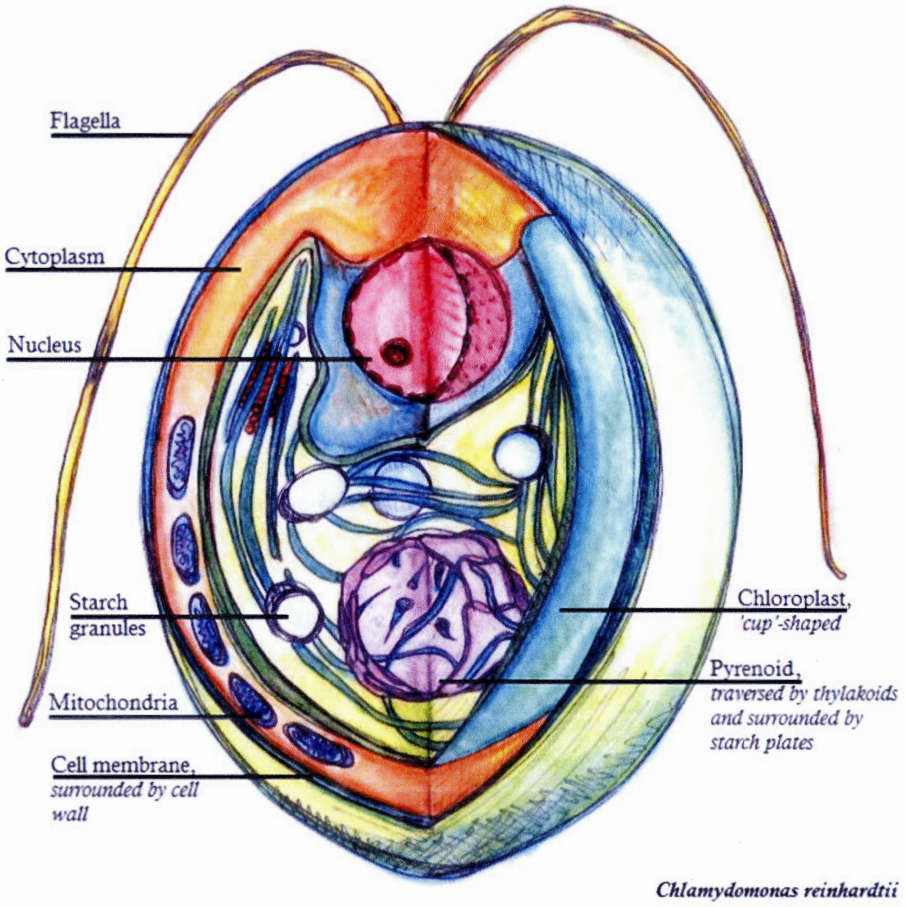


Figure 3.3 Cross section of a *C. reinhardtii* (*C. reinhardtii*) (en.wikipedia.org/wiki/ Chlamydomonas_reinhardtii).

C. reinhardtii have a cell wall, a chloroplast, an eyespot or stigma, for perceiving the light and two flagella. In terms of shape, cells of *C. reinhardtii* are spherical to oval, and the length is variable between 8 to 22 μm, a pair of flagella expand *via* a collar. The length of flagella is about 2 times of body length. Inside the cell, the chloroplast surrounds the nucleus. The cell wall is made of glycoproteins with high hydroxyproline content. Cells can divide quickly, and under optimal condition, the number of cells can reach about 1 to 2 10^7 cells per ml (Rochaix *et al.*, 2006).

3.4. Cell response to heavy metals exposure

Effects of toxic metals (copper, iron, mercury, lead, cadmium, and zinc) in green *C. reinhardtii* has been investigated (Hanikenne, 2003b). Cadmium and copper affected the growth, photosynthetic activity, and chlorophyll content. Copper, cadmium, and zinc can inhibit the uptake of nutrients as the nitrate into the cell (Hanikenne, 2003c), (Devrise *et al.*, 2001). Indeed, the cell wall of *C. reinhardtii* represents a protection against metals. Also, phytochelatins can be synthesized by *C. reinhardtii* as a result of heavy metals stress for the detoxification of excess intracellular metals (Harris, 2001b). Plus, for *C. reinhardtii* exposed to Hg^{2+}, the accumulation of Hg^{2+} and the toxicity effect of Hg^{2+} has been previously reported (Samadani *et al.*, 2018). It was shown that Hg^{2+} can affect the growth rate due to a strong accumulation of Hg^{2+} in algal biomass. Also, this author did report the toxicity effect of Cd^{2+} on *C. reinhardtii* (Samadani *et al.*, 2017).

CHAPTER IV
MATERIAL AND METHODS

4.1. Chemicals and materials

The solution of metals was prepared with metal salts from Fisher Scientific, New Jersey, USA. The zinc Nanopowder (particle size: 35 nm, purity > 99 %, appearance: black, melting point: 419.53°C, molecular weight: 65.37, boiling point: 907°C) was purchased from MTI Corporation, Richmond, Canada. Phytochelatin standards (purity > 98 %) were purchased from Canpeptide, Montreal, Canada. The others chemicals in this project were purchased from Sigma Aldrich, such as trifluoroaceticacid (TFA), methane sulfonic acid (MSA), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), thris(2-carboxyethyl) phosphine hydrochloride (TCEP), glutathione (GSH), and monobromobimane (mBBr), diethylenetriamine penta acetic (DTPA), and HPLC-grade acetonitrile (ACN) from Caledon Laboratories LTD (Georgetown, Ontario, Canada. The water was filtered by (Thermo scientific barnstead nanopure ultra water purification system). Containers used in this project were made of polycarbonate, polypropylene or glasses. Al glass flasks were soaked for a day in a solution of 10 % nitric acid (Sigma-Aldrich) and rinsed six times with Milli-Q water from a purification system of Millipore. Then, al of flasks and materials needed for cultivation were sterilized by autoclave. Erlenmeyer flasks (50 ml) were handled for stock cultures, and provided Pyrex storage dishes were handled for long-term cultures.

4.2. Biological material

Al tests were applied with the unicellular green alga *C. reinhardtii* (CC-125). The Wild-type of *C. reinhardtii* (CC-125) was purchased from the *Chlamydomonas*

resource center (Duke University, Durham, NC, USA). The cultivation of algal cells was prepared in 1-L batch culture of HSM (high salt medium). The composition of HSM was based on Harris (1989): NH_4Cl 5gr/500ml, $MgSO_4.7H_2O$ 0.2 gr/500ml, $CaCl_2$ 0.1 gr/500ml, KH_2PO_4 14.8 gr/500ml, K_2HPO_4 28.8 gr/500ml, H_3BO_3 92.76 mgr/500ml, $MnCl_2.4H_2O$ 207.69 mgr/500ml, $ZnCl_2$ 1.64 mgr/500ml, $FeCl_3.6H2O$ 79.89 mgr/500ml, $Na_2EDTA.2H_2O$ 150 mgr/500ml, $CoCl_2.6H_2O$ 1.3 mgr/500ml, $Na_2MoO_4.2H_2O$ 3.63 mgr/500ml, $CuCl_2.2H2O$ 0.006 mgr/500ml. The pH of medium was adjusted at 6.8, by using 1N HCl and 1N NaOH. Then, the culture was sterilized by autoclave for 1.5 hours at 80 °C. After that, the medium will stand overnight before use, and the medium was equilibrated for 24 h to reach chemical equilibrium.

The condition of growth consisted of a continuous illumination with an intensity of 80 µmol photons m^{-2} s^{-1} provided by white fluorescent lamps (sylvania Grolux F36W) and temperature of 24 ± 1 °C. The cell density at the exponential growth phase was fixed with a cell counlter multisizer Z3 (Beckman counlter Inc). To prevent the metal contamination, al glasswares and polycarbonate containers were acid-presoaked in 10 % HNO_3 for 12 h before use with Nanopure water (thermo scientific barnstead Nanopure ultra water purification system).

4.3. $ZnCl_2$ and Zn-NP treatments

This project was carried out by preparing fresh standard solutions of $ZnCl_2$ and Zn-NP at a concentration of 500 µM, and testing solutions of 30 µM and 60 µM were prepared. The experiments were performed on algal samples having an initial cell density of 5×10^5 cells per ml. The experimental algal cultures were exposed from 24 h to 72 h to different concentrations (30-60 µM) of Zn^{2+} and Zn-NP (in a final volume of 25 ml). The control for this test did not have additional Zn^{2+} and Zn-NP. The experiments were done in triplicate for each tested concentration of Zn-NP and $ZnCl_2$. Al Erlenmeyer flasks were placed on shaken platforms (at a rate up to 80 rpm) at 24 °C under a

continuous illumination of 80 ± 10 µmol photons m^{-2} s^{-1}. At 24, 48, and 72 h, bioanalytical measurements were performed for the cell density, the accumulation of total zinc in algal cells, and the induction of glutathione and Phytochelatins according to their retention time by UHPLC.

4.4. Determination of the relative cell division rate

The number of cell was determined by using Countess II FL automated cell counter (life technologies Thermo Fisher Scientific Brand). The evaluation of cell number for each sample was performed in triplicate. 10 µl of algal culture was diluted with 15-20 µl of Trypan blue stain 0.04 % in order to increase the contrast of the cells. The change in cell number (cells per ml) was determined daily during 72 h of growth.

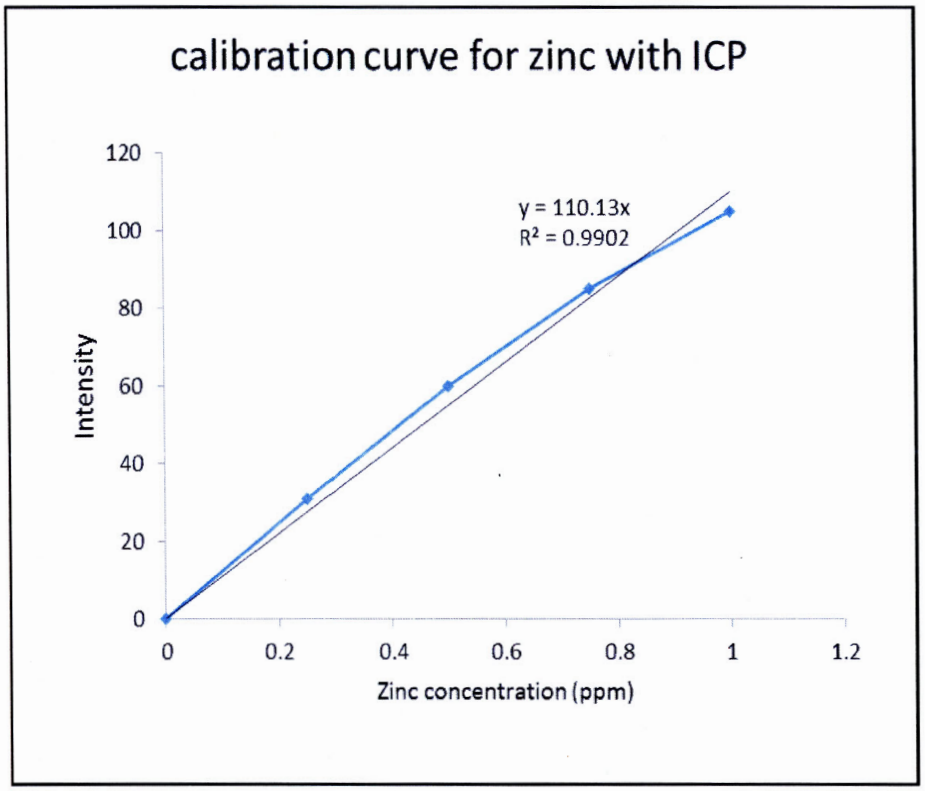
4.5. Total zinc accumulation

For the determination of the accumulation of total Zn^{2+} in algal cells, we used the following methodology: 1. Each sample was filtered by using acid-washed filters (cellulose nitrate filter paper - Millipore 0.45 µm); 2. To remove the excess of adsorbed Zn^{2+} and Zn-NP in the cell wall, each sample was washed with 5 ml of EDTA (1 mM); 3. Each filter was dried at 60 °C during 24 h, then each filter was digested with 1 ml of HNO_3 (80 %) and 125 µl of H_2O_2 (30 %) at 120 °C during 12 h.
Prior measurements, each sample was diluted in HNO_3 (10 %) by filling 10 ml of Nano pure water. Then intracellular Zn^{2+} and Zn-NP was measured by using the inductively coupled plasma emission spectroscopy (ICP-OES model: 5200, Agilent technologies), and the quantity was presented as Pico gram per cell. For making the calibration curve, standard solutions were prepared in 10 % HNO_3.

4.6. Preparation of ICP-OES standard calibration solutions

A calibration curve was handled to define the concentration of Zn^{2+} in the digested solution. The instrument was calibrated by using several solutions of known concentration of $ZnCl_2$. Five standard calibration solutions were used as 0, 0.25, 0.5, 0.75, and 1 ppm (Figure 4.1).

a)



b)

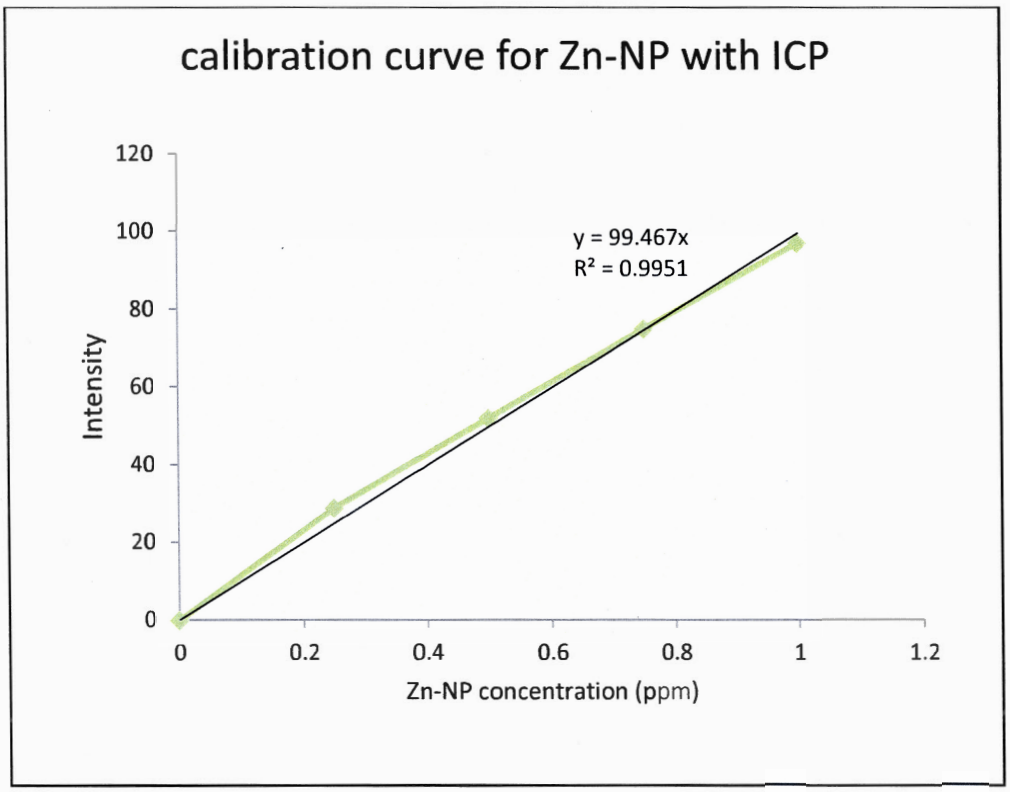


Figure 4.1 Standard calibration curve for a) Zn^{2+} and b) Zn-NP.

4.7. Characterization and quantification of induced thiol compounds

For the analysis of thiol compounds, our methodology consisted in four steps:
The first step consisted in the preparation of algal extracts. Total amount of algal cells (25 ml) from the control and the treatment was harvested by centrifugation for 15 min at 3000 *g*. The next step was the oxidation of each pellets, which was suspended in 1.2 ml of 0.12 mM HCl and 5 mM DTPA (the acid was added for denaturation of the enzymes). Then each sample was broken by ultra sonication for 5 min at 0 °C. Each sample was harvested by centrifugation (40 °C, 20 min, 13 000 *g*). After that, the

supernatant was used for the reduction step: 650 µl of 200 mM HEPES and 5 mM DTPA (pH 8.2) was added to 250 µl of supernatant, then 25 µl of 20 mM TCEP was added to prior medium. TCEP is responsible for reducing the disulfide bridge. HEPES was added as buffer (pH 8-9). The next step is the derivatization: 10-20 µl of 100 mM monobromobimane (mBrB) in acetonitrile (ACN) at the final concentration (1-2 mM) in excess was added to prior medium under dim light condition. Then each medium was transferred to a dark place for 15 min in order to the reaction take place. Then, 100 µl of 1 mM Metasulfonic acid MSA (at the final concentration of 0.1 mM) was added to the medium. MSA was used to stop the reaction and the final volume was 1 ml for each medium. The mBrB was used as a fluorescent marker that can label peptide compounds (containing -SH group). We used a UHPLC (ultra-high-pressure liquid chromatography) (Agilent technologies, united states) equipped with fluorescence detector (Agilent technologies, 1260 infinity, with 8 µl FLD cell and 2 mpa maximum pressure). We used a light excitation at 380 nm and the fluorescence emission was measured at 470 nm. Each sample was separated on a reverse phase using C-18 column (50 mm x 2.1 mm, eclipse plus 18 with RRKD 1.8 mm particle size) at 40 °C with pre-column (Agilent technologies, 1290 infinity) and a quad-pump (Agilent technologies, 1290 infinity) with flow rate of 0.1 ml/min. Solvent A was 0.1 % trifluoroacetic acid (TFA) as the aqueous mobile phase and solvent B was acetonitrile. The gradient was as followed: from 0-1.7 min, 10-21 % B: 1/7-4.20 min, 21-35 % B: 4.20-5 min, 35-100 % B: 5-6.30 min, isocratic 100 % B: 6.30-8.20 min 100-10 % B. Between each measurement, the column was cleaned by 100 % acetonitrile for 5 min, followed by 10 min at 10 % acetonitrile for equilibration at a flow rate of 0.1 ml/min. The retention time was checked with PCs standards. Also, for the quantification of PCs and GSH, the calibration curve was used based on PCs and GSH standards. Fresh stock standard solutions were prepared at 0.01 M and 10-100 µM, in the same way as the biological sample solution of 0.12 M HCl and 5 mM DTPA which was used in order to minimize the oxidation. Glassware and plastic bottles were soaked in acid bath (1M HCl) for at

least 24 h and then was rinsed 3 times with de-ionized water. Also, the mobile phase 0.1 % TFA was filtered before use. A chromatogram is presented in figure 4.2.

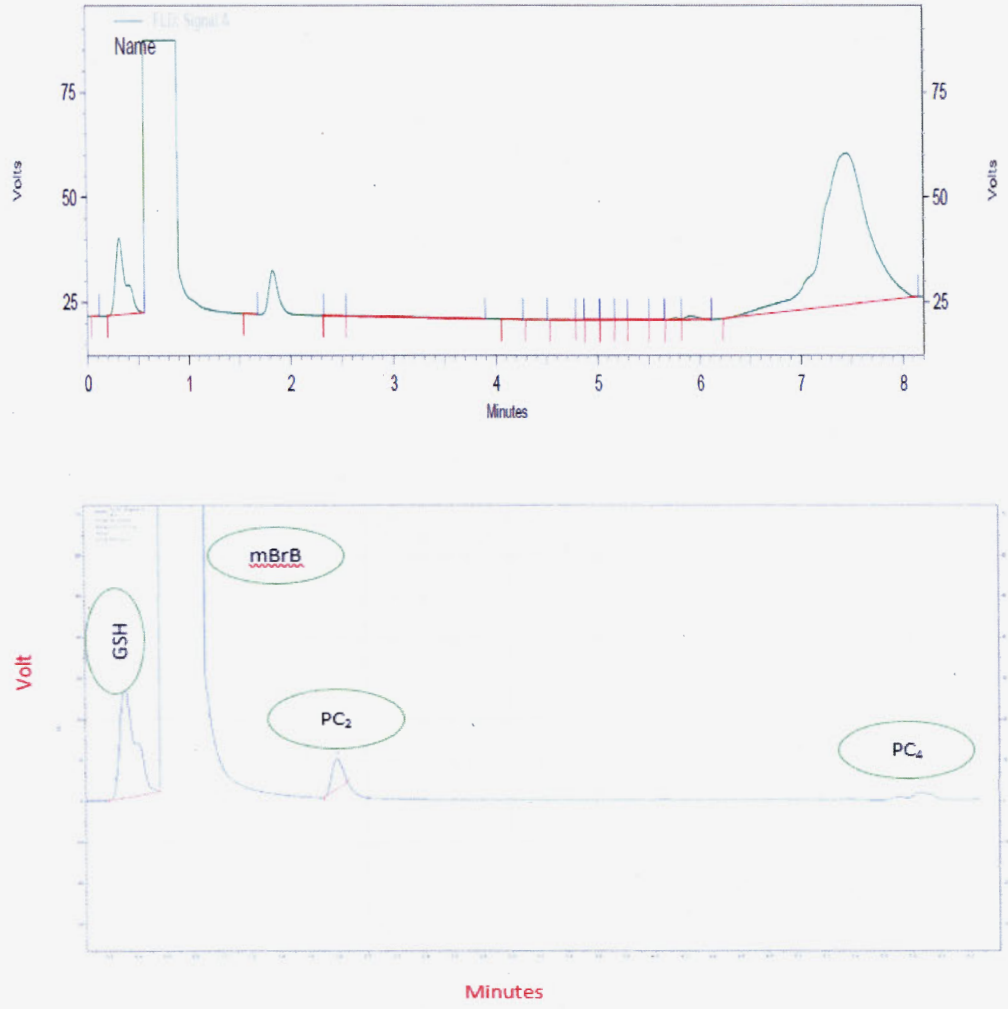
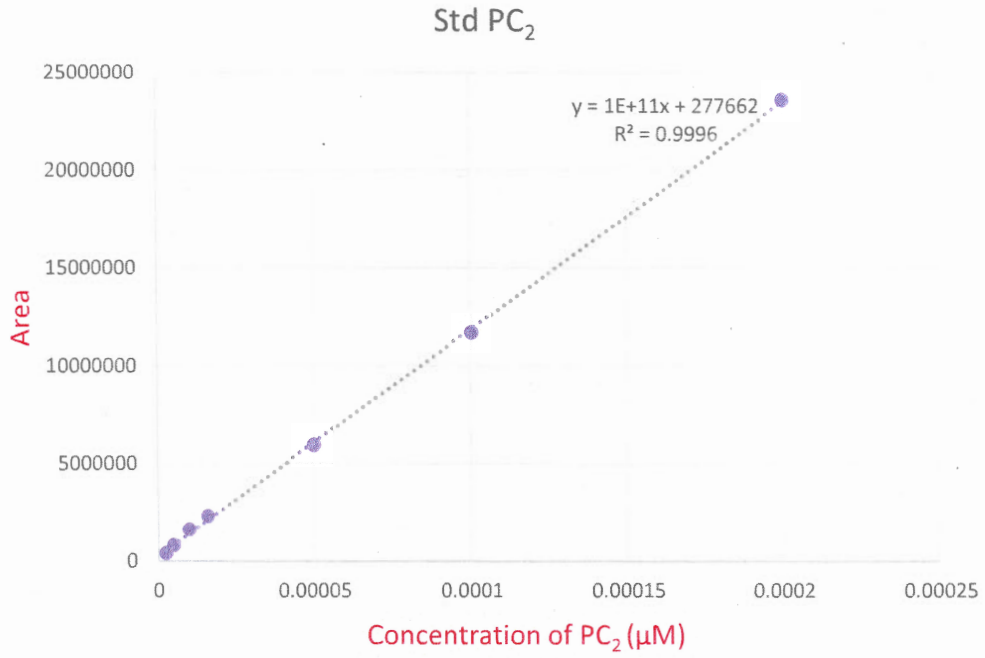


Figure 4.2 Chromatogram showing the retention time of glutathione and phytochelatins (PC_2 and PC_4) for Zn-NP treated-cells (60 µM) of *Chlamydomonas reinhardtii.*

4.8. Calibration standard curve for phytochelatins

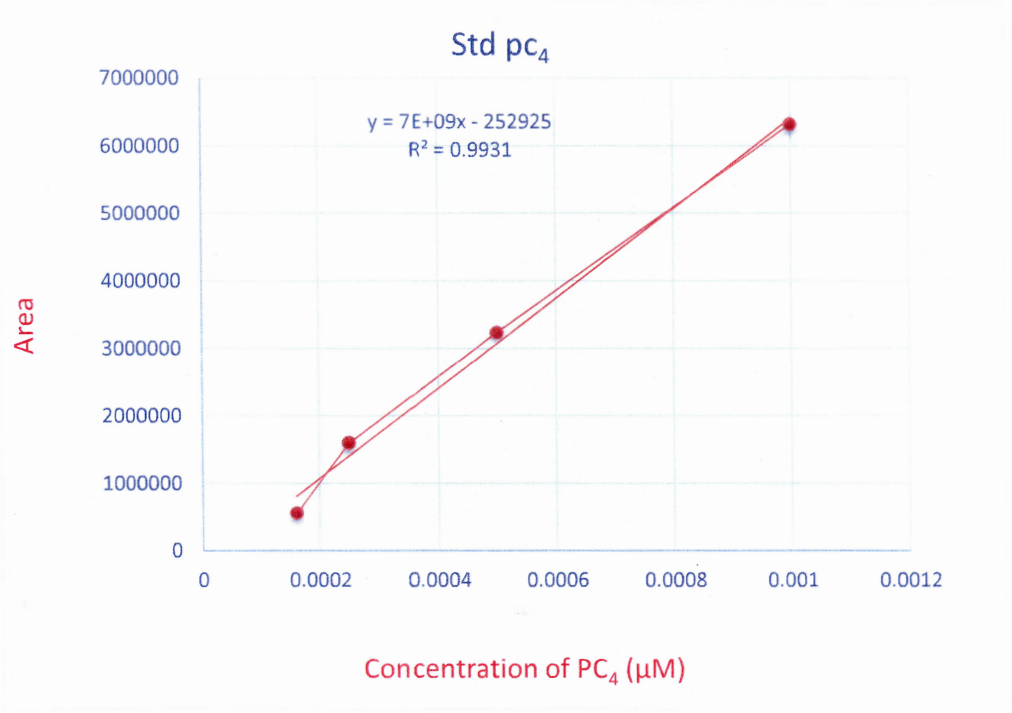
The calibration curve of phytochelatins was provided with different concentration of PCn prepared from a stock solution of 1 mg/ml in HSM, these solutions were analyzed by means of UHPLC. The assay was linear over the concentration range examined (Figure 4.3).

a)



AVRAGE	STDEV	RSD%	LOD=3Sb/m	LOQ=10Sb/m
828271.3333	21257.33326	2.566469755	0	0

b)



Average	STDEV	RSD%	LOD=3Sb/m	LOQ=10Sb/m
6324067.667	199629.1324	3.156657123	0	0

Figure 4.3 Standard calibration curve for a) PC_2, b) PC_4, analyzed by UHPLC.

4.9. Calibration standard curve for glutathione

The calibration curve of glutathione was provided with different concentration of glutathione prepared in H_2O. These solutions were analyzed by means of UHPLC. The assay was linear over the concentration range examined (Figure 4.4).

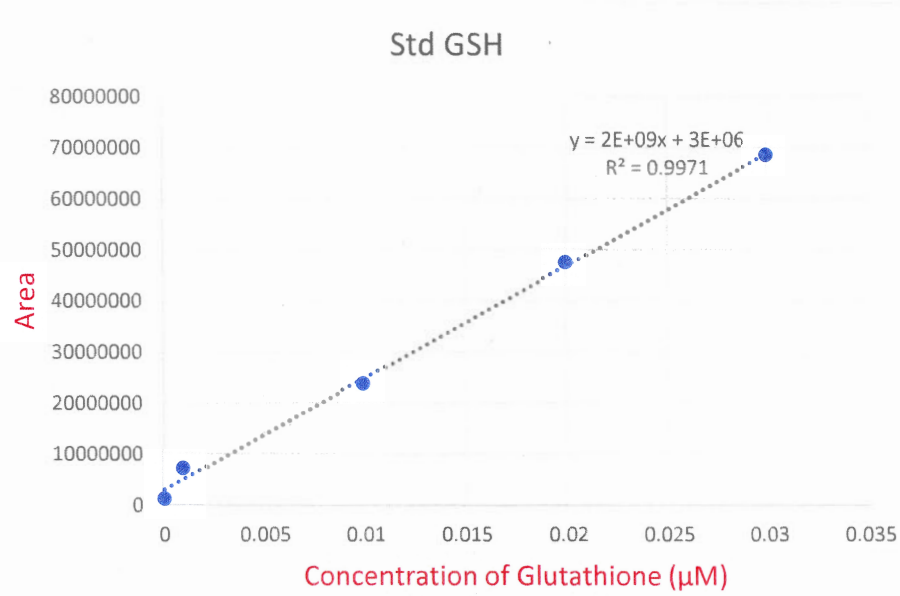


Figure 4.4 Standard calibration curve of glutathione (GSH) analyzed by UHPLC.

CHAPTER V
RESULTS AND DISCUSSION

5.1. The effect of Zn^{2+} and Zn-NP on the cellular division

In the industrial world, the intensive use of zinc contributed significantly to the contamination of the aquatic environment (Hirata *et al.*, 2005e). For this reason, it was important to study the toxicity effect of Zn^{2+} and Zn-NP in aqueous solution by using the biological model *C. reinhardtii*. In my project, I evaluated the relative cellular division rate in order to have a specific indication of the tolerance range of algal cells to the exposure of both Zn^{2+} and Zn-NP. Therefore, the effect of Zn^{2+} and Zn-NP on the growth rate (μ) of algal cells was monitored by measuring the change of the cell density under two concentrations (30 μM and 60 μM) at different time of exposure (24 h, 48 h, and 72 h).

5.1.1. The effect of Zn^{2+} on the cellular division

Algal cells of *C. reinhardtii* were exposed to $Zncl_2$ at the concentration of 30 μM and 60 μM at different time of exposure from 24 h to 72 h. As shown in the Figure 5.1, the algal cell density for each tested concentration was measured to calculate the growth rate (μ) as cell number per day for each time of exposure. Our results showed that the maximum toxicity effect of Zn^{2+} was induced at 24 h. For the concentration of 30 μM at 24 h, there was a slight decrease on the relative cellular division rate compared to the control by an amount of 30 %. Plus, for the concentration of 60 μM at 24 h, there was a strong decrease on the relative cellular division rate compared to the control by an amount of 60 %, which indicated an increase of toxicity level for the concentration of 60 μM compared to 30 μM. Our results strongly indicated that the maximum

inhibitory effect of Zn^{2+} was observed for the concentration of 60 µM at 24 h. According to previous report (Huang *et al.*, 2009a), the green alga *C. vulgaris* was exposed to different concentrations of Zn^{2+} (from 5 µmol/L to 80 µ mol/L) during 7 days, and the results showed that Zn^{2+} was toxic for the concentration of 60 and 80 µM. they determined Zn^{2+} by inductively coupled plasma mass spectrometry (ICP-MS. However, for the concentration of 30 µM and 60 µM at 48 h and 72 h, there was no significant differences in comparison to the control. These results on the growth ascertain that *C. reinhardtii* was tolerant to the inhibitory effect of Zn^{2+} under this condition. According to (Suarez *et al.*, 2010c), there is a direct relation between the preventative effect and the concentration of the metal.

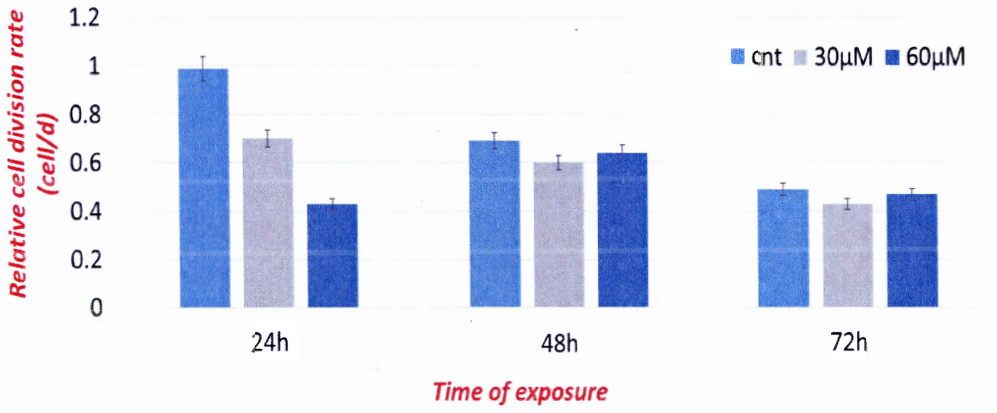


Figure 5.1 The effect of 30 and 60 µM of Zn^{2+} on the relative cellular division rate of *C. reinhardtii* at different time of exposure (24 h, 48 h, 72 h). The cnt represents the Control. Each data indicated the average of 6 replicates, n=6.

5.1.2. The effect of Zn-NP on the cellular division

When *C. reinhardtii* was exposed to Zn-NP under the concentration of 30 and 60 µM at different time of exposure from 24 h to 72 h, the algal cell densities for each tested

concentration were calculated as cell number per day, as shown in Figure 5.2. Our obtained results showed that the growth rate (μ) was related to the toxicity effect of Zn-NP, which was dependent to the tested concentration and the time of exposure. In fact, the toxicity effect of Zn-NP was the highest at 24 h. For the concentration of 30 μM of Zn-NP at 24 h, there was a slight decrease on the relative cellular division rate by an amount of 30 % compared to the control. Also, for the concentration of 60 μM at 24 h, there was a strong decrease on the relative cellular division rate by an amount of 60 % compared to the control. Plus, at 48 h and 72 h, for both tested concentrations (30 and 60 μM), the results demonstrated a decrease of the relative cellular division rate compared to the control, which was higher in comparison to 24 h. The results showed that, at these times of exposure, the growth rate decreased due to an increase of the toxicity impact on the cellular system of *C. reinhardtii*. Compared to the effect of Zn^{2+}, the effect of Zn-NP was different on the growth rate of alga *C. reinhardtii*, under tested conditions.

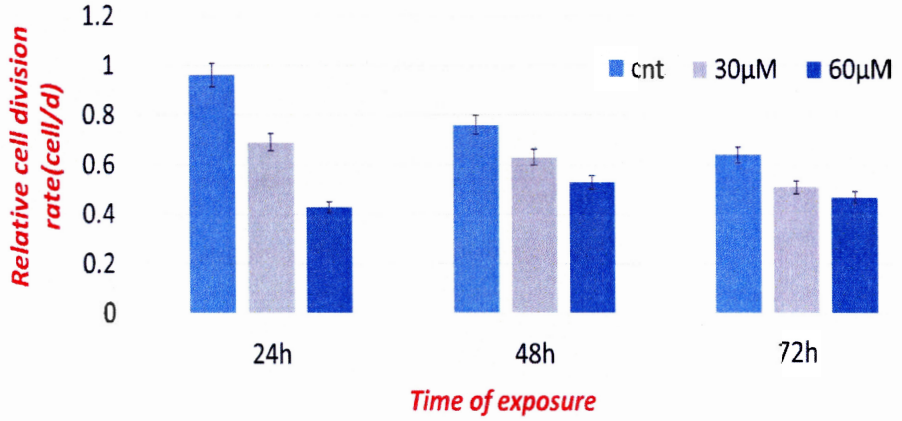


Figure 5.2 The effect of 30 and 60 μM of Zn-NP on the relative cellular division rate of *C. reinhardtii* at different time of exposure (24 h, 48 h, 72 h). The cnt represents the Control. Each data indicated the average of 6 replicates, n=6.

According to obtained results on the inhibition of the growth rate, we could determine the tolerance capacity of *C. reinhardtii* against the toxicity effect of Zn^{2+} and Zn-NP suspension in aqueous solution. This knowledge can be used for the development of a new bioremediation technology for these metallic contaminants using *C. reinhardtii.* For alga *C. reinhardtii*, under tested conditions, the toxicity of Zn-NP was more important than Zn^{2+}, for the same concentration, during 48-72 h. According to former study (Huang *et al.*, 2009b), the green alga *C. vulgaris* was exposed to different concentrations of Zn^{2+} during 7 days, and the results showed that Zn^{2+} was toxic for the concentration of 60 µM. In my project, results showed that Zn^{2+} caused a stronger toxicity effect in *C. reinhardtii* for the concentration of 60 µM compared to the concentration of 30 µM.

5.2. Bioaccumulation of Zn^{2+} and Zn-NP in algal cells

The bioaccumulation of Zn^{2+} and Zn-NP was measured in algal cells to better understand the effect of Zn^{2+} and Zn-NP on the *C. reinhardtii* for two tested concentrations (30 µM and 60 µM) at different times of exposure (24 h, 48 h, and 72 h). The previous study of (Klaine et Ward, 1993) reported that the concentration of the essential metallic nutrients in the growth of medium can have a significant efficiency on the bioaccumulation of Zn^{2+}.

5.2.1. Bioaccumulation of Zn^{2+} in algal cells

The algal cells of *C. reinhardtii* were exposed to $Zncl_2$ for the concentrations of 30 and 60 µM at different time of exposure from 24 h to 72 h. The intracellular concentration of Zn^{2+} for each tested concentration were calculated as pico gram per cell. As shown in Figure 5.3, the bioaccumulation of Zn^{2+} in algal cells was determined. As shown by the obtained results, there were significant differences in accumulated Zn^{2+} between

algal cells exposed to the concentrations of 30 μM and 60 μM. The maximum capacity of accumulated Zn^{2+} in algal cells, under tested conditions, was for the concentration of 60 μM at 48 h. For the testing concentration of 60 μM, there was an increase in accumulated Zn^{2+} in algal cells from 24 h to 48 h, by an amount of 43 %.

In addition, the results demonstrated a decrease in accumulated Zn^{2+} in algal cells from 48 h to 72 h, by an amount of 57 %. In a prior study (Gaither et Eide, 2001), it was reported that for algae the Zn^{2+} can be uptake in the cell through two ways, a transport with low affinity and a transport with high affinity. According to (Hassler *et al.*, 2005a), *Chrorella kessleriiwas* exposed to Zn^{2+} for the concentration from (20 μM to 50 μM). The authors showed that two factors can affect the intracellular concentration of metals, such as the rate of metal uptake and the rate of metal expulsion. In addition, light and temperature can reduce the amount of accumulated Zn^{2+} in algal cells. According to a former study (Scheidegger *et al.*, 2010d), *C. reinhardtii* was exposed to Pb^{2+} for a short term of exposure (6 h). They determined the amount of Pb^{2+} by inductively coupled plasma mass spectrometry (ICP-MS). They reported that there was an increase in the intracellular concentration of Pb^{2+} in algal cells of *C. reinhardtii* during 6 h. In another study (Huang *et al.*, 2009c), the alga *C. vulgaris* was exposed to different concentrations of Zn^{2+} during 7 days, and their results showed an increase of bioaccumulation in algal biomass during the first three days under the exposure to the low concentrations (5 μM and 10 μM). However, for high concentrations (60 μM and 80 μM), there were no significant differences in algal biomass of *C. vulgaris*.

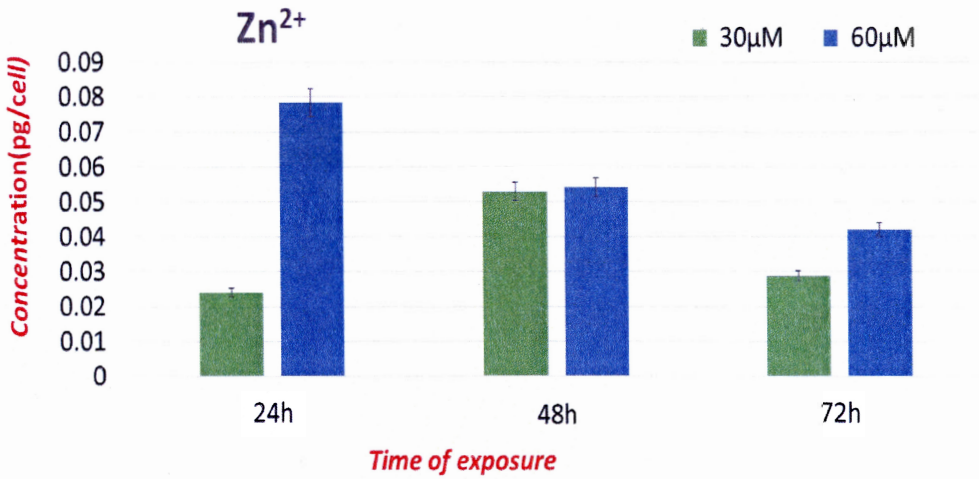


Figure 5.3 Bioaccumulation of Zn^{2+} in algal cells at different time of exposure (24 h, 48 h, and 72 h) for the tested concentrations of 30 and 60 µM. Each data indicated the average of 6 replicates, n=6.

5.2.2. Bioaccumulation of Zn-NP in algal cells

When *C. reinhardtii* was exposed to Zn-NP for the concentrations of 30 and 60 µM at different time of exposure from 24 h to 72 h, the intracellular concentration of total Zn^{2+} for each tested concentration were calculated as pico gram per cell. In Figure 5.4, the bioaccumulation of total Zn^{2+} in algal cells is shown. The results demonstrated that there were significant differences between accumulated total Zn^{2+} during the exposure time from 24 h to 72 h. There was a decreasing trend in the intracellular concentration of total Zn^{2+} by amount of 33 % from 24 h to 72 h for both concentrations (30 µM and 60 µM) of Zn-NP. The maximum capacity of accumulated total $Zn^{2+}$was reached at 24 h under the concentration of 60 µM of Zn-NP. During 48 h and 72 h, the bioaccumulation of total Zn^{2+} in algal cells was lower than at 24 h, which can be explained by the toxicity effect of Zn-NP.

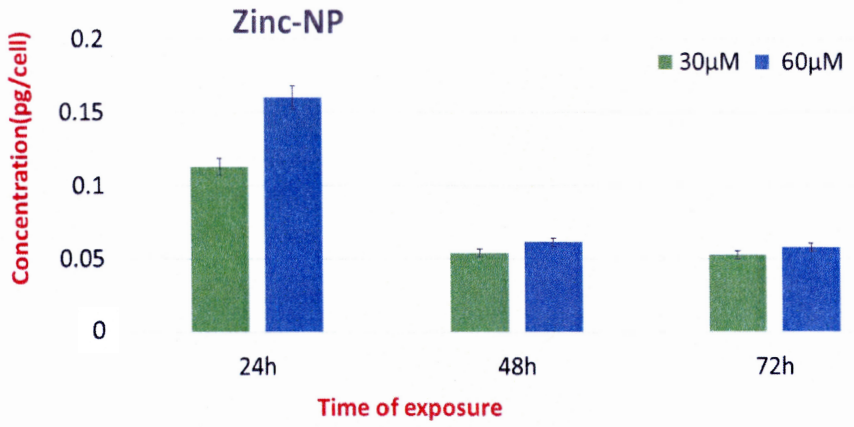


Figure 5.4 Bioaccumulation of total Zn^{2+} in algal cells exposed to 30 and 60 µM of Zn-NP at different time of exposure (24 h, 48 h, and 72 h). Each data indicated the average of 6 replicates, n=6.

5.3. The effect of Zn^{2+} and Zn-NP on the induction of thiol compounds

The concentration of thiol compounds was measured in algal cells by considering the bioaccumulation effect of Zn^{2+} and Zn-NP when *C. reinhardtii* was exposed to two concentrations (30 µM and 60 µM) at different times (24 h, 48 h, and 72 h). According to (Torricelli *et al.*, 2004g), the glutathione and phytochelatins synthesis is a significant mechanism for the detoxification of heavy metals in plants and green algae, by forming of complex with heavy metal and the thiol group (SH).

5.3.1. The effect of Zn^{2+} on the phytochelatins synthesis

The figure 5.5 and figure 5.6 show us the amount of thiol compounds, when algal cells were exposed to Zn^{2+} for the concentrations of 30 µM and 60 µM at different time of exposure from 24 h to 72 h. Among the analyzed thiol compounds (including GSH,

PC_2, PC_3, PC_4, and PC_5), we could detect and quantify only the PC_2 and PC_4 under al treatment condition using the UHPLC. In comparison, these phytochelatins were not detected in the control culture for al time of exposure.

5.3.1.1. The effect of 30 µM of Zn^{2+} on the induction of thiol compounds

For the concentration of 30 µM, we found the maximum induction of PC_2 and PC_4 at 24 h. The induction of PC_2 was more important than PC_4, representing the main inducer under this condition. According to previous studies, the exposure to low concentration of metals favorite the possibility of forming small chains of phytochelatins (PC_2 and PC_3) instead of the long ones (PC_4 and PC_5). Concerning the induction of PC_4 from 24 h to 48 h, there was a decrease in the amount by 44 %. In addition, from 48 h to 72 h, there was an increase in the amount by 55 %. A former study (Kobayashi *et al.*, 2006) about the complexion of PC_n with Zn^{2+} demonstrated that the ratio between Zn^{2+} and PC_2 was 1:1 under the pH of 7.5. Also, the complexion PC_2- Zn^{2+} can be explained from two possibilities: The first is from two atoms of sulfur from Cys, and the second is from two atoms of oxygen from Glu. A prior study (Le faucheur *et al.*, 2006g) demonstrated that Zn^{2+} has a lower toxicity than others metals (such as Cu^{2+}, Ni^{2+}, Pb^{2+}, Ag^{2+}) in alga *Scenedesmus vacuolatus*, which induced the intracellular thiol contents. Moreover, Pb^{2+} has higher toxicity than the other metals under tested condition. Main inducer was GSH and PC_2 when exposed to Pb^{2+} but under exposure of Zn^{2+}, the main inducer was GSH.

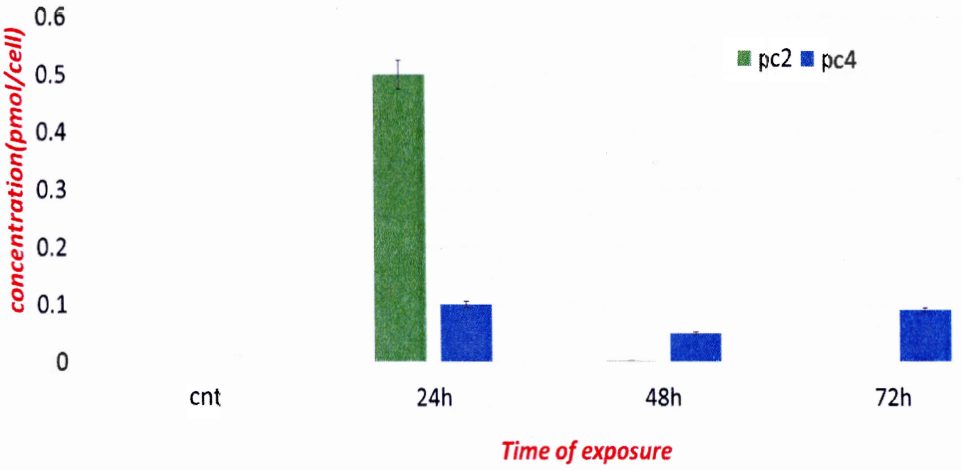


Figure 5.5 Effect of 30 µM of Zn^{2+} on the induction of thiol compounds at different time of exposure (24 h, 48 h, and 72 h). Each data indicated the average of 6 replicates, n=6.

5.3.1.2. The effect of 60 µM of Zn^{2+} on the induction of thiol compounds

As you can see in Figure 5.6, there was a high induction of PC_4 at 24 h, representing the main inducer under the concentration of 60 µM of Zn^{2+}. Under this high concentration, the possibility of forming longer chains of phytochelatins (PC_4 and PC_5) was stronger. In addition, there was a decreasing trend in the concentration of PC_4 from 24 h to 72 h. This can be due to a decrease of toxicity impact in algal cells from 24 h to 72 h, according to the level of the relative cellular division rate (showed previously). The cellular uptake of Zn^{2+} is known to induce phytochelatins in different species of algae such as *C. reinhardtii* and *stigeoclonium tenue* (Gekeler *et al.*, 1998; Skouronska, 2002a). In addition, the phytochelatins play an important regulatory role for the cellular tolerance of Zn^{2+} toxicity in algae (Skouronska, 2002b). According to (Suarez *et al.*, 2010d), two parameters play important role in the amount of PC_n: 1) The amount of GSH; 2) the activity of PC_n synthase. In my study, obtained results showed that algal

cells of *C. reinhardtii* produced small and long chains of phytochelatins, when *C. reinhardtii* was exposed to Zn^{2+} at different times of exposure.

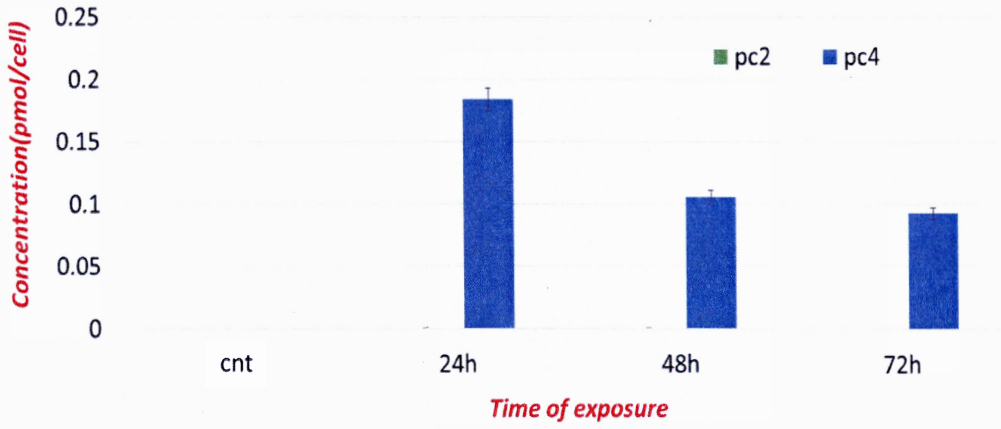


Figure 5.6 Effect of 60 µM of Zn^{2+} on the induction of thiol compounds at different time of exposure (24 h, 48 h, and 72 h). Each data indicated the average of 6 replicates, n=6.

5.3.2. The effect of Zn-NP on the induction of thiol compounds

The Figures 5.7 and 5.8 show the amount of thiol compounds in algal cells, when *C. reinhardtii* was exposed to the concentrations of 30 µM and 60 µM of Zn-NP at different time of exposure from 24 h to 72 h. Among the analyzed thiol compounds (GSH, PC_2, PC_3, PC_4, and PC_5), we could detect only the PC_2, PC_4, and GSH by using UHPLC. These thiol compounds were not detected in the control culture for the same procedures.

5.3.2.1. The effect of 30 µM of Zn-NP on the induction of thiol compounds

The main inducer under the concentration of 30 μM of Zn-NP was GSH, with the highest induction at 24 h. The PC_4 was also induced significantly, but at a lower level. However, the maximum induction of PC_4 was at 72 h. These results demonstrated that the maximum induction of GSH at 24 h was related to the highest toxicity effect of Zn-NP on the relative cellular division rate. From 24 h to 48 h, there was a decrease in the induction amount of GSH, due to the toxicity impact in algal cells from 24 h to 48 h. There were no significant differences in the amount of PC_2, but there was an increasing trend in the amount of PC_4 from 24 h to 72 h. In according to (Cristina Suarez et al., 2010e), *C. reinhardtii* was exposed to Pb^{2+} (10^{-11}-10^{-7} M) under short-term (6h) and long-term (45h) of exposure, small phytochelatins (short chain) are a result of low intracellular concentration of metals and long phytochelatins are a result of a high intracellular concentration of metals. Recently, studies reported that phytochelatins are an indicator for the toxic effect of Zn^{2+} (Le Faucheur *et al.*, 2006h), since they represent a defensive mechanism in response to metals. For example, in a previous study (Skowronska, 2002c), two types of algae were exposed to Zn^{2+}, and for short term of exposure (17 h), there were no changes in the level of GSH and phytochelatins. Nevertheless, in the long term of exposure (48 h), there were some changes in the level of GSH and phytochelatins.

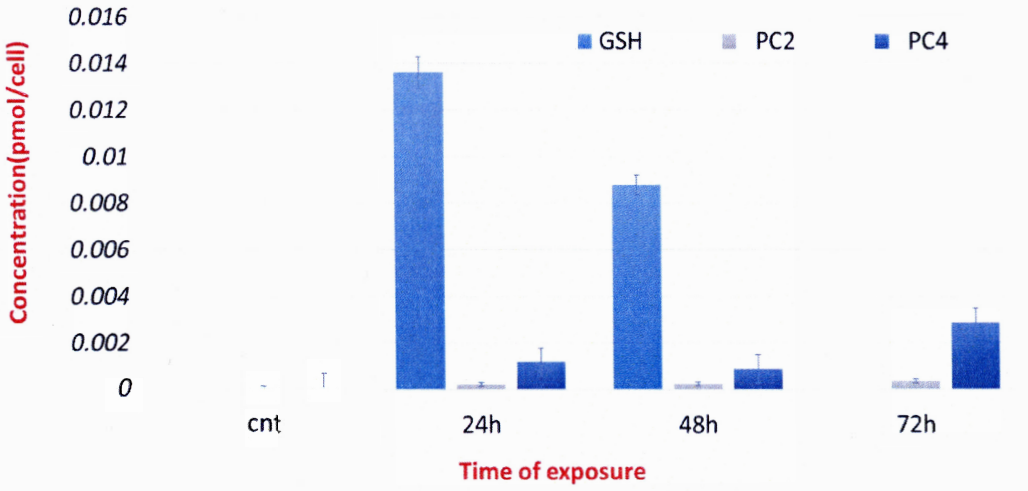


Figure 5.7 Effect of 30 µM of Zn-NP on the induction of thiol compounds at different time of exposure (24 h, 48 h, and 72 h). Each data indicated the average of 6 replicates, n=6.

5.3.2.2. The effect of 60 µM of Zn-NP on the induction of thiol compounds

As shown by our results, the main inducer for the concentration of 60 µM of Zn-NP was GSH, and there was high induction of GSH at 24 h. The maximum induction of PC_4 was reached at 72 h, but the maximum induction of PC_2 was at 24 h. There were no significant differences in the concentration of PC_4, but there was a decreasing trend in the concentration of PC_2 from 24 h to 72 h. In fact, the most important difference between the effect of Zn^{2+} and Zn-NP on the induction of thiol compounds was the detection of GSH when we tested the effect of Zn-NP. In accordance to (Hirata *et al.*, 2001b), GSH plays a fundamental role in PC_n synthesis for algae in response to metal and others environmental stresses.

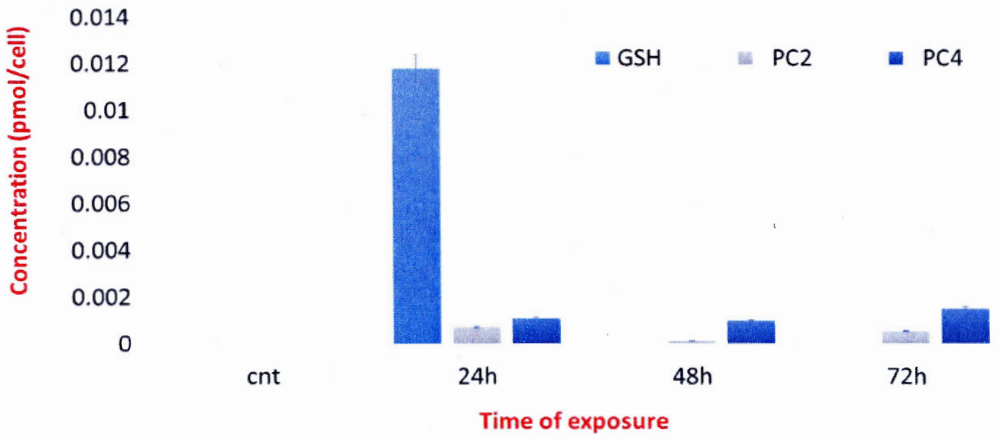


Figure 5.8 Effect of 60 µM of Zn-NP on the induction of thiol compounds at different time of exposure (24 h, 48 h, and 72 h). Each data indicated the average of 6 replicates, n=6.

Based on our results, three parameters can affect the induction of thiol compounds such as: The chemical nature of the metallic contaminant, the concentration, and the time of exposure. Our results showed the bioaccumulation of both Zn^{2+} and Zn-NP in algal cells of *C. reinhardtii* under tested conditions. Depending on the chemical nature of Zn^{2+} and Zn-NP, peaks of PC_2, PC_4, and GSH were detected in algal culture of *C. reinhardtii* during 24 h to 72 h. Our chromatogram results showed clearly well-separated peaks for GSH, PC_2, and PC_4 with a retention time of 1 min, 2 min, and 4 min, respectively. Chromatograms of GSH and PCs were obtained by HPLC with *Chlorella kesslerii* after being exposed to Zn^{2+} (Hassler *et al.*, 2005b), and similar pics were reported for thiol compounds by means of HPLC. In our study, chromatograms were obtained by means of UHPLC with fluorescence detection using the molecular probe monobromobimane (mBrB). This fluorescent probe can detect efficiently GSH and PCs since it selectively reacts with thiol group. An important difference between chromatograms of HPLC and UHPLC is the retention time, which is faster by using our UHPLC for testing the same condition. In another study (Hunaiti *et al.*, 2016) for determination of thiol compounds in plant tissue extracts. In fact, our obtained results showed that the detected thiol compounds and their quantity were correlated with the chemical nature of the metallic contaminants, Zn^{2+} or Zn-NP. Therefore, the induction of thiol compounds can be used as a physiological stress indicator of different metallic contaminants using the green alga *C. reinhardtii.*

5.4 General discussion

The main objective of this work was to 1) investigate the Investigate the toxicity of Zn^{2+} and Zn-NP on the growth rate of *C. reinhardtii.* 2) Determine the accumulation of Zn^{2+} and Zn-NP in algal cells of *C. reinhardtii.*3) Determine the induction of GSH and PCn in algal cell exposed to Zn^{2+} and Zn-NP. 4) Evaluate the correlation between of GSH and PCn, the accumulation of total Zn^{2+} in algal cells, and the toxicity effect

on growth rate. Our obtained results concerning the change of these cellular parameters are summarized in the tables 5.1 to 5. 5. The variation of these parameters authorized to explain the highest efficiency of Zn^{2+} and Zn-NP bioaccumulation and to compare treatment conditions at 24 h, 48 h, and 72h. According to our obtained results, we found in this study that Both Zn^{2+} and Zn-NP induced an inhibition effect on the cellular division of algal cells. The cell division for Zn^{2+} decreased only by 30 % compared control at the concentration of 30 µM in 24h. Also at the concentration of 60 µM in 24h, the cell division for Zn^{2+} decreased only by 60 % compared control. The cell division for Zn-NP decreased only 32 % compared control at the concentration of 30 µM in 24h. In addition, at the concentration of 60 µMin 24h, the cell division for Zn-NP decreased only 58 % compared control (Table 5.1).

The highest efficiency of Zn^{2+} bioaccumulation by algal cells of *C. reinhardtii* was under the treatment condition of 60 µM Zn^{2+} in 24h (table 5.2) and for Zinc-NP the highest efficiency of Zn^{2+} bioaccumulation was at the concentration of 60 µM in 24h (table 5.2). Under this experimental condition, at the concentration of 60 µM of Zn^{2+} in 48h, the bioaccumulation of Zn^{2+} decreased only 38% compared to 24h. In addition, at the concentration of 60 µM of Zn^{2+} in 72h, the bioaccumulation of Zn^{2+} decreased only 50% compared to 24h. As well as, at the concentration of 60 µM of Zinc-NP in 48h, the bioaccumulation of Zn^{2+} decreased only 63 % compared to 24h (table 5.2). In addition, at the concentration of 60 µM of Zinc-NP in 72h, the bioaccumulation of Zn^{2+} decreased only 68 % compared to 24h, (table 5.2).

The synthesis of thiol compounds was determined, when alga cells exposed to Zn^{2+} and Zn-NP at the concentration of 30 µM and 60 µM in different time of exposure from 24h to 72h, according my results Zn-NP and Zn^{2+} affected differently the induction of glutathione and phytochelatins. At the concentration of 30 µM for Zn^{2+}, we had maximum induction of PC_2 and PC_4 in 24h. Also at the tested concentration, the main inducer was PC_2, because at low concentration, the possibility of forming small phytochelatins (PC_2 and PC_3) is more than long phytochelatins (PC_4 and PC_5), (table

5.3). As well as at the concentration of 60 µM for Zn^{2+}, we had maximum induction of PC_4 in 24h, (table 5.4). Also at the tested concentration, the main inducer was PC_4, because at high concentration, the possibility of forming long phytochelatins (PC_4 and PC_5) is more than small phytochelatins (PC_2 and PC_3). We did not detect any PC_3 and PC_5. This may indicate that the protective molecular mechanisms involving GSH and PCs were not induced against Zn^{2+} cellular toxicities. Glutathione was only induced by the effect of Zn-NP (table 5.5), the main inducer was GSH and the maximum GSH level was found at 24h under 30 µM of Zn-NP. Nevertheless, for PC_4, the maximum induction was at the concentration of 30 µM in 72h. For the tested concentrations, Zn-NP was more toxic than Zn^{2+} on algal cells of *C. reinhardtii*. This study demonstrated a different physiological effect of Zn-NP compared to Zn^{2+}.

Table 5.1 Effect of Zn^{2+} and Zn-NP on cellular division in 24h compared to control.

Parameter	Zn^{2+} 30 µM	Zn^{2+} 60 µM	Zn-NP 30 µM	Zn-NP 60 µM
Cellular division	30% ↓	60% ↓	32% ↓	58% ↓

Table 5.2 Effect of Zn^{2+} and Zn-NP on cellular division in 48h compared to control.

Parameter	Zn^{2+} 30 µM	Zn^{2+} 60 µM	Zn-NP 30 µM	Zn-NP 60 µM
Cellular division	----------	----------	23% ↓	33.3% ↓

Table 5.3 Effect of Zn^{2+} and Zn-NP on cellular division in 72h compared to control.

parameter	24h	48h	72h
Main induction	PC_2	---	---

Table 5.4 Effect of 30 µM Zn^{2+} on thiol compounds in different time of exposure.

parameter	24h	48h	72h
Main induction	PC_4	PC_4	PC_4

Table 5.5 Effect of 60 µM Zn^{2+} on thiol compounds in different time of exposure

Parameter	Zn^{2+} 30 µM	Zn^{2+} 60 µM	Zn-NP 30 µM	Zn-NP 60 µM
Cellular division	----------	----------	25%	37.5%

Table 5.6 Effect of 30 µM Zn-NP on thiol compounds in different time of exposure.

parameter	24h	48h	72h
Main induction	GSH	GSH	---

Table 5.7 Effect of 60 µM Zn-NP on thiol compounds in different time of exposure.

parameter	24h	48h	72h
Main induction	GSH	---	---

.5.5 Conclusion

For determining the maximum capacity of algal cells of *C. reinhardtii* to accumulate Zn^{2+} the bioaccumulation efficiency was investigated by estimating the content of Zn^{2+} in algal biomass when algal cells of *C. reinhardtii* was exposed to the concentration of 30 µM and 60 µM of Zn^{2+} and Zn-NP from 24h to 72h. The cellular toxicity induced by the bioaccumulation effect of Zn^{2+} was determined by monitoring several cell physiological parameters that we mentioned. Based on our results the highest efficiency of Zn^{2+} bioaccumulation by algal cells of *C. reinhardtii* was under the treatment condition of 60 µM of Zn^{2+} in 24h, and for Zinc-NP the highest efficiency of Zn^{2+} bioaccumulation was at the concentration of 60 µM in 24h. These parameters are indicators of Zn^{2+} toxicity inside the cell. In addition, our results specified for us the induction of thiols group (glutathione and Phytochelatins) was significant for algal cells of *C. reinhardtii* to avoid the inhibitory effects of Zn^{2+}. Glutathione is only induced by the effect of Zn-NP, and the maximum GSH level was found at 24 under 30 µM of Zn-NP. In conclusion, this study demonstrated a different physiological effect of Zn-NP compared to Zn^{2+} on algal cells of *Chlamydomonas reinhardtii.*

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