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BIOACUMULATION AND TOXIC EFFECT OF ZINC ON THE GREEN ALGA *CHLORELLA VULGARIS*

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LIST OF ABREVIATIONS

AAS	Atomic Adsorption Spectrometry
ABS	Absorption by chlorophyll
ACN	Acetonitrile
ATP	Adenosine tri-phorphate
BLM	Biotic ligand model
Chl a	Chlorophylle *a*
Chl b	Chlorophylle *b*
Chl	Chlorophylle
Cys	Cystein
EDTA	Ethylene diamine tetra acetic acid
ET	Electron transport
F_M	Maximum fluorescence (at approx. T = 1 sec)
F_o	Initial fluorescence
F_S	Satationnaire Chl fluorescence
F_V	Variable Chl fluorescence
Glu	Glutamic acid
Gly	Glycine
GSH	Glutathion
HEPES	4 - (2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HNO_3	Nitric acid
HPLC-FL	High performance liquid chromatography coupled fluorescence detector
ICP-MS	Mass Spectrometry inductively coupled plasma source
LED	Light Emitting Diode
mBrB	Monobromobimane
Me-PC	Metal complexes phytochelatine
MeOH	Methanol

MSA	Metasulfonic acid
NADPH	Nicotinamide adenosine diphosphate (reduced)
PC	Phytochelatine
PCn	Phytochelatine with n = 2, 3, 4...
PCs	Phytochelaines
PEA	"Plant Efficiency Analyser" = fluorescence measuring apparatus
PI	Photosynthetic index
PQ	Plastoquinone
PSI	Photosysteme I
PSII	Photosysteme II
Q_A	First quinone electron acceptor of PSII
Q_B	Second quinone electron acceptor of PSII
R_2	Correlation coefficient
RC	Reaction center
SH	Thiol functional group
TCEP	Tris (2-carboxyethyl) phosphine
Zn	Zinc

RÉSUMÉ

La pollution des milieux aquatiques par les métaux traces représente un problème majeur qui nécessite l'évaluation du risque de toxicité pour plusieurs espèces aquatiques. En particulier, le zinc est un oligo-élément qui peut être toxique à des concentrations élevées pour les organismes vivants, et la libération de Zn *via* les eaux usées représente un important problème de qualité d'eau douce au Canada. Dans le développement d'une nouvelle technologie de bioremédiation, les microalgues peuvent être utilisées pour le traitement des eaux usées, car elles ont la capacité de bioaccumuler les métaux. Le problème principal au niveau cellulaire est l'implication des mécanismes moléculaires au cours du processus de bioaccumulation des métaux dans les cellules d'algues. Afin de déterminer la capacité maximale des cellules d'algues de *Chlorella vulgaris* à accumuler le Zn, l'efficacité de la bioaccumulation a été étudiée par l'estimation de la teneur en zinc dans la biomasse algale quand l'algue *C. vulgaris* a été exposée pendant 72 h aux différentes concentrations de $ZnCl_2$. La capacité de la biomasse algale de bioaccumuler le Zn a été déterminée par spectrométrie d'absorption atomique et l'effet toxique du Zn bioaccumulé a été évalué sur l'activité photosynthétique en utilisant l'émission de fluorescence de la chlorophylle. L'induction des phytochélatine a été analysée qualitativement et quantitativement par chromatographie liquide à haute performance. Selon nos résultats obtenus, le rendement de bioaccumulation le plus élevé a été atteint pour la plus forte concentration de $ZnCl_2$ (100 mM) à 24 h de temps d'exposition. Dans ces conditions expérimentales, la bioaccumulation de Zn était cinq fois plus par rapport au témoin. La division cellulaire a diminué seulement de 25 % par rapport au témoin. L'inhibition de la photosynthèse, liée à l'activité de la photochimie et du transport des électrons du PSII, a seulement diminué de 60 % par rapport au témoin. L'induction de la teneur en glutathion dans les cellules d'algues a été 6 fois plus par rapport au témoin. La synthèse de phytochélatines a été fortement induite où le PC_4 a dublé par rapport aux PC_2. En outre, les résultats ont démontré que le Zn bioaccumulé induit un effet de toxicité beaucoup plus fort dans les cellules d'algues prés 48 h par rapport à 24 h d'exposition, comme en témoigne le nombre croissant de cellules mortes, une plus forte action inhibitrice sur la photosynthèse, une plus faible absorption et capacité de séquestration en Zn. Enfin, nos résultats ont montré que, pour maintenir l'efficacité optimale de bioaccumulation, l'induction des groupes thiols était importante afin d'éviter les effets inhibiteurs du Zn. En conclusion, cette étude a permis de déterminer les limites d'utilisation de *C. vulgaris* pour la bioaccumulation en Zn dans le développement d'une technologie de phycoremédiation.

ABSTRACT

The pollution of aquatic environments by trace metals represents a major problem which requires the toxicity risk assessment for several aquatic species. Particularly, zinc is a micronutrient that can be toxic at high concentrations for living organisms, and the release of Zn through wastewater represents an important water quality problem in Canada. In the development of a new bioremediation technology, microalgae can be used for wastewater treatment since they have the ability to bioaccumulate metals with high efficiency. Therefore, the main problem at cellular level is the involvement of the molecular mechanisms during the bioaccumulation process of metals in algal cells. In order to determine the maximum capacity of algal cells of *C. vulgaris* to accumulate Zn, the bioaccumulation efficiency was investigated by estimating the content of Zn in algal biomass when *C. vulgaris* was exposed during 72 h to different concentrations of $ZnCl_2$. The capacity of algal biomass to bioaccumulate Zn^{2+} was determined by atomic absorption spectrometry, and the toxic effect of bioaccumulated Zn was evaluated on the photosynthetic activity by using chlorophyll fluorescence emission. The induction of phytochelatins was analyzed qualitatively and quantitatively in relation to the bioaccumulated Zn by high performance liquid chromatography. According to our results, the highest efficiency of bioaccumulation was reached for the highest concentration of $ZnCl_2$ (100 mM) at 24 h of exposure time. Under this experimental condition, the bioaccumulation of Zn was five times more compared to the control. The cell division decreased only by 25 % compared to control. The inhibition of photosynthesis, related to PSII primary photochemistry and electron transport activity, decreased only by 60 % compared to control. The induction of glutathione content in algal cells was 6 times more compared to control. The synthesis of phytochelatins was highly induced where the PC_4 increased by 2 times in comparison to PC_2. Moreover, these results demonstrated that the bioaccumulated Zn induced a much stronger toxicity impact in algal cells at 48 h in comparison to 24 h of exposure, as indicated by the increasing number of dead cells, a stronger inhibitory effect on photosynthesis, a lower uptake and sequestration capacity of Zn. Finally, our results showed that, to maintain optimal bioaccumulation efficiency, the induction of thiols group (glutathione and phytochelatins) was important for algal cells in order to avoid the inhibitory effects of Zn. In conclusion, this study permitted to determine the capacity and limitations of using *C. vulgaris* for the bioaccumulation of Zn in the development of phycoremediation technology.

GENERAL INTRODUCTION

Metals are found naturally in the earth and can be distributed into the environment through several sources such as erosion of the crust land, precipitation and volcanic emissions. However, anthropogenic activities can increase the release of metals in the environment through metallurgy and industrial wastes, and consequently changing metal concentration in different ecosystems. Indeed, metals are closely linked to human civilization since the revolution of iron. The growth of the world population and the importance of metals in economies augment the needs for the production of metals in developing countries. The increasing production for metals will induce their dispersal and contact with the environment. In particular, industrial effluents are responsible to the pollution of aquatic ecosystems due to their high level in metals. Therefore, the quality of the environment and human health are at risk of harmful effects caused by the exposure to metals, considering both their speciation and bioavailability. Moreover, the remediation of wastewater represents one of the most important environmental problems for the preservation of water quality as a natural ressource.

In particular, zinc is a micronutrient which can be toxic at high concentrations in water due to its solubility (Roberts *et al.*, 1997; Coleman, 1998). Although zinc is an essential requirement for health of organisms, excess zinc can be harmful (Roberts *et al.*,1997). The free zinc ion in solution can be highly toxic to plants and animals (Coleman, 1998). In human health, the ingestion of zinc salts can provoke the release of phosphine gas which can enter the blood stream and affect the lungs, liver, kidney, heart and central nervous system (Eliott JL, 2006). Furthermore, the strong affinity of zinc for aquatic particles and organic matters may result in its deposition into sediments. Indeed, the release of large quantity of Zn through wastewater represents an important water quality problem in Canada. There are several methods available for the removal of metals, with advantages and disadvantages, which are defined according to their effectiveness, necessity and quantity of chemical reagents as well as energy. In the development of a new bioremediation technology, microalgae can be used for wastewater treatment since they have the ability to bioaccumulate metals (Levy *et al.*, 2007). Therefore, the main problem to study at cellular level is the molecular mechanisms of

detoxification involved during the bioaccumulation process of metals in algal cells. Indeed, it is important to determine the efficiency of algal cells to bioaccumulate metals in relation to specific environmental conditions (pH, light irradiance and temperature) and the algal species.

The main objective of this Master study is to determine the bioaccumulation efficiency and the cellular effects induced by metal ions of zinc in green algal cells of *Chlorella vulgaris*. Our main hypothesis is that the efficiency of Zn bioaccumulation depends on the metal concentration and the exposure time, and also on the induction of molecular mechanisms of metal sequestration. Our specific objectives are: 1- The determination of the bioaccumulation capacity and efficiency when algal cells of *C. vulgaris* are exposed to different concentrations of $ZnCl_2$ during 72 h; 2- The determination of the bioaccumulation toxic effects of Zn on cellular division and photosynthetic processes (Photosystem II photochemistry activity); 3- The identification and quantification of synthesized thiolated peptides related to the induction of molecular detoxification mechanisms, especially the synthesis of glutathione and phytochelatins.

This Master thesis is organized into four main chapters. In the first chapter, the environmental context is defined, such as the problem of metal pollution, the preservation of the quality of freshwater, the case of zinc, used wastewater treatment technologies and the development of the phycoremediation technology. In the second chapter, the biochemistry of green algae is presented, the criteria for the selection of algal species *Chlorella vulgaris*, the mechanisms of photosynthesis, the speciation and interaction of metals at cellular level, the induction of cellular and biochemical indicators of toxicity and mechanisms of cellular detoxification of metals. In the third chapter, methodological approaches are presented such as cell count, chlorophyll fluorescence emission and liquid chromatography. Finally, the results obtained in this study are discussed in the fourth chapter.

CHAPTER I

1. Introduction to water quality

Water is a basic source of life representing an essential substrate to support all life forms such as plants, animals and humans (Vanloon and Duffy, 2005). There are two main natural sources: surface water such as fresh water lakes, rivers, streams, and ground water such as springhead water and well water (McMurry and Fay, 2004; Mendie, 2005). One of the most important environmental threats today is ground water contamination (Vodela *et al.*, 1997) and between the large varieties of contaminated water sources, some metals such as mercury, cadmium play a keyrole with their strong toxicity even at low concentrations (Marcovecchio *et al.*, 2007). Groundwater becames polluted when anthropogenic various sources release substances which dissolved or mixed in waters canals (Environment of Canada, 2010-08-05). Therefore, aquatic pollution has become one of the most important environmental problems. Each year, the industries produce for $ 1.5 trillion in global chemicals, and tens of thousands of these substances are discharged into the environment. The sources of water pollution in Canada are acid rain, soil drifting, sedimentation, ground water contamination and wastewater (Environment Canada). Wastewater is a complex mixture of organic and inorganic materials with also man-made compounds. Huge amounts of organic carbon that is present in the sewage are carbohydrates, fats, proteins, amino and volatile acids. The major its of inorganic matter consists of large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulfur, phosphate, bicarbonate, ammonium salts and heavy metals (Tebbutt, 1983; Horan, 1990; Lim and Phang, 2010). Every day, people are exposed to a large amount of chemicals, from industrial pollutants in the air, to pesticide residues in foods, to heavy metals in drinking water.

Heavy metals such as Cd, Hg, Cu and Zn in wastewaters are dangerous for the environment. These ions may cause toxic and harmful effects to aquatic organisms (Aksu and Kutsal, 1990). Indeed, when these metals are released in the aquatic environment, they can be bioaccumulated in the food chain from various ways such as phytoplanktons and fish (Figure 1.4). Then birds nourish from fish and up to human as last consumers. Therefore, the concentration of pollutants will be increased from one link to another link in the food chain, a

phenomena named biomagnification. For these reasons, the development of technology for the removal of metals from polluted zone is necessary (Torres *et al.*, 1998).

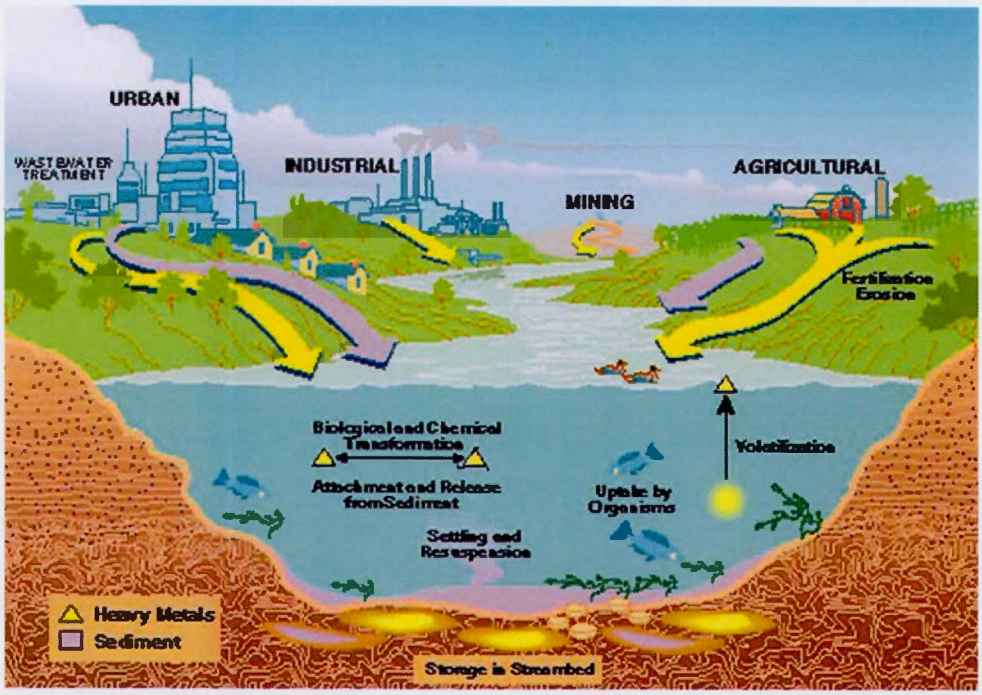


Figure 1.1 Heavy metals are released into the marine system from various sources. Typical sources are municipal wastewater-treatment plants, manufacturing industries, mining and agricultural cultivation and fertilization. Heavy metals may be volatilized to the atmosphere or stored in riverbed sediments. Toxic heavy metals are taken up by organisms; the metals dissolved in water have the greatest potential of causing the most injurious effects (Mead and Leenher,1995).

Today the effect of the release of wastewater pollutants on aquatic ecosystems and human health increased considerably. These impacts will have negative effects on fish and wildlife populations, oxygen discharge, limitation on fish and shellfish harvesting, consumption and drinking water consumption (Environment Canada). The pollution of the aquatic environment by inorganic and organic chemicals has been identified as a serious threat to the quality of aquatic ecosystems (Samir, 2008).

1.1 Heavy metals

Heavy metals are metallic chemical element with high range of atomic weights. They have a density higher than 5 g ml^{-1}. Some of them such as mercury, lead and cadmium are toxic, dangerous to health and to the environment, even at low concentrations, but some bio-essential elements such as zinc, copper and iron become toxic only at high concentrations (Hogan, 2010). Approximately fifty-three of the ninety naturally elements are called heavy metals. Since the middle of the 19^{th} century, the production of heavy metals increased severely for more than 100 years, with simultaneously emissions to the environment (Figure 1.1). At the end of the 20^{th} century, however, emissions of heavy metals tend to reduce in developed countries (Järup, 2003).

Heavy metals are toxic when they are not metabolized and accumulate in the body. They can enter in the human body via food, water, air or absorption through the skin. Industrial exposure is common in adults and consumption is more incident in children (Roberts, 1999). Iron, for example, prevents anemia and zinc is a cofactor in over 100 enzymatic reactions. However, some heavy metals such as Cd, Cr, and Pb have no special activity, and are harmful. Long time exposure to heavy metals can damage the nervous system and also may cause physical, muscular and neurological degenerative processes like Alzheimer's disease, Parkinson's disease, muscular dystrophy, cancer and multiple sclerosis (Environment Healh center, 1999).

1.1.1 Sources of heavy metals

Heavy metals are from natural geological processes and anthropogenic activities. The most important natural geological sources of heavy metals are soils and rocks while anthropogenic sources include industrial, agricultural, municipal and residential activities (Audry *et al.*, 2004; Singh *et al.*, 2005; Hang *et al.*, 2009). Industrial processes use metals as raw materials (e.g. smelting, mining, electroplating) and they release from oil/coal gasification and other oil production, which are the major source of heavy metals contamination (Singh et al., 2005; Hang et al., 2009).

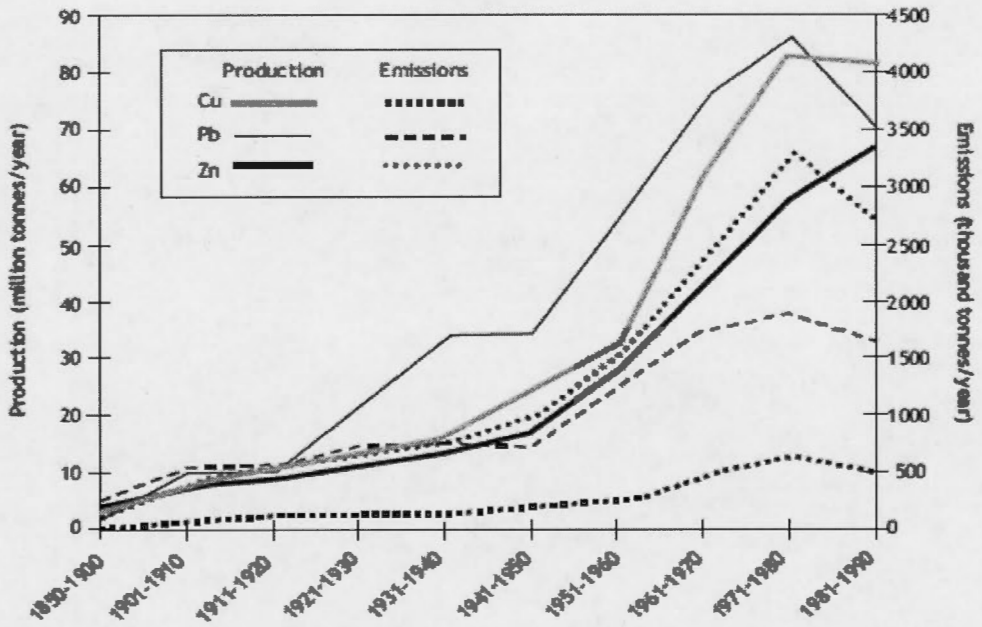


Figure 1.2 Global production and consumption of certain toxic metals, 1850–1990 (Nriagu Jerom, 1996) reproduced with permission.

1.1.2 Heavy metals in the environment

Metals can enter easily into the environment through human activities, such as agricultural processes, industrial activities and waste sewage (Hani and Anal, 1990). Trace metals are common in water without representing a danger at low dose. Indeed, some metals

are essential for life. Calcium, magnesium, potassium and sodium are fundamental for normal body functions. Zinc, copper, iron, manganese, molybdenum, selenium and cobalt are necessary at low amount as catalysts for enzyme activities. Drinking water containing high concentrations of these metals, or toxic metals such as aluminum, arsenic, barium, cadmium, chromium, lead, mercury, selenium, and silver, may be harmful to our health (Gadd, 2009). Metals may be as free metal ions or as complex ions, chelated with inorganic ligands such as Cl^-, OH^-, CO_3^- or NO_3^-, and can also create complexes with organic ligands such as amines, humic acids and proteins. The chemical forms of metals in the aquatic environment is depend on environmental variables such as salinity, temperature, pH, organic and particulate matter and biological activities (Lobban and Harrison, 1994).

The speed of industrialization and urbanization increases the chance for those heavy metals of being released into the natural environment. There are some major heavy metals with high toxicity risk (e.g. arsenic, cadmium, chromium, lead, mercury) which have been identified in various ecosystem compartments such as water, soil and sediments (Audry *et al.*, 2004; Singh *et al.*, 2005; Zhang *et al.*, 2009). Due to the wide distribution and critical health impacts, those heavy metals are in the list of pollutants to be monitored by the (WHO, USEPA), European Union and Health Canada.

Heavy metals in the aquatic environment can exist in many forms, dissolved, colloidal and particulate (Audry *et al.*, 2004). They can be link to sediments in different ways, including physio-chemical adsorption on sediments, clogging in amorphous materials and bioaccumulation in aquatic organisms (Tessler *et al.*, 1979; Singh *et al.*, 2005). Major of heavy metals are cations. For example zinc and copper, both carry [e^{+2}] charge. This soluble form of metals is more dangerous because it is quickly transported and more easily available to plants and animals. Metal behavior in the marine soft and salty (streams, lakes and rivers) environment is similar to that outside an aquatic system. Actually, many heavy metals tend to be collected at the bottom of water bodies and some of these metals will dissolve. The aquatic environment is more sensitive to the damaging effects of heavy metal pollution because aquatic organisms have more contact with the soluble metals (Nriagu, 1996).

1.2. Zinc

1.2.1 Physical and chemical properties

Zinc is the first element of group 12 in the periodic table and also is the 24th most abundant element in the Earth's crust. It is found in nature and cover up 0.02 % by weight of the earth's cortex. Normally zinc is grey due to coating with an oxide or basic carbonate. It is very rare to find free zinc metal in nature. Pure zinc is bluish-white and shiny when polished. It has the atomic number 30 and the standard atomic weight of 65.38 amu. The metal present hard and fragile at most temperatures but becomes soft and melting between 100 and 150 °C. There is a variety of zinc forms, such as zinc carbonate, zinc chloride and zinc acetate or zinc sulfide (Environment Health Criteria, 2001). Chemical and physical properties of zinc and selected compounds are presented in Table 1.1 and Table 1.2 (O'Neil *et al.*, 2010).

Table 1.1 Some chemical forms of zinc (O'Neil *et al.*, 2001).

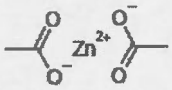
Characteristic	Zinc	Zinc acetate	Zinc chloride
Synonyms	Zinc dust; zinc powder	Acetic acid, zinc salt; acetic acid, zinc(II) salt; dicarbomethoxyzinc; octan zinecnaty [Czech]; zinc diacetate; zinc(II) acetate	Butter of zinc; chlorure de zinc (French); zinc (Chlorure de) (French); zinc butter; zinc chloride ($ZnCl_2$); zinc dichloride; zinco (cloruro di) (Italian); zinkchlorid (German); zinkchloride (Dutch)
Registered trade name(s)	Asarco; L 15; Blue powder; CI 77945; CI pigment Metal 6; Emanay zinc dust; Granular zinc; JASAD; Merrillite; PASCO	No data	Tinning flux (DOT)[b]; AI3-0440; Zintrace
Chemical formula	Zn	$Zn(C_2H_3O_2)_2$	$ZnCl_2$
Chemical structure	Zn		Cl-Zn-Cl
Identification numbers:			
CAS registry	7440-66-6	557-34-6 (anhydrous) 5970-45-6 (dihydrate)	7646-85-7
NIOSH RTECS	ZG8600000	AK1500000 (anhydrous) ZG8750000 (dihydrate)	ZH1400000
EPA hazardous waste	No data	No data	No data
OHM/TADS	7216955	No data	7216957
DOT/UN/NA/ IMCO shipping	Zinc, powder or dust, UN 1436; zinc, powder or dust, zinc ashes, IMO4.3; zinc ashes, UN 1435	Zinc acetate, environmental hazardous substance, solid, NOS, UN 3077	Zinc chloride, anhydrous, UN 2331; zinc chloride, solution, UN 1840; zinc chloride, anhydrous, solution, IMO 8.3
HSDB	1344	1043	1050
NCI	No data	No data	No data

Table 1.2 Physical and chemical properties of zinc and selected compounds (O'Neil *et al.*, 2001).

Property	Zinc	Zinc acetate	Zinc chloride
Molecular weight	65.38	183.48	136.29
Color	Bluish-white, lustrous	White granules	White granules[b]
Physical state	Solid metal	Solid	Solid
Melting point	419.5 °C	237 °C (decomposes)	290 °C
Boiling point	908 °C	No applicable	732 °C
Density (g/cm^3)	7.14 at 25 °C	1.735	2.907 at 25 °C
Odor	No data	Faint acetous odor[c]	Odorless; fume has acrid odor[c]
Odor threshold:			
Water	No data	No data	No data
Air	No data	No data	No data
Solubility:			
Water	Insoluble[d]	4.0×10^4 mg/L at 25 °C; 6.7×10^4 mg/L at 100 °C[c]	4.32×10^6 mg/L at 25 °C; 6.14×10^6 mg/L at 100 °C
Other solvent(s)	Soluble in acetic acid and alkali	33 mg/L in alcohol	1 g/1.3 mL alcohol; 1 g/2 mL glyderol; 1 g/0.25 mL 2% hydrochloroacetic acid
Partition coefficients:			
K_d (mL/g)	0.1–6,000[e]; 40 (average)[f]; 39 in sandy loam soil; 12.2 in sandy soil[g]	No data	No data

1.2.2 Zinc in the environment

Zinc is part of nature and exists in many rocks and minerals. The natural level of zinc in the earth's cortex is nearly 70 mg/kg (dry weight) and ranging between 10 and 300 mg/kg (Malle, 1992). Canada produced 693000 tones of zinc per year, and about 19000 kg of zinc is released into freshwaters yearly (Environment Canada, 1998; Gouverment du Canada, 2011). More than half of this amount (58 %) is produced for galvanizing steel to protect it from corrosion (Figure 1.2). About 14 % of zinc is used as manufacture of dry cell batteries and die casting and 10 % goes to the production of alloys such as brass or bronze, and significant amount of zinc is used in many industrial applications. In natural surface waters, the concentration of zinc is usually under 10 μg/L, and in ground waters, 10–40 μg/L. The main sources of zinc pollution in the environment are fertilizers, sewage sludges and mining (Bradi, 2005).

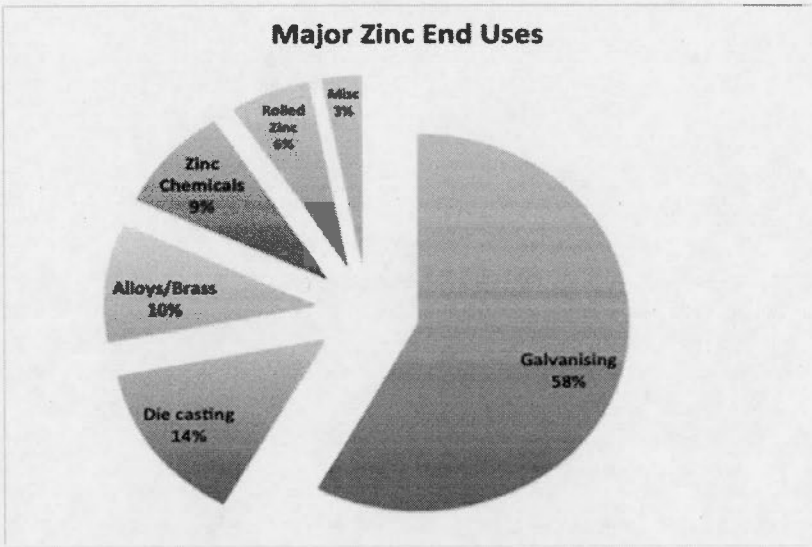


Figure 1.3 Major zinc end uses in Canada (Bradi, 2005) reproduced with permission.

Today's zinc concentrations are increasing unnaturally, due to addition of zinc through human activities. Most zinc is entered to environment during industrial activities, such as mining, coal and waste combustion and steel processing. Some soils are completely contaminated with zinc, and these are to be found as sewage sludge from industrial zone. they have been used as fertilizer or where zinc has to be mined or refined (Environment Health Criteria, 2001). The major zinc mining areas are Canada, Russia, Australia, USA and Peru'. The world production of Zinc is over than 7 million tons for year. At present more than 30% of the world's need (zinc) provide by zinc recycling. . The bioavailability of zinc in water depends on many factors such as temperature, water hardness, pH and dissolved organic carbon content.Zinc is a recyclable material, and 2 millions tones of zinc are recycled every year (European Zinc Institute 1990). Natural zinc level in environment is show in Table 1.3.

Table 1.3 Natural zinc level (total zinc) in the environment (Van Assche *et al.*, 1996).

		Range
Air (rural) (µg/m3)		0.01 - 0.2
Soil (general) (mg/kg dry weight)		10 - 300
Rocks (ppm)		
	- basaltic igneous	48 - 240
	- granitic igneous	5 - 140
	- shales and clays	18 - 180
	- sandstones	2 - 41
	- black shales	34 - 1500
Ore bodies (%)		5 - > 15
Surface waters (µg/l)		
Habitat-type:		
- Open ocean (surface)		0.001 - 0.06
- Coastal seas/inland seas		0.5 - 1
- Freshwater:		
	- Alluvial lowland rivers rich in nutrients and oligo-elements (e.g. European lowland)	5 - 40
	- Mountain rivers from old, strongly leached geological formations (e.g. Rocky Mountains)	< 10
	- Large lakes (e.g. Great Lakes)	0.09 - 0.3 (dissolved)
	- Zinc-enriched streams flowing through mineralization areas	> 200

1.2.3 Estimation of zinc in drinking water

It has been reported in studies that the daily intake of zinc isabout 5-22 mg in different areas of the world (Elinder, 1986). The zinc content of typical diets of North American adults is between 10 and 15 mg/day. The recommended dietary for adult men is about 15 mg/day, for adult women 12 mg/day, for formula-fed infants 5 mg/day, and for preadolescent children 10 mg/day (Cousins and Heempe, 1990). Drinking-water usually makes a little contribution to zinc intake unless high concentrations of zinc occur as a result of corrosion of pipings and fittings. Under certain condition, tapwater can provide up to 10 % of the daily intake (Gillies and Paulin, 1982).

1.2.4 Effects on human health

Critical toxicity happens from the consumption of excessive amounts of zinc salts, either accidentally or as a dietary supplement. Vomiting usually happens after the consumption of more than 500 mg of zinc sulfate. Most poisoning has been reported after the drinking of acidic liquor kept in galvanized containers: fever, nausea, vomiting and stomach cramps occurred 3–12 h after ingestion. Food poisoning happens due to the use of galvanized zinc containers in food preparation, and symptoms occur within 24 h including nausea, vomiting and diarrhea, sometimes with bleeding and abdominal cramps (Elinder, 1986; Kok, 1988). The high concentrations of zinc can cause adult respiratory distress syndrome, acute renal tubular necrosis, chemical pneumonitis, interstitial nephritis, irritation and corrosion of the gastrointestinal tract (Environment Health Criteria, 2001). Figure 1.3 shows effect of zinc in excess on body organs.

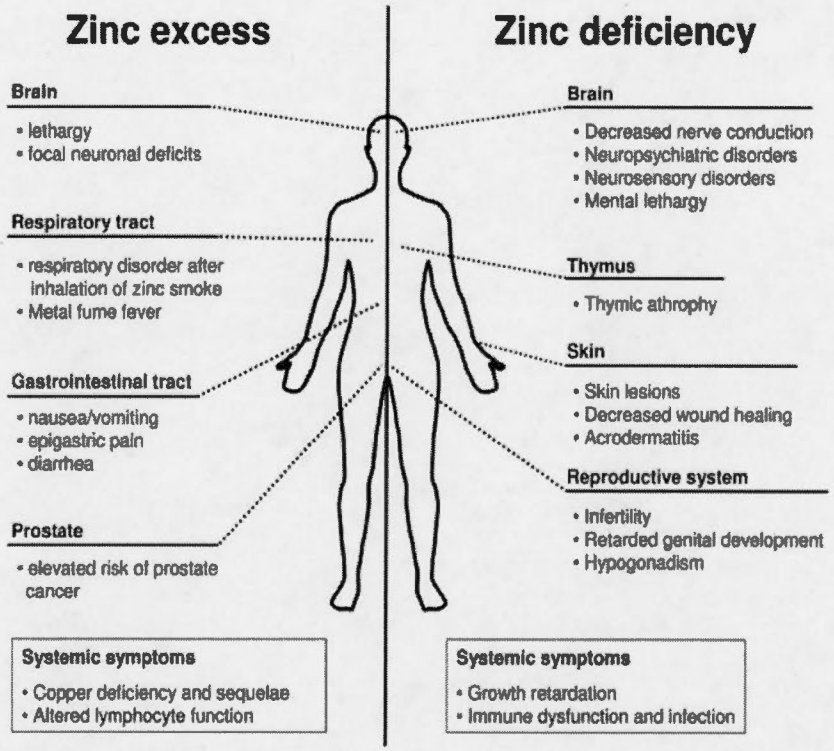


Figure 1.4 Comparison of zinc effects intoxication versus deficiency. Intoxication by excessive exposure to zinc (left side), and deprivation of zinc by malnutrition or medical conditions (right side), has detrimental effects on different organ systems. (Plum, Rink and Haase, 2010).

1.2.5 Environmental toxicity of zinc

Zinc is an essential ion playing a major role in over 300 enzymes (Vallee and Falchuck, 1993). However, Zinc is harmful to organisms at higher levels, by for example disrupting Ca homeostasis in freshwater fish through the induction of hypocalcaemia (Waurnau *et al.*, 1996). Fish can accumulate zinc gently over the time. Incomplete data are available to evaluate or predict the short term and the long-term effects of zinc and its compounds to plants, birds, or land animals (Gillies and Paulin, 1982). Soils with an excessive zinc problem can be toxic to plants. Soil pH is one the most factors that influences zinc availability to plants. The toxicity of zinc in water is influenced by water hardness and pH. In lower toxicity of zinc, waters face with higher water hardness and lower pH, and in

higher toxicity of zinc the water has lower hardness and higher pH. Generally, zinc and its salts have high acute and chronic toxicity to aquatic system in polluted waters.

1.3.1 Wastewater treatment technology

Today, wastewater treatment technology is composed on three different treatments for the remediation of water: Primary (physical) treatment is designed to remove big, suspended and floating solids from sewage. Primary treatment can reduce the BOD(biochemical oxygen demand) of the incoming wastewater by 20-30 % and the total suspended solids by some 50-60 % (Horan, 1990). Primary treatment (filtration and sedimentation) is usually the first level of wastewater treatment. The goal of this step is to remove debris that could damage the treatment facility's equipment. Depending on the size of the particle, different types of filtration membrane such as ultra-filtration, nano-filtration and reverse osmosis can be used for heavy metal removal (Juang, 2000).

Secondary (biological) treatment removes the dissolved and suspended organic matter that escapes primary treatment. This step aims to reduce the BOD exerted by reducing organic matter. This step used the microbes for consuming the organic matter as food, and they converting it into carbon dioxide, water and energy for their own reproduction. The biological process (aerobic and non-aerobic pond) used some additional settling tanks to remove more of the dissolved solids. Secondary treatment can remove about 85 % of the suspended solids and BOD (Horn, 1990). Secondary treatment technologies include the basic activated sludge process, the deferent of pond and wetland, dribbling filters and other forms of treatment, which use biological process to break down organic matter (Kott *et al.*, 1974).

Tertiary (chemical) treatment is kind of complementary stage for the secondary treatment. Chemical technologies are applied to decrease the mobility of metals by converting them into inactive states. Oxidation, reduction and neutralization reaction are normally used for remediation. The complete tertiary process can be applied to remove ammonium, nitrate and phosphate which is estimated to be about four times more expensive than the primary treatment (De la Noue *et al.*, 1992). Chemical treatment can remove more than 99 % of all the impurities such as nitrogen and phosphorus from sewage. There are some examples of chemical treatment such as coloration, ozonation, chemical or electrochemical

rain (Ozdemir *et al.*, 2005), and ion-exchange treatments, which are not economical (Pehlivan & Altun, 2006). Figure 1.5 is showing a wastewater treatment plant in Montreal.

Primary, secondary and even tertiary treatment cannot by expected to remove 100 % of the pollutant materials and as a result, many microorganisms still remain in the waste stream. To prevent the spread of waterborne diseases and also to minimize public health problems, disinfection treatment with chlorine is required for the destruction of pathogenic organisms in wastewaters. However, some environmental authorities believe that chlorine residuals in the effluent can cause a problem in their process. Initial cost of wastewater treatment plant including primary, secondary or advanced stages is expensive (Oswaled, 1998), Also, many heavy metals can pass from this process. Therefore, we need cost-effective alternative technologies for the treatment of wastewaters containing metals (Bailey *et al.*, 1998).

Figure 1.5 Montreal's wastewater treatment plant (Spacing Montreal, 2009).

1.3.2 Use of algae in wastewater treatment

Recently several biological methods have been investigated as alternative to physical and chemical methods. Algae have proved to be advantageous because they show several benefits, for example, economic instauration, metal recovery potentiality, lower volume of chemical and high efficiency to dilute effluents (Gupta and Rustogi, 2008). Microalgae are

suitable to maintain water quality because of their nutrient needs, rapid reproduction rate and very short life cycle. Trace metals (such as Co, Mo, Ca, Mg, Cu, Zn, Cr, Pb and Se) can be accumulated in algal cells by active biological transport (Yee *et al.*, 2004). Moreover, algal cells have the ability to detoxify excess metals by producing extracellular and intracellular binding compounds such as phytochelatins (Moffet and Brand, 1996). The history of the commercial use of algal cultures such as *Chlorella* and *Dunaliella* is about 75 years old with the application in wastewater treatment and mass production. Recently, significant countries interested in developing this technology are in industrialized world nations, i.e., Australia, USA, Thailand, Taiwan and Mexico (Borowitzka and Borowitzka, 1988,1989a,b; Moreno *et al.*, 1990; Wong and Chan, 1990).

Microalgal cultures are an elegant solution as a tertiary and biochemical treatment due to their ability to use inorganic nitrogen and phosphorus for growth (Oswald, 1988; Tam and Wong, 1995). Also, they are able to remove heavy metals (Rai *et al.*, 1981), as well as some toxic organic matter from wastewater. Microalgae offer a cost effective approach to removing nutrients.They have a high capacity for inorganic nutrient uptake (Blier *et al.*, 1995). In a previous study, Lau *et al.*, (1996) reported the ability of *Chlorella vulgaris* in the removal of nutrients. Thier the results indicat a nutrient removal efficiency of 86 % inorganic N and 70 % inorganic P. In another paper, Colak and Kaya (1988) demonstrated an elimination of nitrogen (50.2 %) and phosphorus (85.7 %) in industrial wastewater treatment and elimination of phosphorus (97.8 %) in domestic wastewater treated by algae. Therefore, microalgae can be grown in ponds with little nutritional input or maintenance and this is an advantage. Many authors believed that this method.The separation of the metal saturated algae from the medium is an economic method for removing heavy metals from wastewater (Blier *et al.*, 1995). Therefore, metal sequestering processes are dependent on the algal species, the metal ion species and the media conditions.

Today, microalgae can be applied in bioreactors for wastewater treatment (Figure 1.6). The harvested algae (biomass) can be converted into biofuel such as biodiesel and bioethanol as a renewable fuel (Sialve *et al.*, 2009) (Figure 1.7). Another application of microalgae is be for food production. Algae contain high-level amounts of protein, β-caroten and omega-3 (Varfolomeev and Wasserman, 2011). Algae are also a good source of vitamins, minerals and fatty acids and they can improved the immune reaction of animals (Varfolomeev and

Wasserman, 2011). Therefore, microalgae have the posibility to simultaneously put fuels in our vehicles, recycle CO_2 also provide nutrition for animals and people (Palmer, 1998).

Figure 1.6 Pictures of algal bioreactors (http://www.treehugger.com/20090514-algae-bioreactor.jpg).

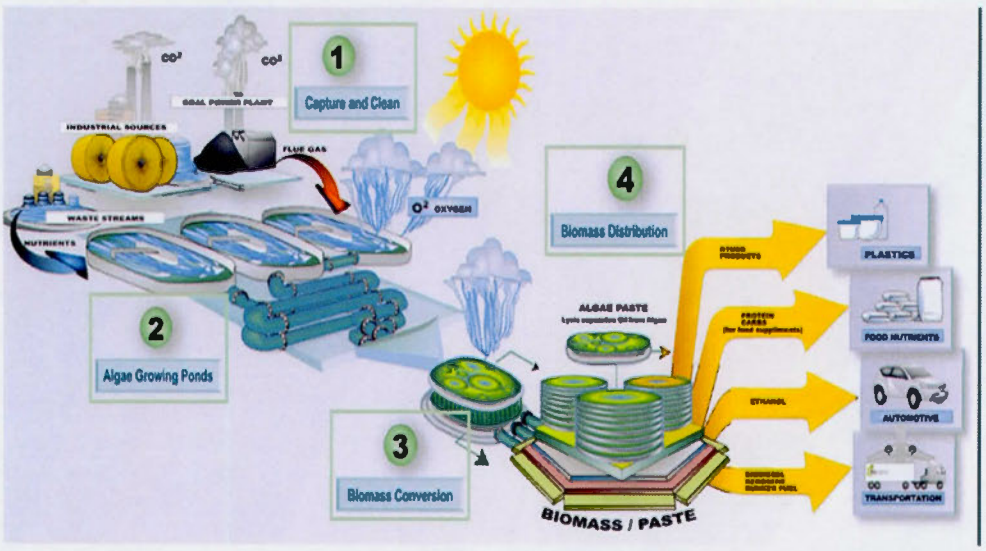


Figure 1.7 Integration scheme of applications of microalgae (Adapted of Bioalgene, 2009).

BIOCHEMISTRY OF GREEN ALGAE

1.4 Microalgal cell

As the first level of biological organization capable of life, the cell is the basic unit of structure and function of organisms. Cells have many membranes for their existence, which define each cell's boundary and help create electrochemical relation within and outside the cell (Buchanan and Gruissem, 2000). Microalgae are microscopic photosynthetic microorganisms which consist only of a single cell or unicellular. Microalgae are generally efficient in utilizing sun light, CO_2, water and nutrients to produce high biomass yields. They can also be grown without the use of fertilizers and pesticides, which results in less waste and pollution (Gouveia and Springer, 2011). In order to find the best way to cultivate algal strains and maximize carbon dioxide consumption, several parameters are necessary, including light intensity, temperature and media requirements (Richmond, 2004).

The Figure 1.8 shows an image of alga *Chlorella vulgaris*, which was used on this study. It has been found that this species of microalgae can vary in diameter from 2-8 μm.

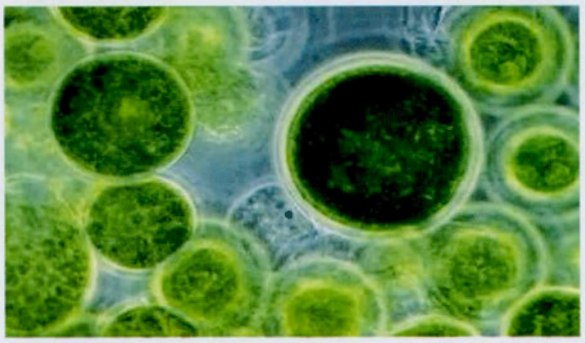


Figure 1.8 Microscopic image of *Chlorella vulgaris* http://algaetechinternational.com/new/chlorella.

1.4.1 *Chlorella vulgaris*

Algal cells of *Chlorella vulgaris* are circular in shape with diameters ranging from 2-10 microns. *C. vulgaris* reproduces very quickly under optimal growth conditions, capable of doubling in 8 hours (0.6 g/L day). *C. vulgaris* needs only sunlight, carbon dioxide, and small amounts of minerals such as phosphate and nitrogen to reproduce. *C. vulgaris* is also good for mass production because photosynthetic efficiency is approximately near 8 % (Morita *et al.*, 2001). For comparison, the photosynthetic efficiency of most trees is nearly 0.5 % and solar panels are almost 10-20 %. The lipid content of *C. vulgaris* is about 20 % by weight of the dried cell mass (Sheehan *et al.*, 1998). Figure 1.9 show a micrograph of a *C. vulgaris* cell (Stead *et al.*, 1995). Therefore, *C. vulgaris* has great potential for cultures and tolerate 10-15 % of carbon dioxide (Lee *et al.*, 2000). It has been shown that *C. vulgaris* can also grow in environments under high temperatures of 30-35 °C (Converti *et al.*, 2009) and acidic environments such as pH of 3 (Mayo, 1997).

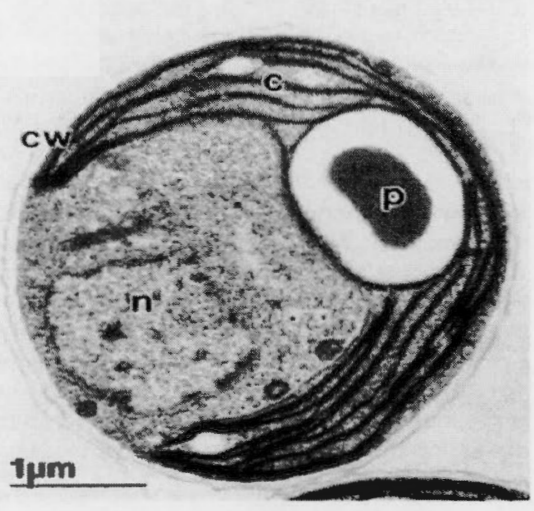


Figure 1.9 TEM (transmission electron microscopy) micrograph of an individual *Chlorella* cell (Stead *et al.*, 1995). C: chloroplast, p: pyrenoid, n: nucleus, and cw: cell wall.

1.4.2 Microalgae growth dynamic

The change in cell density of micro-algal cultures is composed in four growth phases, which are illustrated in Figure 1.10:

A: Induction phase or adaptation, when an algal culture is transferred from a plate to a liquid culture, we can observe a little increase in cell density.

B: Second phase (log phase), the cell density increases in time according to a logarithmic function. The specific growth rate is normally dependent on algal species, light intensity, media composition and temperature.

C: Stationary phase. Since the limiting factor and the growth rate are balanced, the cell density will reach a constant level. Cell division slows down when nutrients, light, pH, carbon dioxide or other physical and chemical factors begin to inhibit growth.

D: Death or "crash" phase. During the final stage, the water quality decayed and nutrients are decreased to a feeble level of standing growth. Cell density decreases quickly and the culture finally falls (Barnabé, 1990).

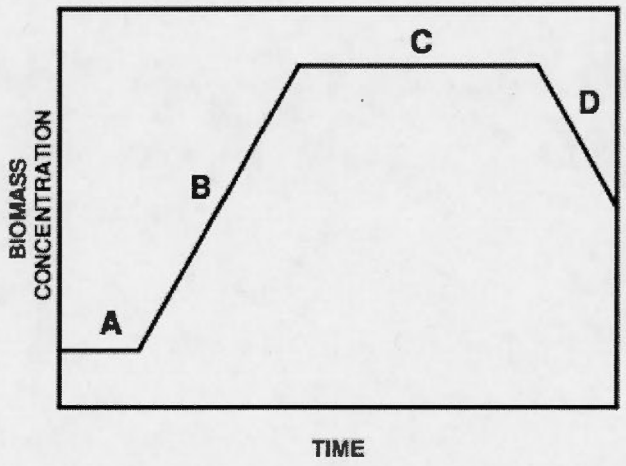


Figure 1.10. Four growth phases in microalgal cultures. A: Adaptation (lag phase); B: Exponential growth phase (log phase); C: stationary phase; D: Logarithmic death phase (Barnabé, 1990).

1.5 Photosynthesis

Photosynthesis is the reduction of light energy of the sun into chemical energy by plants and algae. Plants can absorb water and burn sugars to releas energy for growing (Milan and Librairie, 1970). The primary materials are carbon dioxide (CO_2) and water (H_2O), and the end-products are oxygen and carbohydrates like sucrose, glucose or starch. This modification of carbon dioxide into organic matter and production of oxygen make the composition of our atmosphere providing for all life forms with essential food and fuel (Nelson and Ben-Shem, 2004). The overall equation for these reactions can be presented as

$12\ H_2O + 6\ CO_2 +$ Light energy $\Rightarrow C_6\ H_{12}\ O_6 + 6\ O_2 + 6\ H_2O$

Photosynthesis consists of two different phases. The first phase, dependant of light (mainly photochemical process of photosynthesis) which happened in the thylakoid membrane. The photochemical reactions are linked to transport of electrons and protons for the synthesis of adenosine tri-phosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH). The second phase, is independent of light and it is called biochemical level in the stroma. This phase consists of biochemical reactions related to the Calvin Cycle, where the ATP and NADPH are used for the transformation of CO_2 into carbohydrates (Shackleton and Robinson, 1991). In plants and algae, photosynthesis happens in organelles named chloroplast. Normally, the plant cell has about 10 to 100 chloroplasts. The chloroplast can have a diameter of 5-10 μm and a dept of 3-4 μm (Buchan and gruissem, 2000).

The chloroplast is surrounded by a membrane composed of a phospholipids inner membrane, a phospholipids outer membrane and an inter membrane space between them (Figure 1.11). There is an aqueous fluid within the membrane, called the stroma. The stroma contains stacks of discs that are named thylakoids, and also stacks of thylakoids named grana, which are the site of photosynthesis. The interior of thylakoid membranes, composed of lipids, are called lumen (Staehlin, 1986).

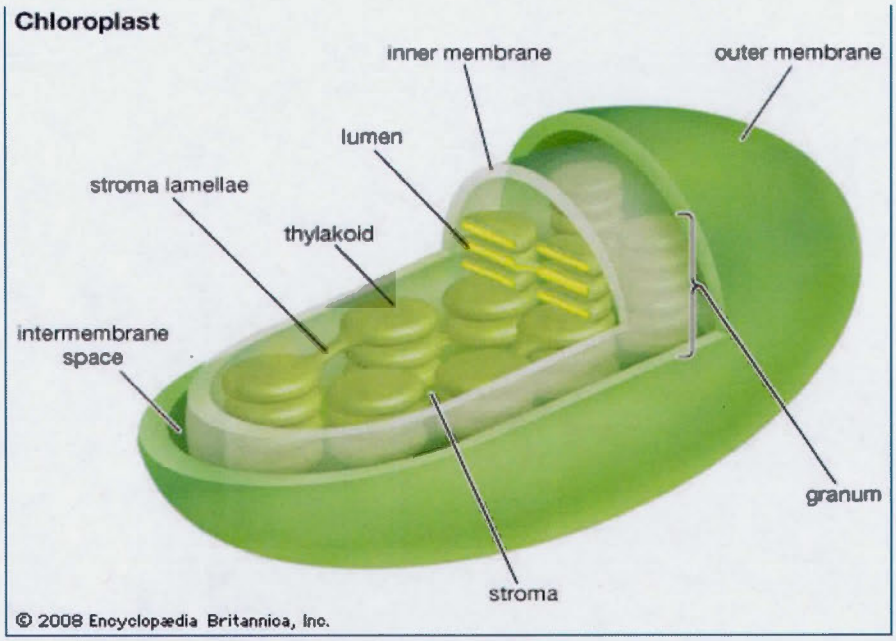


Figure 1.11 Structure of a chloroplast (www.britannica.com).

In thylakoid membranes, four types of protein complexes are involved in the conversion of light energy into chemical energy (Figure 2.5).

These protein complexes include (Malkin and Niyogi, 2000):

1. Two light collector antennas (LHCI and LHCII);

2. Two reaction centers as PSI and PSII (P680 and P700);

3. A transmembrane enzyme system, named ATP synthase;

4. Electron transport carriers between PSI and PSII, as plastoquinones (PQ), cytochrome-b_6f complex and plastocyanines (PC).

The hotosynthesis system requires the collaboration of two photosystems (Figure 1.12). Photosystem I (PSI) includs iron-sulfur reaction centers joined with proteins as terminal electron acceptors and many polypeptides that play a major role in the connection between plastocyanin and P700. Photosystem II (PS II) contains four manganese atoms and a dozen polypeptides. The antennae have many pigments proteins complexes involved as light harvesting collector (LHC) near the heart and trimetric LHC II in the periphery. D1 and D2

proteins (about 33 kDa) are the active center of PSII for charge separation (Buchanan and Gruissem, 2000).

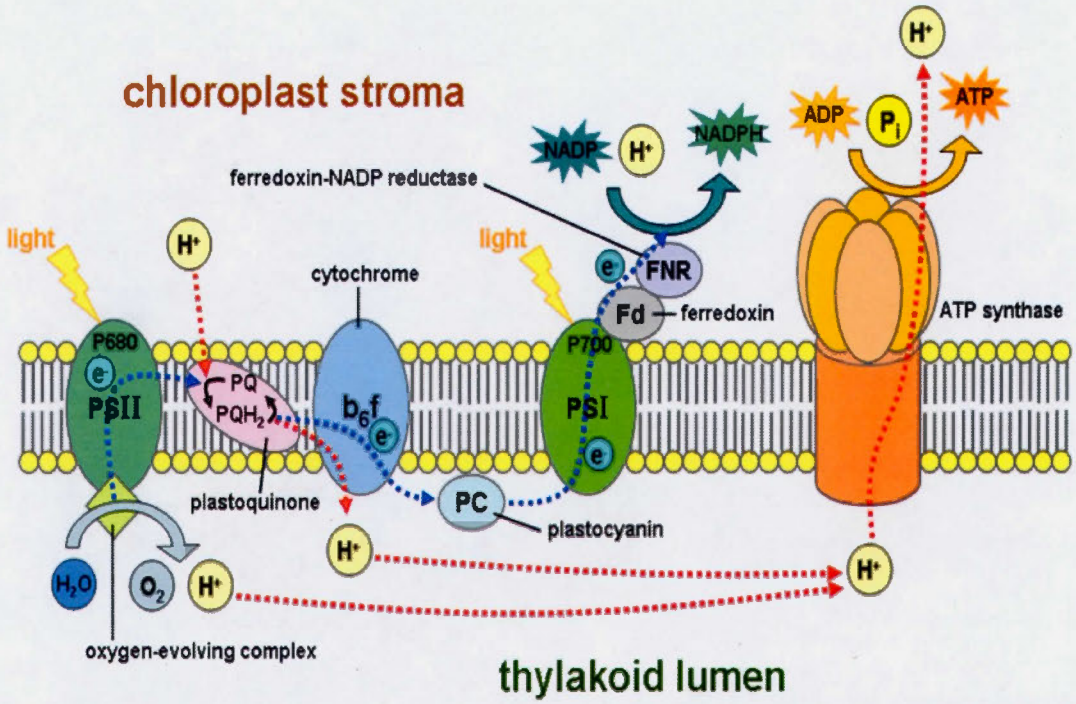


Figure 1.12 Schematic organization of the photosynthetic protein complexes involved in the transport of electrons in the thylakoid membrane of plants. Cyt, cytochrome; Fd, ferredoxin; b_6f, dimeric integral membrane; FNR, ferredoxin-NADP reductase; PCm plastocyanin; PSI and PSII, photosystem I and II; P680, the PSII reaction center; P700, the PSI reaction center (Malkin and Niyogi, 2000).

1.5.1 Structure and function of PSII

The primary photochemical reactions of photosynthesis happens in the Photosystem II. Photosystem II has several key parts such as light harvesting chlorophyll-binding proteins, a pair of chlorophyll molecules known as the P680 reaction center, pheophytin molecules, and plastoquinones (Figure 1.13). The light-harvesting protein complexes include hundreds of pigment molecules, essentially chlorophyll *a*, *b* and carotenoids (Jansson, 1994). There are two group of antenna complex in PSII: The internal antenna consists of many pigments-

proteins complexes, CP43 and CP47, CP29, CP26 and CP24 which are linked 40 to 50 molecules of chlorophyll (Chl) and have 10 molecules of β-carotene. Both complexes allow the transfer of light energy to the reaction center chlorophyll (P680). The device antenna called LHCII contains 50-60 % of the total chlorophyll pigment (Duysens, 1989). Each of these pigments absorbs light of specific wavelengths (Figure 1.14). When one of the many photons of light absorb by chlorophyll molecules surrounding the reaction center, it creates resonance energy. That resonance or vibrational energy is passed to another chlorophyll molecule until it reaches the P680 reaction center (Bricker and Ghanotakis, 1996).

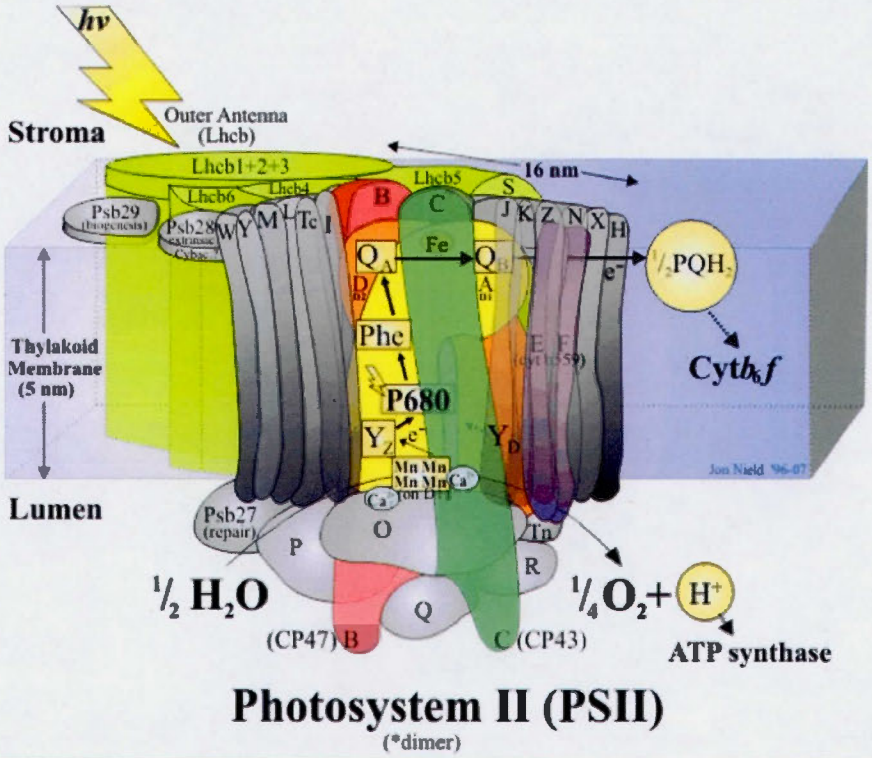


Figure 1.14 The Photosystem II. Q_A and Q_B, quinones A and B; Tyr: tyrosine; b_6f: dimeric integral membrane (Rawan, 1990).

The PSII reaction center consists of two major proteins, D1 and D2 (the molecular weight 34 kDa and 32 kDa, respectively) (Heller *et al.*, 1998). The energy captured by the antenna is moved toward a special pair of chlorophylls called P680 (or pigment 680) because

it has a maximum absorption of light at 680 nm. After reduction of PSII pheophytin, electron is passed to Q_A and Q_B, and then to the PQ, the cytochrome b_6f and the plastocyanin. These plastoquinone molecules are located in the D2 and D1 proteins. The P680 is reduced by the oxygen-evolving complex, and two water molecules are needed for the water splitting to provide electrons to reduce the P680. (Diner and Babcock, 1996).

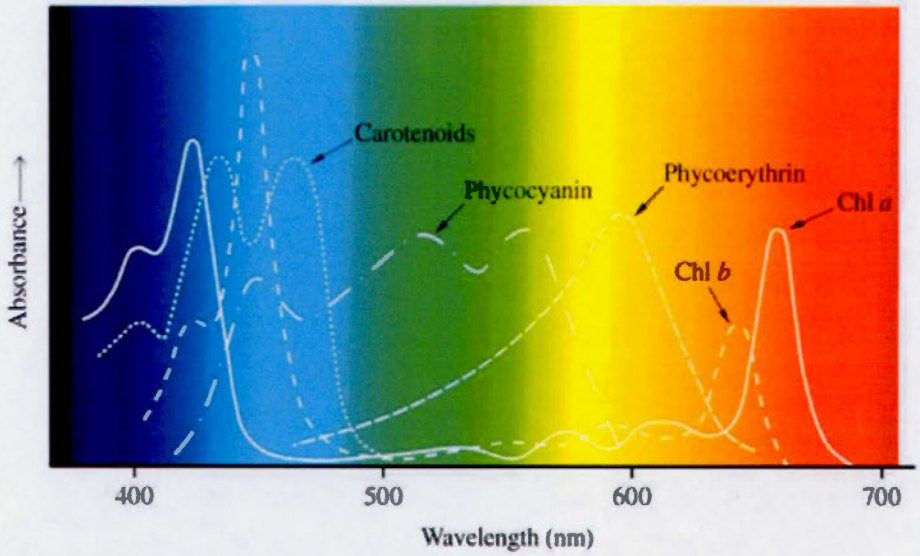


Figure 1.14 Absorption spectra of different photosynthetic pigments (Rawn, 1990).

1.5.2 Chlorophyll fluorescence

Light energy that is absorbed by chlorophyll in plants has three fates: a) it can be used to run photosynthesis (photochemistry), b) dissipated as heat or c) re-emitted as red fluorescence (Baker, 2008). The chlorophyll (Chl) fluorescence is a rich and complex signal. When chlorophyll molecules absorb a photon *via* an excess light, the molecule if is excited from the ground (Chl) to the first electronic excited state (Chl^*) within < 10-15 s^{-1} (Figure 1.15). These excited state molecules can reach higher energy level ($^3Chl^*$). The molecule will return to the ground state by re-emitting its energy as light in the form of fluorescence, as heat or by photochemistry.

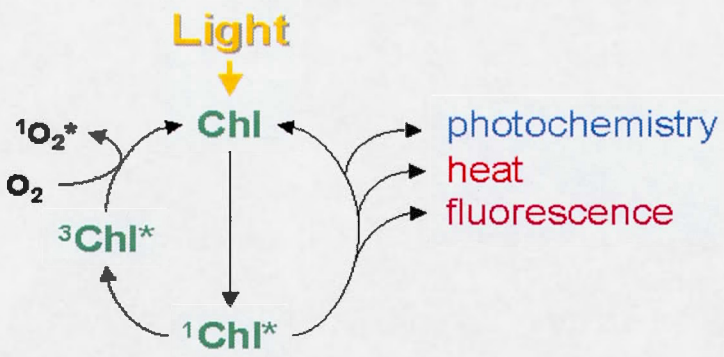


Figure 1.15 Photochemistry and ways of excited chlorophyll in PSII (Müller, Li and Niyogi, 2001).

Dissipation energy in form of fluorescence was discovered in 1931 by Kautsky and Hirsh (1931).The chlorophyll fluorescence is mainly derived from the Chl *a* of LHCII (Lazar, 1999). By measuring the chlorophyll fluorescence yield, information related to changes in the efficiency of photochemistry and heat dissipation can be obtained. Chlorophyll fluorescence kinetics measurement is an important tool for the molecular analysis of state of the PSII photochemical reactions and electron transport between PSII and PSI.

1.5.3 OJIP Test

For the first time, Strasser, Srivastava and Govindjee (1995) showed that there are different stages in the growth curve of the rapid induction of chlorophyll fluorescence from the initial level (F_O) to the maximum level (F_M). When presented on a logarithmic time scale, this fluorescence kinetic show several transitions: Fo, F_J, F_I and F_P ($F_P = F_M$). In fact, they showed that transitions O-J-I-P is related to the redox state in the reaction center of PSII (Figure 1.16). Over a period of 1s , the fluorescence level from Fo reaches the maximum level F_M (Strasser and Ştirbeţ, 2001). The OJ phase is the reduction of the primary electron acceptor, Q_A. The fluorescence level of the transition I is (FI) the first reduction of Q_B ($Q_A^- Q_B^-$) (Strasser *et al.*, 1995). Finally, IP represents the reducing of the electron acceptor side of PSI (Schansker *et al.*, 2005).

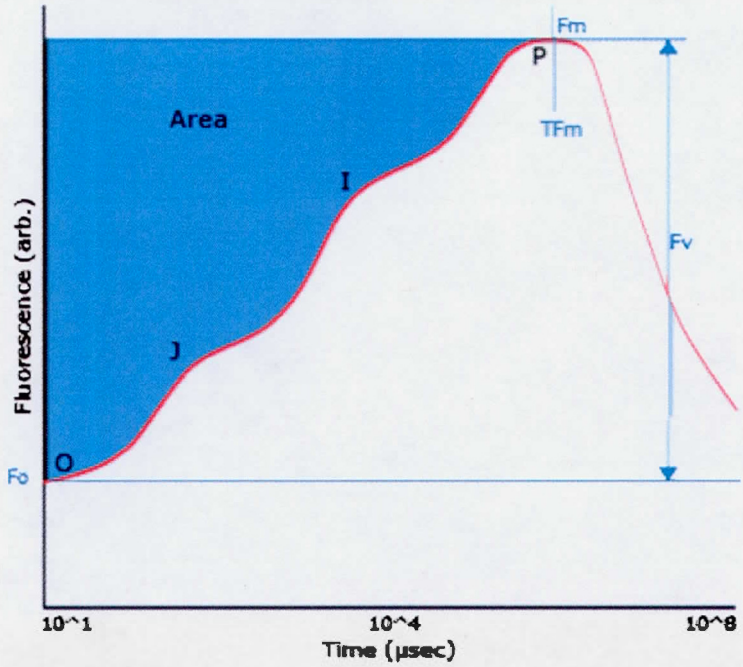


Figure 1.16 Fluorescence induction curve representing the transitions OJIP measured on a logarithmic time scale (Strasser, Srivastava and Govindjee, 1995).

1.6 Speciation and interaction of metals with living organisms

1.6.1 Metal speciation and bioavailability to algae

Many studies have shown that the bioavailability of metals was dependent on the activity of free ion rather than their total concentration (Tessier and Turner, 1995; Campbell *et al.*, 2002). Metal bioavailability is adjusted by kinetic factors. Inside the cell, metals are used in metabolism or accumulated in the cell (Tessier and Turner, 1995). Inside the cell, zinc like others metals can be uptakes by intracellular ligands that are present in the cell (Van Montagu, 1995).

Metals generally exist in the aquatic environment in polar, hydrophilic forms, which are hydrated and are unable to pass cell membranes by simple diffusion. There are few exceptions such as some organometallics and neutral metal complexes. In Figure 1.17, three differents mechanisms of metal uptake are shown: In the mechanism (i), represent transport

of hydrophilic metal ion species by ionic binding of metal ion to a cellular ligand on the cell surface; In the mechanism (ii), the metal transport across membrane involves either membrane carriers or channels. Such transport is depending on the free metal ion concentration. In the mechanism (iii), some metal complexes involve an assimilable ligand, such as thiosulphate, the metal transport is by passive diffusion of lipophilic species. Once metals have entered the intracellular environment, a variety of mechanisms exist for their detoxification (Vijver *et al.*, 2004).

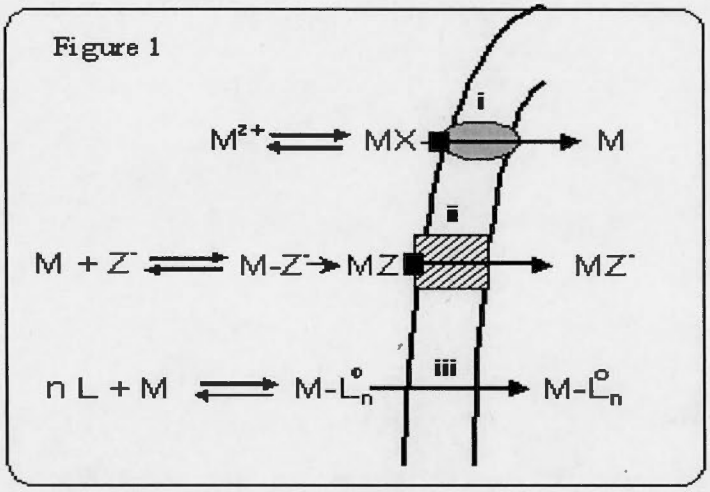


Figure 2.17 Three mechanisms of metal transport across the cell membrane. Mechanism i represents transport of hydrophilic metal ion species by ionic binding of metal ion to a cellular ligand on the cell surface. Mechanism ii represents passive diffusion of lipophilic species. Mechanism iii represents transport of metal complexes involving an assimilable ligand (Campbell and Hare, 2009).

1.6.2 Biosynthesis of glutathione and phytochelatin

Glutathione (GSH) is known to be efficient in the detoxification of cell. GSH protects cells by reducing reactive oxygen species (ROS) and also by binding to metals. GSH is a tripeptide that is made by glutamic acid (Glu), cysteine (Cys) and glycine (Gly), and also it is the main thiol compound produced by animals, plants, algae and bacteria (Giovanelli *et al.*, 1980, Noctor and Foyer, 1998). The GSH synthesis is shown in Figure 1.18. The synthesis of

Y-glutamylcysteine from L-glutamate and L-cysteine is catalyzed by the enzyme Y-glutamylcysteine synthetase. Then, the addition of glycine is catalyzed by the enzyme glutathione synthetase to produce GSH. It is generally synthesized in the cytosol, chloroplast, vacuole and mitochondria where amino acids are present (Noctor and Foyer 1998).

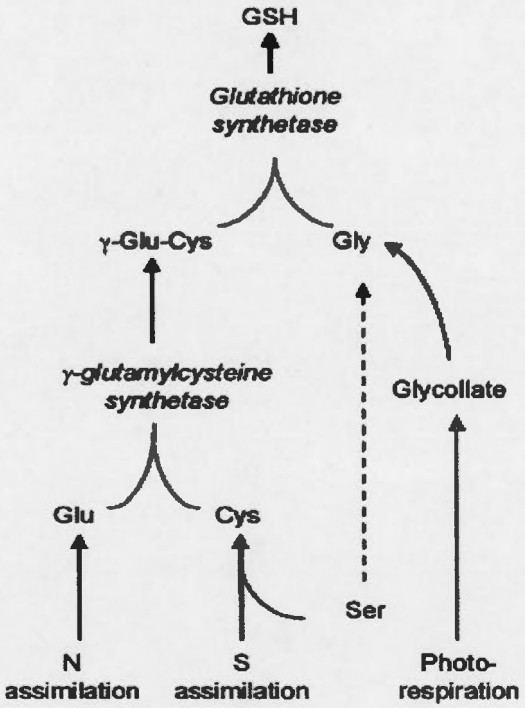


Figure 1.18 Synthesis of glutathione from its constituent amino acids. Glu: glutamic acid; Cys: cysteine; Ser: serine; Gly: glycine (Noctor and Foyer 1998).

Phytochelatins (PCs) are known as small polypeptides with the amino acid structure y-(Glu-Cys)n-Gly, where n ranges from 2 to 11, and which can be found in plants, algae and many fungi (Cobbett, 2000; Robinson, 1989). Phytochelatin is produced enzymatically by phytochelatin synthase from the glutathione, a tripeptide of glutamate, cysteine and glycine. The final glycine is clipped and the y-Glu-Cys is joined to another glutathione to form the n = 2 dimer. The extra Glu-Cys can be added to the peptide to create longer chains (n = n +1). In higher plants, the peptide can reach up to n = 11 (Grill *et al.*, 1989). Phytochelatin is able to protect from metal toxicity by chelating metal ions in the cells. As well as it plays a major

role in protecting macromolecules from the detriment of free radicals by trapping them in an aqueous phase (Freedman *et al.*, 1989). Therefore, GSH is essential for the synthesis of phytochelatins, which concentration is well controlled in microalgae (Rijstenbilet and Wijnholds, 1996; Morelli and Scarano, 2001; Ahner *et al.*, 2002). The production of PC depends on the specific metal ion and its concentration in plants and algae. The chemical structures of PCs, GSH and Y-Glu-Cys are shown in Figure 1.19.

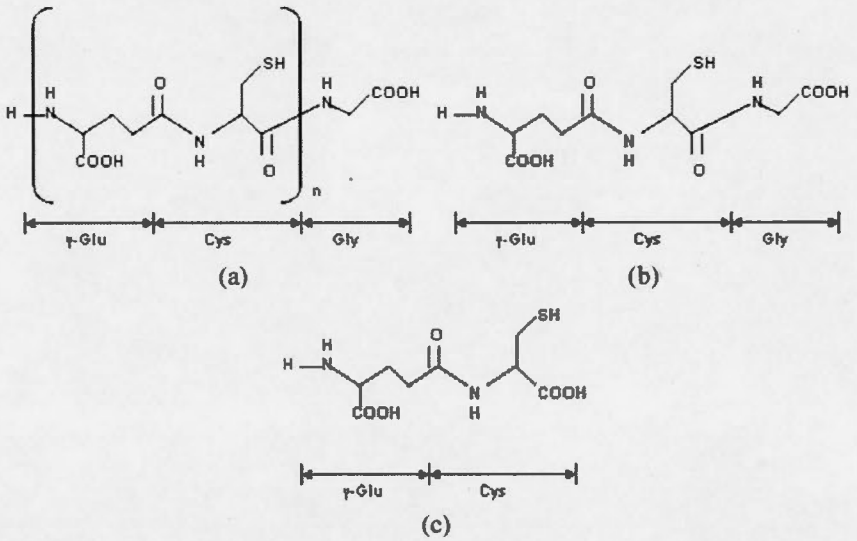


Figure 1.19 Chemical structures of (a) phytochelatin, (b) glutathione and (c) gamma-glutamylcysteine (Rama and Rai, 2009).

The PCs bind with metals through the thiol (-SH) group of cysteine. With the increasing metal concentration, the level of polymerization in PCs can augment indicating an increase binding stability of metal–PCn complexes (Faucheur, Behra and Sigg, 2005). The metal–PC complex is formed by many factors such as the availability of ligand, the kinetics of complex formation and steric factor. The interaction of some heavy metals (Cd, Zn) with PCs is shown in Figure 1.20. It shows binding stoichiometry of sulfur atoms from single or multiple PC molecules to be 4 to 1 (Cruz *et al.*, 2002; Kobayashi and Yoshimura, 2006). While in PC-Zn complex observed an interaction of 1:1 complex (one Zn to one PC) (Kobayashi and Yoshimura, 2006).

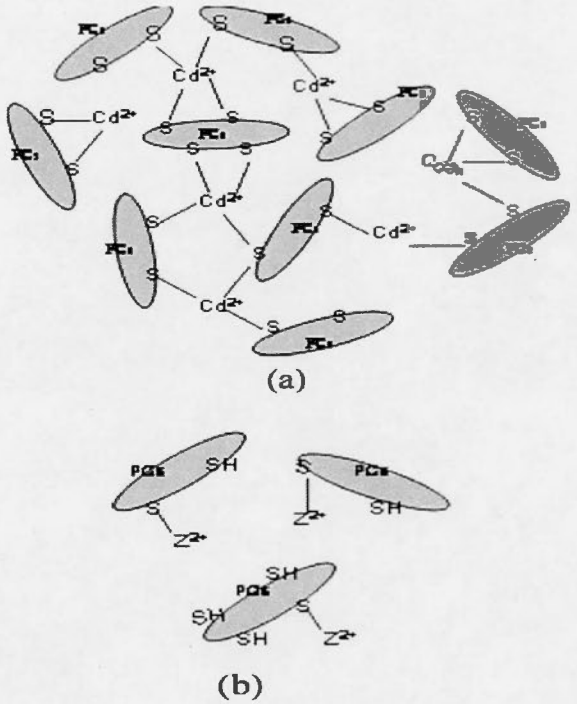


Figure 1.20 Interaction of PCs with different heavy metals. (a) Cd^{+2} and (b) Zn^{+2} binding with sulfur atoms of group (SH) in Cys residues from either single or multiple PC molecules, forming different complexes (Rama and Rai, 2009).

1.6.3 Detection of phytochelatins as biomarkers

As GSH is involved in many biochemical processes, it is not very effective as a biomarker for metal stress in phytoplankton and algae (Kawakami, 2006). On the other hand, PC production has been suggested as a biomarker due to its specificity as a detoxification against metal stress in aquatic system (Ahner *et al.*, 1997). Researchers believe that the use of PC induction can be more suitable than others biological responses. However, nutrient limitation and antagonistic effects of metal combinations may play important roles in PC determination in natural waters (Ahner *et al.*, 2002; Wei *et al.*, 2003).

Several analytical techniques have been used for the characterization of PCs or PC-metal complex in different matrices. In order to analyze PC induction in microalgae, a post-column derivatization reversed-phase HPLC coupled to an UV-VIS detector or fluorescence

detector is generally used (Thumann *et al.*, 1991; Di Toro *et al.*, 2001). The application of derivatisation step for PCs and GSH increased the sensitivity of the analysis. With a fluorescence detector, mBrB is the most commonly used sulfur-specific fluorescent tag (Kawakami, Gledhill and Achterberg, 2006). Other analytical methods have been used to analyse PCs and PC–metal complexes such as chromatographic separation (gel filtration or HPLC) coupled with UV detection, flame atomic absorption spectrometry, radio-active labeling, and inductively coupled plasma–mass spectrometry (ICP-MS), electrospray–MS (ESI-MS) or ESI-tandem MS (Vacchina *et al.*, 1999; Yen *et al.*, 1992; Campbell, 1995). Such as of coupling offer good sensitivity and good information on the structure of PCs, but they are expensive (Nolan, Lombi and Mclaughlin, 2003). The quality of the chromatographic separation can be affected by several factors: the stationary phase, the type of ligand, the composition of the mobile phase, the diameter of the pore of the stationary phase, the particle size, the size of the column the column temperature and additives to the mobile phase. Thiol groups are considered polar groups, so they are soluble in polar solvents such as water, methanol and acetonitrile (Martin and Guiochon, 2005; Liu and Lee, 2006).

This study was focused on the examination, from a quantitative point of view, of a relationship between the algal exposure to zn and its biological response and bioaccumulation of zn in different time. Metal ions pass across biological membranes, afterward increasing or inhibiting the cellular activities of microorganisms. After of the exposure of microorganisms to trace metal ions they are adsorbed on the cellular surface (binding of metal ions to transport sites at the cell surface) and the subsequent transport into the cell.

CHAPTER II
MATERIAL AND METHODS

2.1 Preparation of materials

Containers used in this study were made of polycarbonate, polypropylene or glasses. All 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 ($R \geq 18.2$ MΩ cm, TOC ≤ 2 mg/L). Then, all of flasks and materials needed for cultivation were sterilized by autoclave.

2.2 Selection of algal species

Experiments were performed with the unicellular green algae *Chlorella vulgaris*. The strain was obtained from the algae collection of the Canadian Phycological Culture Centre (CPCC, University of Waterloo, ON). The name *Chlorella* is taken from the Greek ***chloros***, meaning *green*, and the latin ***ella***, meaning *small*.

Chlorella is a tiny, unicellular green alga, 2-8 (μm) in diameter, which can grow most in large quantities in South East Asia and Australia giving lakes and rivers a green tint (Figure 2.1).

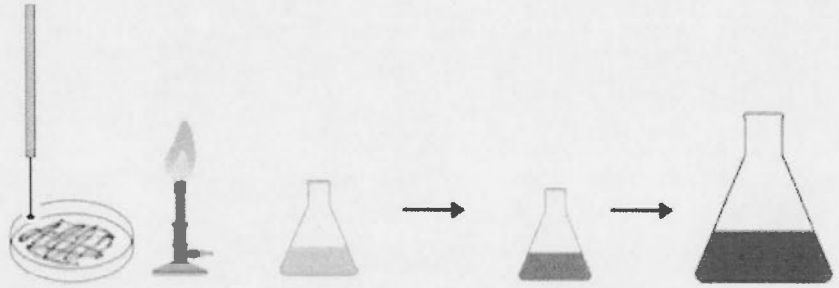
Figure 2.1. Photo of algae *Chlorella* taken by Victoria Lambert BST 2009 (www.telegraph.co.uk).

The alga *C. vulgaris* was chosen for this study since it can be grown easily in the laboratory. Moreover, it is well known in the field of research ecotricity since it has been

used to taste trace metals and it can produce phytochelatins in response to metals (Parent and Campbell, 1994). The alga *C. vulgaris* is useful in biomass production at commercial level since it contains proteins and minerals (Sankar and Ramasubramanian, 2012).

2.3. Culture condition

We used Erlenmeyer flasks (2 L) for stock cultures; while Pyrex storage dishes were used for long-term cultures. Each flask holds about 2 L, but it was used about 500 mL to allow better gas exchange with the liquid culture. For enhancing growth, shaking was used at the speed of 80-90 rpm. Light irradiance was of 80 ± 10 µmol of photons m^{-2} s^{-1}.



1 2 3 4

Figure 2.2. Steps in the preparation of liquid algal cultivation from solid plate cultures.

Step for liquid cultivation (Figure 2.2) one as follows:

1- *C. vulgaris* in agar plate (sterile condition);

2- BBM (bold's basal media) medium;

3- Inoculation of algal sample + media (25 mL);

4- After 7 days, algal culture + BBM (500 mL).

Agar solid plate cultures can be stored for a maximum of one month before being transferred to a new agar plate. Plate cultures were kept at room temperature for one week before being used for liquid inoculation. All steps were performed in sterile condition near the flame and in a biological flow hood.

2.4. Composition of growth media

2.4.1 Bold's Basal Media (BBM)

A modified version of Bold's Basal Medium was used for algal cultures and experiments, and the formulation is shown in Table 2.1. This media contains macro and micronutrients necessary for the growth of microalgae. Stocks solutions were prepared and stored at 4 °C in the fridge until needed. The pH was adjusted with NaOH 0.5 M. Then, culture media was sterilize by autoclave (1.5 hours at 80 °C). Before its use, the medium was equilibrated for 24 h to reach chemical equilibrium.

Table 2.1 Concentration of macro and micro nutriments in culture medium (BBM).

Macro nutrients (M)		Micro nutrients (M)	
$NaNO_3$	2.94×10^{-3}	H_3BO_3	4.62×10^{-4}
$CaCl_2 \cdot 2H_2O$	1.70×10^{-4}	$ZnSO_4 \cdot 7H_2O$	7.67×10^{-5}
$MgSO_4 \cdot 7H_2O$	3.04×10^{-4}	$MnCl_2 \cdot 4H_2O$	1.82×10^{-5}
K_2HPO_4	4.31×10^{-4}	$Co(NO_3)_2 \cdot 6H_2O$	4.21×10^{-6}
KH_2PO_4	1.29×10^{-3}	$CoCl_2 \cdot 6H_2O$	6.67×10^{-6}
NaCl	4.28×10^{-4}	$NaMoO_4 \cdot 5H_2O$	1.23×10^{-5}
$FeSO_4 \cdot 7H_2O$	4.48×10^{-5}	$CuSO_4 \cdot 5H_2O$	1.57×10^{-5}

2.4.2 Preparation of experimental condition

Algal cultures in the exponential growth phase were used for the experiments with a cell density of 10^6 cells/ml. Before the experimental setup, the exposure medium was prepared in triplicate for each tested concentration of $ZnCl_2$ in Erlenmeyer flasks of 50 ml. Under sterile conditions under a biological flow hood, an aliquot of algal sample was inoculated in BBM at a total volume of 20 ml. Algal cells were exposed to different concentrations of Zn^{2+} for 24, 48 and 72 hours. During treatments, Erlenmeyer flasks were placed on an agitator and shaken at 90 rpm.

2.5 Cellular division

The cell density of alga was determined by counting a sample suspension using a cell counter Beckman Coulter Counter Multisizer III. The principle of this cell counter is relatively simple. It provides number, volume, mass and surface area size distributions in one measurement, with a range of 0.4 to 1200 µm. Dilution was done with 1 ml of algal suspension and 9 ml of isotonic solution (a solution that has the same salt concentration as cells) in final volume 10 ml. Then, after washing with pure Isotone, measurement of cell density for each sample was done in triplicate.

In order to monitor the change of cell density by using the change of the absorbance at 750 nm of algal cell suspension, a standard curve was determined. In three flasks, having different initial cell densities of 250×10^3, 650×10^3 and 1×10^6, absorbance at 750 nm and algal cell density were measured during 72 h. Figure 2.3 shows the correlation between the change of cell density and the absorbance. A Perkin Elmer Lambda 40 UV-Visible Spectrophotometer was used to analyze the algal cell growth. As the photosynthetic pigments absorb the light energy from 470 to 750 nm, a wavelength at 750 nm was used for the estimation of cell density in suspension. Fisher-Scientific® Fisherbrand Standard 10-mm path length quartz cuvettes with a sample holding capacity of 1 ml were used to hold the samples in the spectrophotometer.

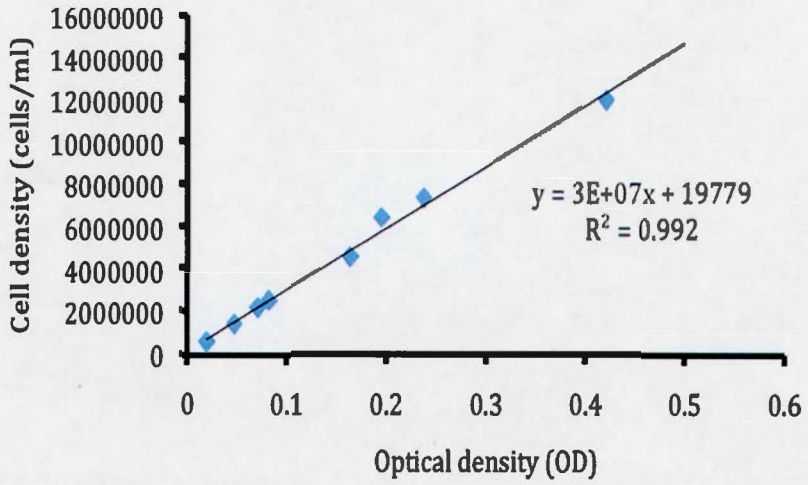


Figure 2.3 Standard curve of algal growth in BBM at 72h.

2.6. Bioaccumulation of zinc by atomic absorption spectrometry

2.6.1. Basic principles of atomic absorption spectrometry

An atomic absorption spectrometer (AAS) is an analytical instrument that measures the concentrations of trace metal elements. AAS is a very sensitive system which can measure down to parts per billion of a gram in a sample. The technique use the wavelengths of light that is specifically absorbed by an element in accordance to the amount of energy needed to promote electrons from one energy level to higher energy level (Welz and Sperling, 1999). The AAS is based on the measurement of the absorption of optical radiation by atoms in gas phase. The signal of the atomic absorption is directly related to the number of ground state atoms in the optical path of the spectrometer. Ground state atoms are produced from the sample material, normally by evaporation of solvent and vaporization of the solid particles followed by analysis of the molecular species into neutral atoms. Usually these steps are performed using an nebulizer and flame (Ingle and Crouch, 1988).

There are five basic parts in an atomic absorption spectrometry instrument:

1. The light source that emits the spectrum of the element (Hollow Cathode Lamp).
2. Nebulizer, sucks up the liquid sample or aspiration and creat a fine aerosol for introduction into flame
3. An "absorption cell" in which atoms of the sample are produced (flame, graphite furnace,...).
4. A monochromator for light dispersion.
5. A detector, which measures the light intensity and amplified the signal.
6. A display that shows the record after it has been stored by the instrument electronics (Perkin, 1996).

2.6.2 Preparation of samples

The culture media and stock solutions were prepared with ultrapure water. All vessels for experiments were sterilized by autoclave for 60 min. The media was autoclaved for 150 min at 121 °C. All chemical compounds (salts) used to make the media and solutions were analytical grade. The containers used were in Pyrex, borosilicate glass or polycarbonate. Zinc solutions and others trace elements were kept in dark polycarbonate containers. The cultur medium culture was kept in Pyrex containers. All containers and reusable materials (pipette tips, magnetic stirrers, etc.) were soaked in nitric acid (10 %) for at least 12 h, washed extensively five times with distilled water and then two times with ultra-pure water.

2.6.3 Estimation of zinc bioaccumulation in algal biomass

In order to determine the bioaccumulation of zinc in *Chlorella vulgaris*, algal cells were exposed to various concentrations of $ZnCl_2$: 0, 100, 1000, 2500, 5000 and 10000 µM. These solutions were prepared from a stock solution of 1 M $ZnCl_2$. The solutions were prepared at least 24 h before treatment so that chemical equilibrium was reached. For all experimental setup, the initial cell density was 10^6 cells/ml. All experiments were done in triplicates, and algal cells were exposed during 24, 48 and 72 hours. Algal biomass was collected by filtration using a filtration unit equipped with a MF-Millipore filter (0.45

microns) and then washed with a solution of 0.001 M EDTA (Ethylene diamine tetraacetic acid disodium salt dihydrate, Sigma Ultra grade, Sigma-Aldrich) to remove adsorbed metals on the cell walls of algae. The filter was rinsed three times with this EDTA solution to a total volume of 10 ml.

2.6.4 Digestion of algal biomass with nitric acid 70%

After filtration, filters were dried in the oven at 80 °C for 24 hours. Thereafter, each filter was placed in a 7ml Pyrex for digestion. Then, 1 ml of 70% HNO_3 was added with 125 µl of hydrogen peroxide (H_2O_2) to digest completely the biomass. The digestion was done over a period of 8 hours at 100 °C in a sand bath. After digestion, 1 ml of nitric acid and 8 ml of ultrapure water was added to the solution to a final volume of 10 ml per sample (Figure 2.4).

Table 2.2. Standard flame emission conditions for Zn.

Wavelength (nm)	Slit (nm)	Flame
213.9	0.2	Nitrous oxide-acetylene

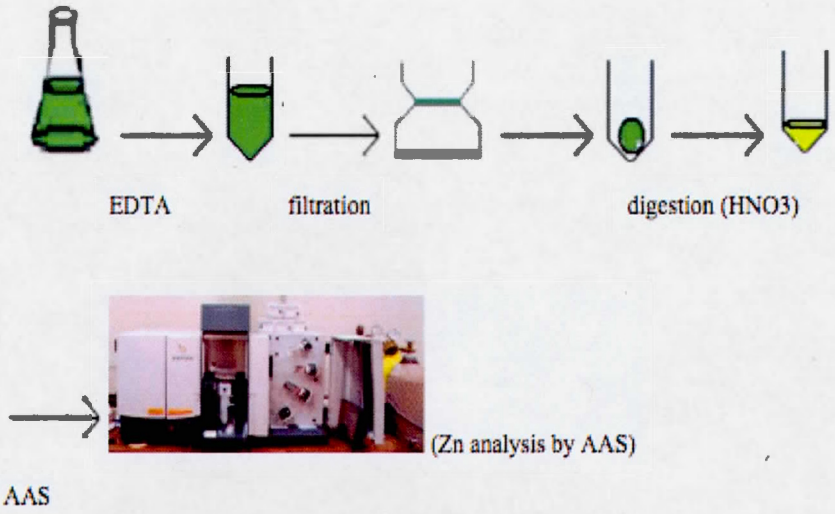


Figure 2.4 Steps in the determination of Zn bioaccumulation in algal biomass.

2.6.5 Preparation of AAS standard calibration solutions

A calibration curve was used to determine the concentration of zinc in the digested solution. The instrument was calibrated using several solutions of known concentration of $ZnCl_2$. The unknown concentration of zinc in each of our samples was estimated from the calibration curve (Figure 2.5). In this study, six standard calibration solutions were used: 0.1, 1, 1.5, 2.5, 5, 10 mg/L. To prepare a stock solution of 100 mg/L of zinc, 0.1 g of $ZnCl_2$ was dissolved in 1 L of media (BBM). The blank was representative of the sample matrix and it contains all reagents used in the sample preparation.

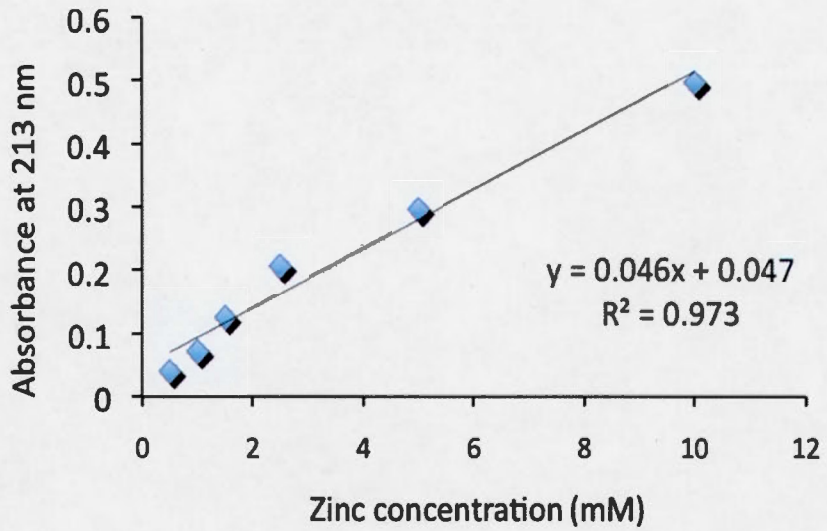


Figure 2.5 Standard calibration curve for zinc at 24-72h, determined by AAS.

2.7 Measurement of chlorophyll fluorescence

2.7.1 Fluorometer instrument

The fluorescence emission of Chl *a* was measured with a Handy PEA instrument (Plant Efficiency Analyser, Hansatech Instruments, King's Lynn, Norfolk, UK). The housing contains a battery, various forms of connection (with the head for measurements, computer and charger) and also contains a mini-computer. The head permit to illuminate the sample and measuring the fluorescence. The illumination consists of a pulse of 1 s red light with a peak at 650 nm (spectral half width of 22 nm) and a maximum intensity of ~ 3000 µmol of photons m^{-2} s^{-1}, and is provided by three light emitting diodes (LEDs). The light is focused on a disk of 5 mm in diameter (Strasser and Srivastava and Tsimilli, 2004). To analyze the data of the fluorescence kinetics, we used the program Biolyzer. The Biolyzer software allows visualization of the fluorescence curves and the calculation of parameters in the JIP-test (Table 2.3). Indeed, the measured parameters are the basis for the calculation of a number of

biophysical and phenomenological states leading to the dynamic definition of a photosynthetic sample at a given physiological state (Strasser, 1992) (Figure 2.6).

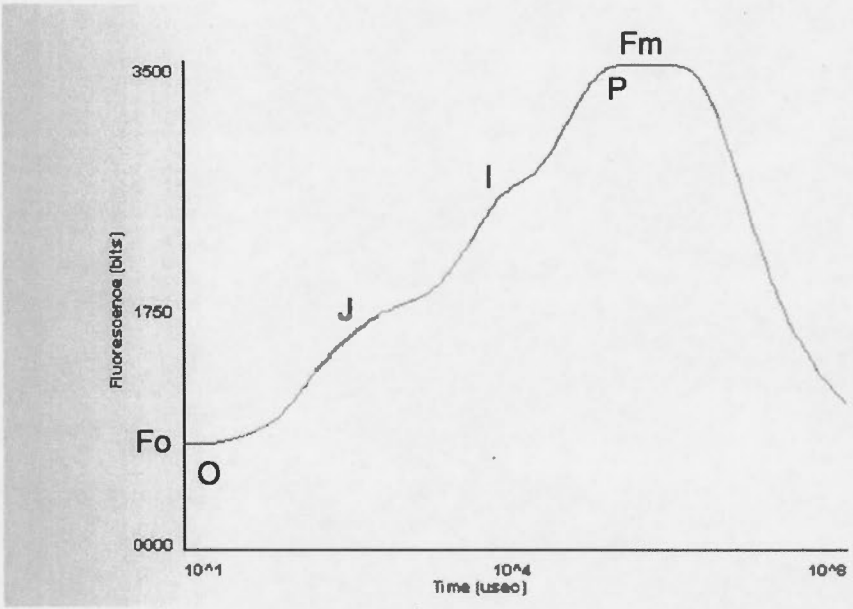


Figure 2.6 Kautsky curve of a healthy green leaf and rephases OJIP (Strasser et al., 2004); F_0 is minimum fluorescence yield and F_M is maximum fluorescence yield.

Table 2.3 Equations and definitions of OJIP parameters (Strasser et al., 1995; 2004).

Parameter	Definition	Equation
O	Fluorescence intensity at 20 μs	
J	Fluorescence intensity at 2 ms	
I	Fluorescence intensity at 30 ms	
P	Maximal fluorescence intensity	
F_V	Maximum variable Chl fluorescence	$F_V = F_M - F_0$
F_M	Maximum fluorescence yield	
M_0		$[4\times(F_{300} - F_{20\mu s})/(F_M - F_{20\mu s})]$
V_J	Relative variable fluorescence at J transient	$(F_{2ms} - F_0)/(F_M - F_0)$
ABS/RC	Effective antenna size per active Reaction center	$(M_0/V_J)/(F_V/F_M)$
P.I.	Index performance of PSII activity	$[1-(F_0-F_M)/(M_0/V_J)]\times$ $[(F_M-F_0)/F_0] \times [(1-V_J)/V_J]$

2.7.2 Sample preparation and fluorescence analysis

For experiments, algal cells were exposed to $ZnCl_2$ for 24, 48, 72 and 96 h to a range of concentrations of 1□100 mM. The experiments were performed at least in triplicates. For the extraction of total chlorophyll, 1 ml of algal sample was centrifuged at 9000 *g* for 5 min and the supernatant was removed. The pellet was suspended with 1 ml of methanol 100% for 2 min in the dark. Tubes were put into a water bath at 65 °C for 10 min, and centrifuge for 5 min before analytical measurements. For the determination of total chlorophyll, the

absorbance was measured at 470, 652, 665.2 nm using a UV-Vis Perkin Elmer Lambda 40 spectrophotometer. The measurement of the rapid fluorescence induction from 10 μs to 1s was performed with a Handy-PEA fluorometer (Hansatech, England). Before fluorescence measurements, treated algal samples of *C. vulgaris* were adapted to darkness for 25 min to induce an equilibrium state of the photosynthetic electron transport.

Algal samples, equivalent to 15 μg of total Chl *a*, were placed on glass fiber prefilters (Millipore #AP20 013 00) by using low-pressure filtration to avoid physiological stress effect and which permitted to obtain reproducible results.

2.8 Methods for determining glutathione and phytochelatins

2.8.1 Basic principle of high performance liquid chromatography

The identification of GSH and PCs was done by high performance liquid chromatography (HPLC) (Vacchina, 1999; Wang, 2000).

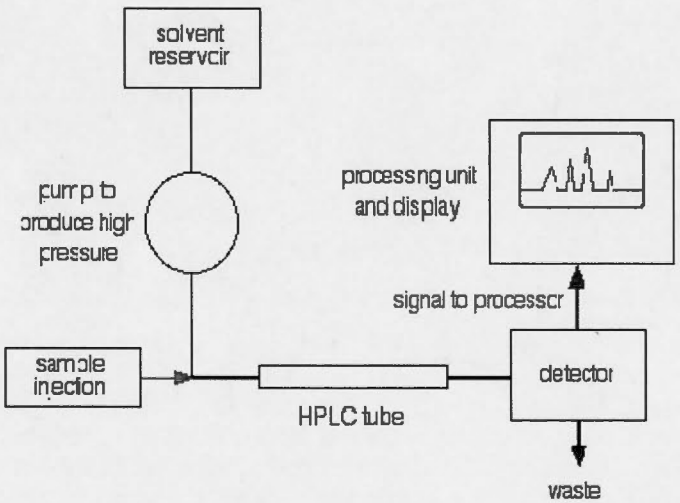


Figure 2.7 Schematic of a high performance liquid chromatograph instrument system (http://www.chemguide.co.uk).

As shown in the diagram in Figure 2.7, HPLC instrumentation includes a pump, an injector, a column, a detector and a display system. The heart of the system is the column

where separation occurs. Since the stationary phase may be composed of micron-sized porous particles, a high-pressure pump is required to move the mobile phase through the column. The chromatographic process runs by injecting the solute into the injector at the end of the column. When the sample and mobile phase are pumped through the column, separation of chemical components happens. Finally, each component elutes from the column and is detected by the detector and displayed as a peak on the monitor. Detection of the eluting components is important, and the method used for detection is dependent to the detector used. In this study we used fluorescence detection. The response of the detector to each component is displayed on computer screen and is known as a chromatogram (Lindsay and Kealey, 1987).

2.8.2 Chemicals

In order to avoid the degradation and loss of compounds, we did a rapid sample preparation. To prevent trace metal contamination on the degradation of reagents and thiol compounds, glassware and plastic bottles were soaked in an acid bath (10% HNO_3) for at least 24 h, and then rinsed five times with ultrapure de-ionized water (Milli-Q, Millipore). All solutions are prepared with ultrapure de-ionized water.

Chemicals used were analytical grade for HPLC. EDTA (99%), trifluoric acetic acid (TFA) ($\geq$ 99%), methane sulfonic acid (MSA) ($\geq$ 99.5%), diethylene triamine pentaacetic acid (DTPA) (99%), monobromobimane (mBrB) ($\geq$ 97%), tris(2-carboxyethyl)phosphine (TCEP) (98%), sodium hydroxyde (NaOH) (98%), glutathione (GSH) (> 97%), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 99%) were obtained from Sigma Aldrich. Analytical standard of zinc, in addition to nitric acid (HNO_3), hydrochloric acid (HCl), L-cysteine (> 99.5%) and sodium nitrate ($NaNO_3$) (99.99%) were purchased from Fluka Analytical. Phytochelatin standards: PC_2, PC_3 and PC_4 (> 95%) were obtained from Canpeptide Inc.(Canada) while cysteine and γ-glutamine were obtained from Sigma Aldrich. Finally, acetonitrile and HPLC grade solution were obtained from Tekni Science Inc. (Qc, Canada).

2.8.3 Sample preparation

The analytical method for PCs and GSH in algae includes a multi-step procedure: 1) Sampling; 2) Sample filtration or centrifugation; 3) Extraction of thiols; 4) Thiol reduction; 5) Thiol derivatization; 6) Analysis of thiol derivatives (Ahner *et al.*, 1995).

2.8.3.1 Sample collection and preparation

Two methods were used to collect algal cells: filtration and centrifugation. When the filtration was used, the tube containing the filter paper was frozen at -80 °C until the extraction of phytochelatins and thiols compounds was performed. The cells were harvested by centrifugation for 10 min (4000 rpm, Multifuge 1SR, Heraeus). Subsequently, the pellet was washed with the media and centrifuged 4 min at the same speed. The pellet was rinsed again and then transferred to a microtube (1.5 ml, Eppendorf). Finally, the supernatant was removed from the microtube and the pellet was stored at -80 °C until the extraction procedure. A schematic overview of the method is presented in Figure 2.8.

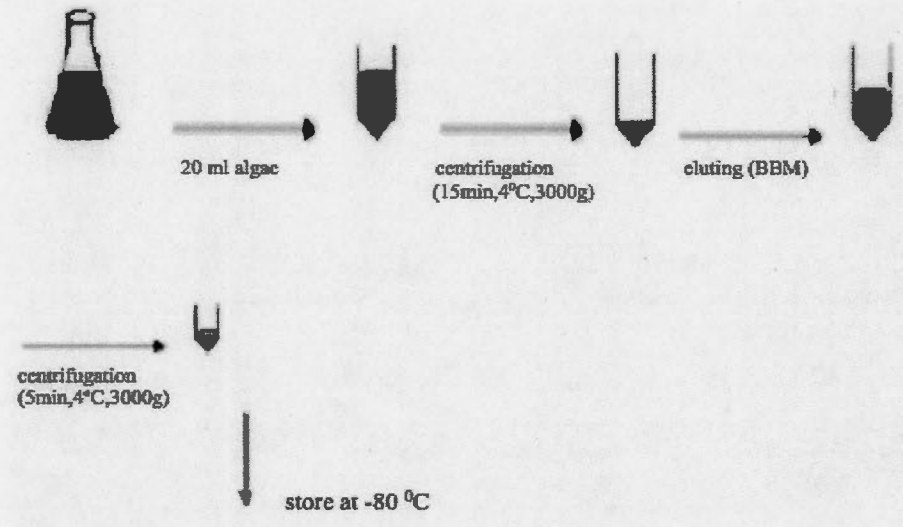


Figure 2.8 Harvesting procedures for collecting algal biomass.

2.8.3.2 Extraction of PCs and GSH

To avoid the degradation of polypeptides by enzymes, the extraction of thiols was made at 4 °C (Figure 2.9). For the extraction process, HCl was added to promote the denaturation of enzymes (capable of degrading peptide-thiols) and DTPA was added as a metal-chelating agent to minimize the oxidation by metals of the SH groups of PCs and GSH. After centrifugation, the supernatant was removed and 1.2 mL of 0.1 M HCl containing 5 mM of DTPA (diethylene triamine pentaacetic acid) was added to the microtube having the pellet of algal cells.

To achieve a better cell breakage, glass beads were added to the mixture which was swirled through a vortex (Digital vortex mixer, 3000 rpm, Fisher Scientific) for 2 min. The extract was disrupted by ultrasonication (0 °C, 5 min) two times of 2.5 minutes and the cell extract was centrifuged at high speed (13000 *g* for 20 min at 4°C) to remove cell debris. The supernatant was used for reduction and derivatization reactions (Silvia, K *et al*, 2006).

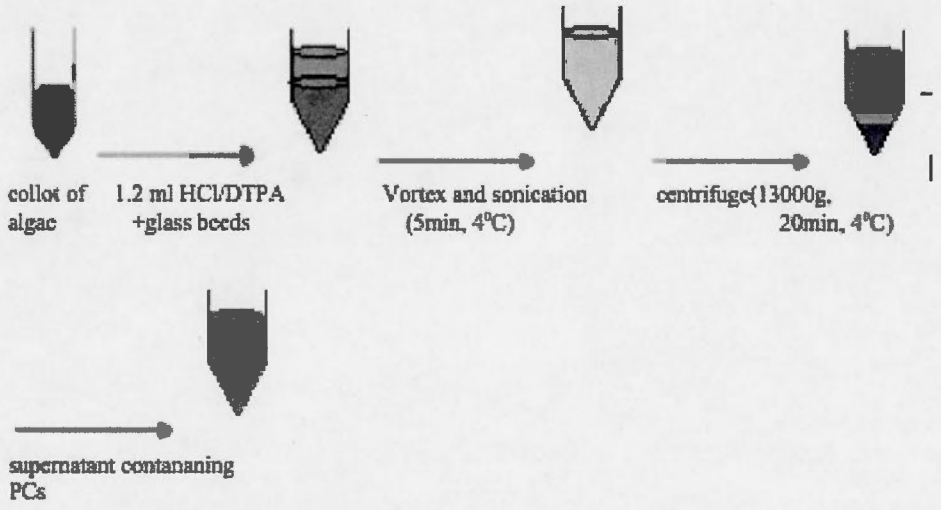


Figure 2.9 Method used for the extraction of thiols.

2.8.3.3 Reduction reaction

Oxidized GSH and PCs (i.e., RS-SR; R = peptide with residual cysteine group) can be converted to free thiols (RS-H) by reduction using TCEP (Rijstenbil and Wijnholds, 1996; Getz *et al.*, 1999). The reduction process of thiols by TCEP is shown in Reaction 1:

$$(CH_3CH_2COOH)_3P + RS\text{-}SR + H_2O \longrightarrow (CH_3CH_2COOH)_3P{=}O + 2\ RS\text{-}H$$

TCEP oxidized thiol reduced thiol

An aliquot of 250 µl of the supernatant was removed and buffered to pH between 8 – 9, by adding 625 µl of 200 mM HEPES and 5 mM DTPA (Figure 2.10). At this pH, thiol groups are deprotonated. The S- is a good nucleophile, and it promotes the derivatization reaction (Rijstenbil.Wijnholds,Biol;1996). Then, 25 µl of 20 mM TCEP (tris (2-carboxyethyl) phosphine) was added to break disulphide bonds before derivatization by mBrB (monobromobimane) fluorescent tag (Tang *et al.*, 2003).

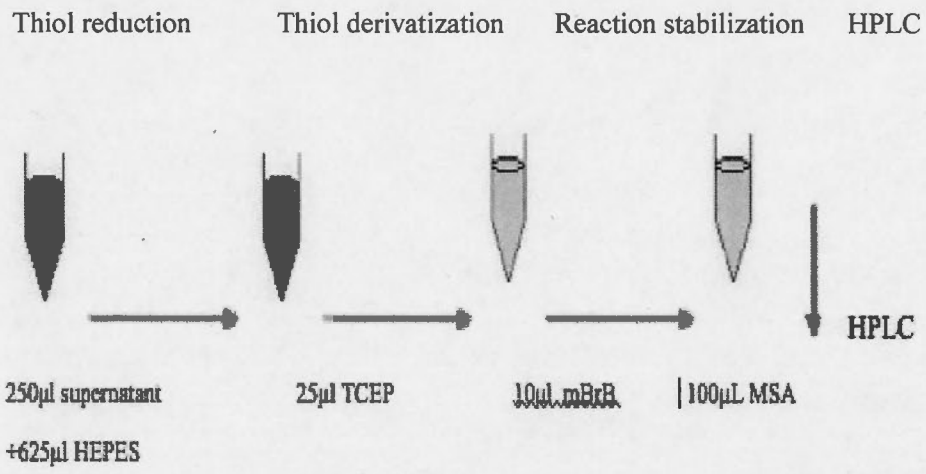


Figure 2.10 Thiol reduction, derivatization and stabilization for HPLC analysis.

2.8.3.4 Derivatization of PCs

The mBrB is the most used fluorescent tag in chromatography although bimane derivatives require the need to eliminate all fluorescent materials from the HPLC column following injection and to reequilibrate the column system prior to a next injection. The mBrB reacts with thiols, but it has slow reaction with amines, phosphates, and carboxylates (Fahey, 1987). The mBrB can be detected at 470 nm using an excitation light at 380 nm. The derivatization of PCs and GSH with mBrB (in acetonitrile, final concentration 1 mM) was done under dim light conditions at room temperature. After 15 min in the dark, 10 µl of mBrB of 100 mM were added and the mixture rested for another 15 min in the dark. The reactions were stabilized with 100 µl of 1 M MSA (methane sulfonic acid). The samples were filtered through nylon filters with a porosity of 0.2 µm, and then stored at 4 °C until analysis was performed by HPLC. The blank was determined by using 250 ml of supernatant of algal cells unexposed to $ZnCl_2$ (Control). The standard curves were obtained by using different concentrations of standard of PCs at 1, 5, 10, 25, 50, 100 and 200 µg/ml.

2.8.3.5 HPLC analysis

Derivative samples (fluorescent thiols) were placed in vials for injection (Jasco Intelligent Autosampler AS-2055). The liquid chromatographic analysis was performed in reverse-phase through a C18 column using a HPLC system consisting of two pumps, an injection valve with a 100 µl loop and a fluorescence detector. The separation was done using a 150 × 4.6 mm C-18 (Econosphere) column with 2.5 µm particle size. Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was acetonitrile (Rijstenbil and Wijnholds, 1996). For the assay, a volume of 100 µl of each sample was injected into the HPLC system, previously settled and balanced (column temperature = 40 °C, flow rate of the mobile phase = 1 ml min^{-1}, λ excitation = 380 nm and λ emission = 470 nm), and a table of the gradient is shown below (Table 2.4).

Table 2.4 Gradient profile of the different phases.

Pumping time (min)	Gradient of solvent	
	ACN (A)	TFA/Water (1 : 1000, v/v) (B)
0 - 13	10 → 21 %	90 → 79 %
13 - 33	21 → 35 %	79 → 65 %
33 - 40	35 → 100 %	65 → 0 %
40 - 50	100 %	0 %
50 - 65	100 → 10 %	0 → 90 %

2.8.4 Optimization of the detection signal

In this chapter, the optimization of the detection signal for the determination of phytochelatins and related thiols are explained.

2.8.4.1 Stability of phytochelatins

The stability of GSH and PCs in derivatized form is important for the sample storage that should be stored for HPLC analysis. The mBrB can be stable for 6 weeks, GSH for 15 days and PCs as bimane derivatives are stable over a 30 days period at 4 °C in the dark (Tang *et al.*, 2003; Rijstenbil andWijnholda, 1996) (Figure 2.11).

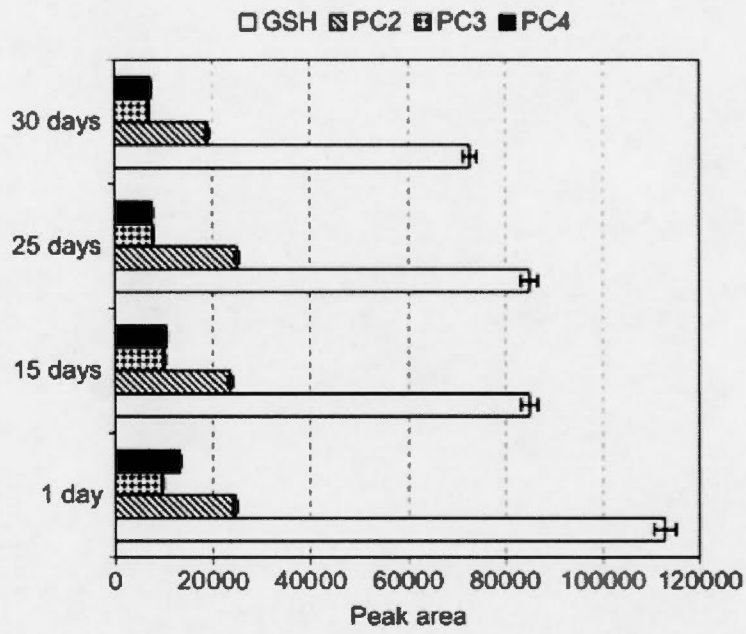


Figure 2.11 Stability of GSH and PCs in bimane form over 30 days (Kawakami, Gledhill, Achterberg, 2006).

2.8.4.2 Determination of matrix effect on the fluorescence signal

The matrix effect was evaluated by the addition of the standards phytochelatins to the algal samples not exposed to the metal. Indeed, in three replicates, 200 µg/ml of PCs were added into aliquots of 250 µl of supernatant of algal sample unexposed to metals. The samples were compared with three other replicates of the same concentration of PCs, but only in the buffer (HEPES, 200 mM, pH 8.2). The solutions were derivatized and analyzed by HPLC. Comparison of standards prepared in HEPES with those prepared in algal extract shows (Figure 2.12) that the matrix does not have an effect on standards. However, after follow-up observations, algae can sometimes produce PCs and especially PC_2 when they were stressed in response to exposure to intense light or sometimes in repetitive centrifugation. For this, we decided to prepare our standards in solutions of our algae control.

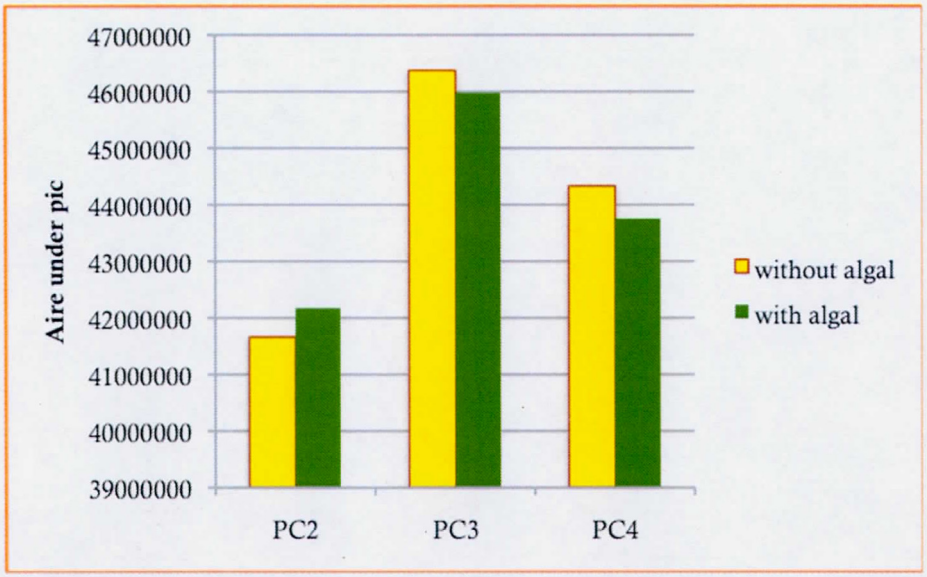
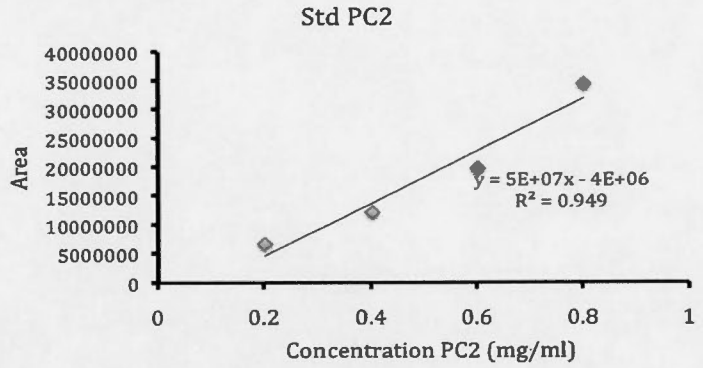


Figure 2.12 Matrix effect (algal) on standards PCs.

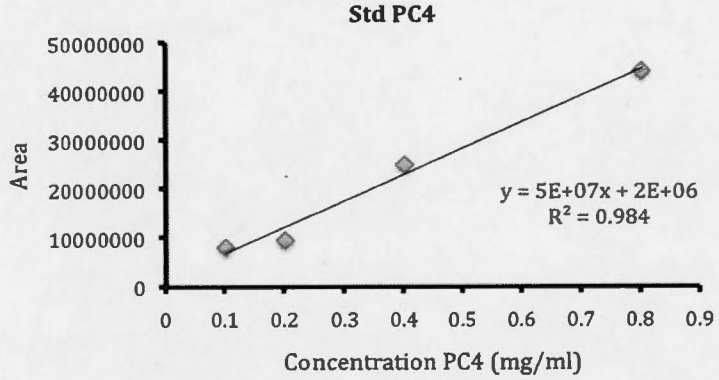
2.8.4.3. Calibration standard curves for phytochelatins

The calibration curve was prepared using different concentrations of PCs prepared from a stock solution of 1 mg/ml in BBM: 0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml. These solutions were derivatized and analyzed by HPLC. The assay was linear over the concentration range tested (Figure 2.13).

a)



b)



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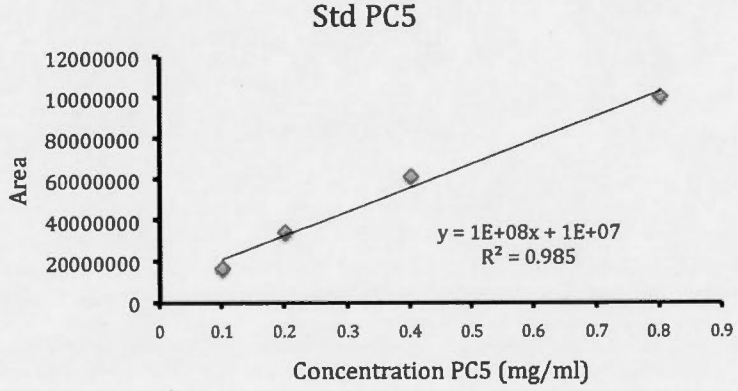
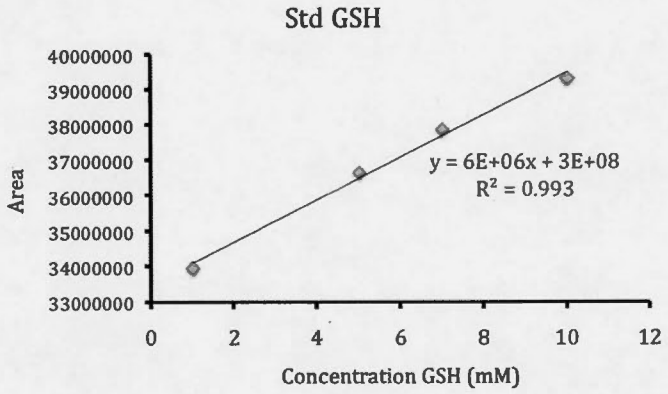


Figure 2.13 Calibration standard curve for a) PC2, b) PC4, c) PC5 performed by HPLC.

2.8.4.4 Calibration standard curves for glutathione and γ-Glutamine-Cysteine

The calibration curve for GSH was determined with different concentrations of standard prepared from a stock solution of 100 mM in BBM: 1, 5, 7 and 10 mM. The calibration curve for γ-Glutamine-Cysteine was determined with different concentrations of standard prepared from a stock solution of 100 mg/ml in BBM: 0.1, 1, 5 and 10 mg/ml (Fig.2.14).

a)



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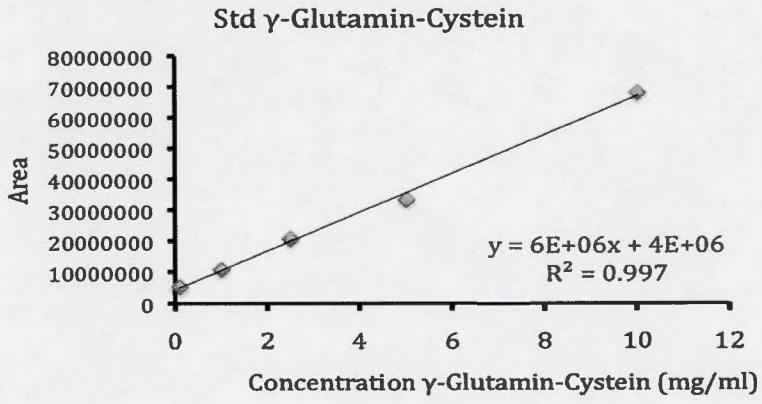


c)

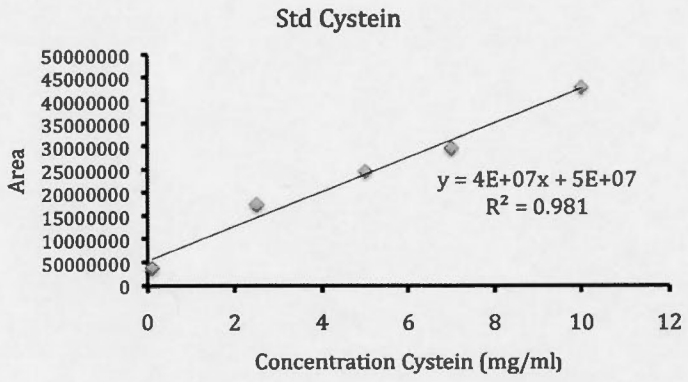


Figure 2.14 Standard calibration curve a) Glutathione (GSH), b) γ- Glutamin-cystein and c) cystein by HPLC.

2.9 Statistical analysis

All experiments were performed in triplicate. Means and standard deviations were calculated for each treatment condition. Significant differences between control and treated samples were determined by one-way analysis of variance (ANOVA) followed by a Dunnett's Multiple Comparison (DMC) test for $p < 0.05$.

CHAPTER IV
RESULTS AND DISCUSION

3.1 Growth-inhibitory effect of $ZnCl_2$ on *Chlorella vulgaris*

3.1.1 Growth inhibition of cell density

One of the objectives of this study was to determine the effect of various concentrations of $ZnCl_2$ on the cell growth of alga *C. vulgaris* during 72 hours of exposure by monitoring the change in cell density. Since it is well known that unicellular green algae are primary producers in the food chain of aquatic ecosystems, algal cells can easily uptake and bioaccumulate trace metals such as Zn. In this context, ecotoxicological studies use the inhibition of cellular division or growth as a reliable biomarker of the toxic action of bioaccumulated metals in algae by indicating the status of the metabolism or physiology of the cell (Toress *et al.*, 1997). Therefore, it was important to study the effect of Zn on cellular division in order to determine the tolerance range of alga *C. vulgaris* to the exposure of this metal.

The effect of $ZnCl_2$ at different concentrations (1-100 mM) on the change of cell density of *C. vulgaris* showed a significant decrease which was dependent to the increasing concentrations of the metal (Figure 3.1). The growth curve of *C. vulgaris* in the absence of $ZnCl_2$ represented the control. Algal growth curve of the control was greater than any of the growth curves of the experimental tested concentrations. Indeed, these results showed a change in cell density of *C. vulgaris* when exposed during 72 h to different concentrations of $ZnCl_2$ (1-100 mM). For algal cells treated to 1-10 mM $ZnCl_2$, a small significant decrease (10-15 %) relative to the control in cell density was observed at 72 h of exposure. Under this treatment condition, the color of the cells was light green compared to dark green for the control. Furthermore, algal cells were forming agglomerations when treated to 30 mM of $ZnCl_2$, indicating a stronger stressful effect of $ZnCl_2$ on cells during 72 h of exposure. Under

50 and 100 mM of $ZnCl_2$, a strong significant inhibitory effect was noticed on the change of cell density already at 24 h of exposure. For algal cells treated to 100 mM of $ZnCl_2$, cellular division were completely inhibited and white dead cells was noticed indicative of a strong cellular toxic effect. In comparison to the control, the rate of cellular division was 23, 14 and 1.4 % at respectively 24, 48 and 72 h of treatment exposure.

Thus, our data present a strong inhibitory effect of $ZnCl_2$ on cellular division of alga *C. vulgaris* already at 24 h for high concentrations (50 - 100 mM). The toxic effect of $ZnCl_2$ on the cellular division was previously demonstrated on different algal species. In one study, the effect of $ZnCl_2$ on marine diatom *Nitzschia closterium* was shown in relation to the increasing concentration of $ZnCl_2$ (20 -100 µM), and a strong decrease in cell division rate (% of control) was noticed over 4 days (Stauber and Florence, 1990). Therefore, based on our results and previous works, it appears that there is a difference in sensitivity to Zn depending to the algal species and the experimental conditions.

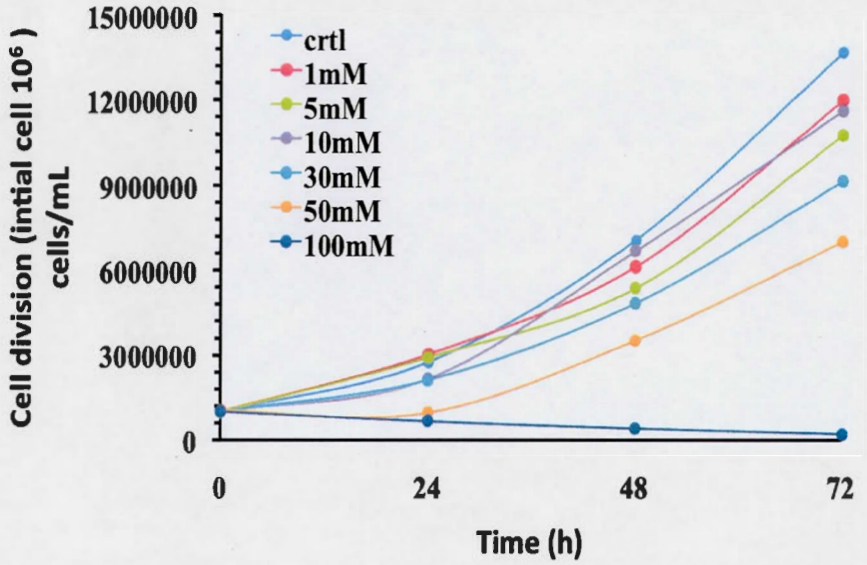


Figure 3.1 Change in the cell density of *Chlorella vulgaris* treated to $ZnCl_2$ at different concentrations (1-100 mM) during 72 h of exposure. For statistical analysis, see Material and Methods section.

3.1.2. Change in growth rate (μ)

When *C. vulgaris* was exposed to $ZnCl_2$ (1-100 mM) during 72 h, algal cell densities for each tested concentration were determined daily as cell numbers (cell ml^{-1}). As shown in Figure 3.2, the growth rate (μ) was calculated, and there was no significant effect of exposure concentrations from 1-30 mM of $ZnCl_2$ on the growth rate of *C. vulgaris* in comparison to the control. However, under the exposure to high concentrations of Zn (50 and 100 mM), there was a strong decrease on the growth rate based on cell density during 72 h. Although Zn is an essential metal for living organisms especially for enzymatic activities, it can be toxic at high concentrations causing cell death. In a previous study, the growth rate (μ) of *C. vulgaris* was monitored during 7 days under the exposure to different concentrations of Zn and Cd (0-80 μM). Authors showed that a stimulating effect of these metals on cell growth rate under the exposure to low concentrations (10, 20 and 40 μM), and under high metal concentrations, there was a strong inhibitory effect on cell growth rate (Huang *et al.*, 2009).

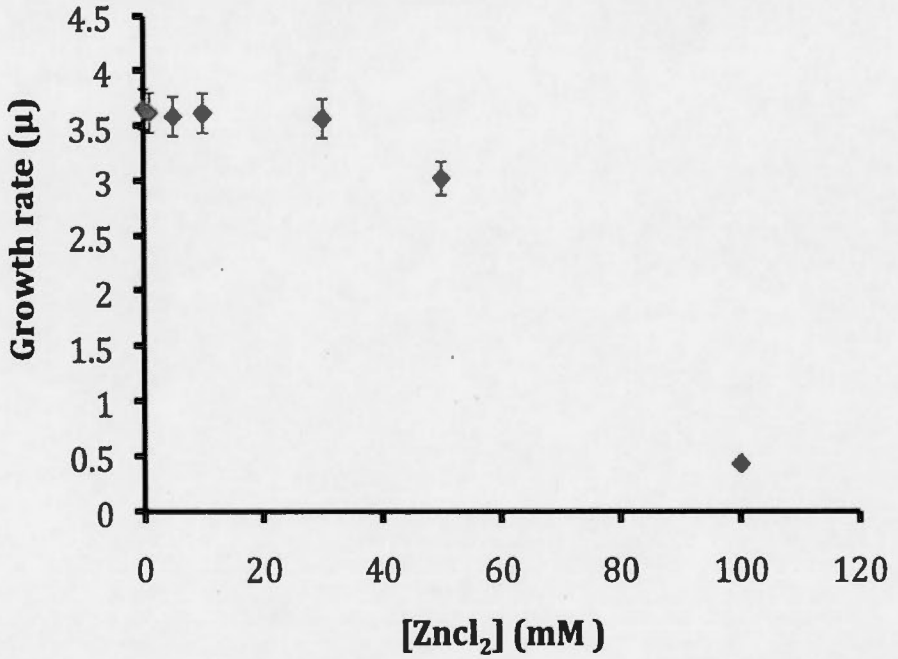


Figure 3.2 Change in growth rate (μ) of *Chlorella vulgaris* exposed during 72 h to different concentrations of $ZnCl_2$ (1-100 mM). The growth rate was calculated according this equation:

$\mu = (\ln N_{t2} - \ln N_{t1}) / (t_2 - t_1)$, where N_{t1} and N_{t2} represented measured initial and final cell densities, respectively. The t_1 and t_2 were the exposed times at initial and final measurement. For statistical analysis, see Material and Methods section.

3.2 Metal accumulation in biomass of *C. vulgaris*

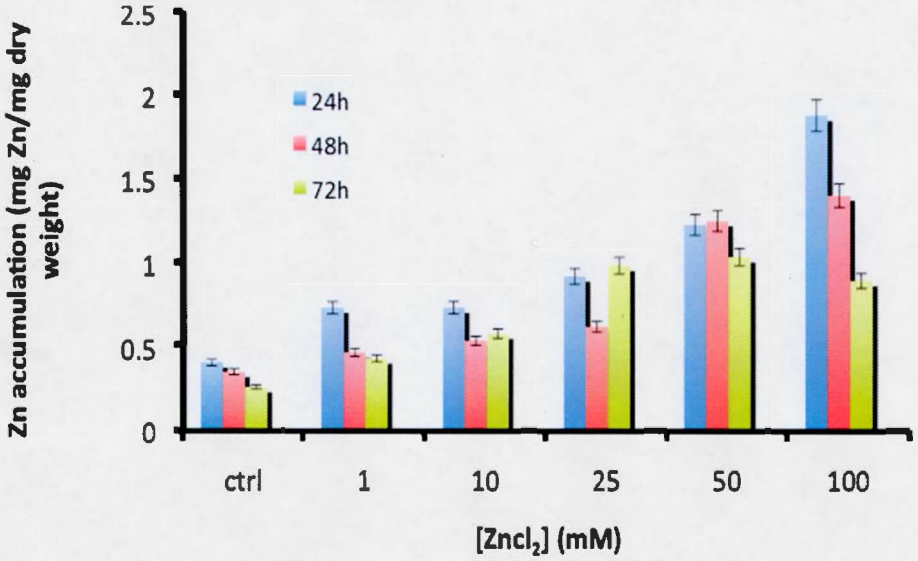


Figure 3.3 The Zn accumulation in algal biomass of *C. vulgaris* exposed during 72 h to various concentrations of $ZnCl_2$ (1-100 mM). Error bars represent standard errors obtained from three biological replicates. For statistical analysis, see Material and Methods section.

Green algae are known to bioaccumulate metals even under high concentrations, representing an important ecological process in the aquatic environment. The ability of microalgae to accumulate metals from aquatic solution is well known and documented (Wilde and Benemann, 1993; Sandau and Pluz, 1996). In algal cell, the overall process of metal uptake and accumulation was demonstrated into two phases: There is a first phase that is not related to cellular metabolism and where metal binds to the cell wall. There is a second phase dependent to transport and sequestration, where metal is accumulated inside the cell (Moreno-Garrido, Lubián and Soares, 2000). Figure 3.3 shows the Zn bioaccumulation in

algal biomass (ratio of metal concentration in mg / mg dry weight of biomass) in relation to the treatment of $ZnCl_2$ at different concentrations (1 - 100 mM) during 72 h of exposure. According to these results, the bioaccumulation of Zn in algal biomass increased significantly with increasing exposure concentrations from 25 mM of $ZnCl_2$ at 24 h. Under the exposure of 50 mM of $ZnCl_2$, the bioaccumulation efficiency increased significantly by about 3 times in comparison to control during 72 h. For the highest concentration of $ZnCl_2$ (100 mM), the bioaccumulation efficiency strongly increased significantly already at 24 h by 4.5 times in comparison to the control. However, there was at 72 h an important decrease in comparison to the other times of exposure. Therefore, these results indicated that the highest bioaccumulation efficiency of Zn in algal biomass was found under the exposure of 100 mM of $ZnCl_2$ at 24 h in comparison to 48 and 72 h. Indeed, it appears that after 24 h the efficiency of the uptake of Zn by algal cells decreased, which may probably be due to serious damages induced by bioaccumulated Zn inside the cellular system. In a previous study, the bioaccumulation of several metals such as Zn, Cu and Co ($10^{-6} - 10^{-9}$ M) by algal cells of *C. vulgaris* was investigated during 7 days, and the results indicated that the metal bioaccumulation capacity was directly related to the tested concentration in the culture medium. The order in the efficiency of bioaccumulation of metal was: $Cu^{+2} > Co^{+2} > Zn^{+2}$ (Afkar, Ababna and Fathi, 2010). Moreover, it was proposed that algal cells can uptake metals *via* two pathways, passively or actively. Several metals such as Pb and Sr are passively absorbed by charged polysaccharides through the cell wall and into the intracellular cytoplasm (El-sheekh *et al.*, 2003). In this study, our results showed that the cell wall may act as a controlled barrier for the uptake of Zn into algal cells.

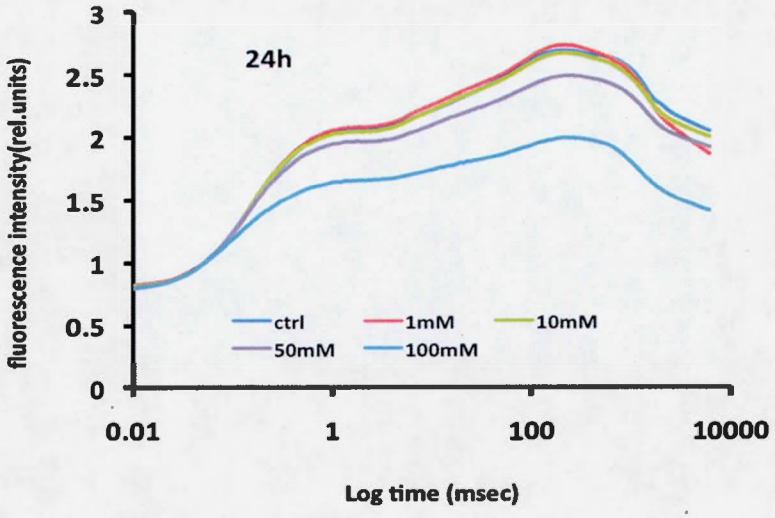
3.3 Photosynthesis-inhibitory effect on algal cells

3.3.1 Change in chlorophyll fluorescence kinetics

In this study, chlorophyll fluorescence was used to assess the toxicity of $ZnCl_2$ on the photosynthetic activity of *C. vulgaris* during 48 h. The bioaccumulation toxic effect of Zn for different exposure concentration of $ZnCl_2$ on the photochemical reactions of PSII is presented in Figure 4.4. The alteration of the photosynthetic activity was determined by the change in

the emission of chlorophyll fluorescence from algal cells. Indeed, the measurement of the rapid OJIP fluorescence emission indicative of the photosynthetic efficiency is a useful and effective method to determinate the physiological status of algal cells under metal stress effect. This method based on fluorescence induction measurements was established by Strasser and Strasser (1995), which permitted to get precise information about the toxic mode of action of pollutants. It was proposed that the fast rise of Chl fluorescence was indicative of the primary electron transport process in PSII complex, and when measured in dark-adapted algae, it was determined by 3 transients named J, I and P related to the reduction state of electron acceptors (Strasser, Srivastava and Tsimilli, 2004). Therefore, we use the chlorophyll fluorescence emission in this study since it represents an excellent indicator of the photosynthetic activity when algal species are exposed to the effect of xenobiotics or environmental stresses.

A)



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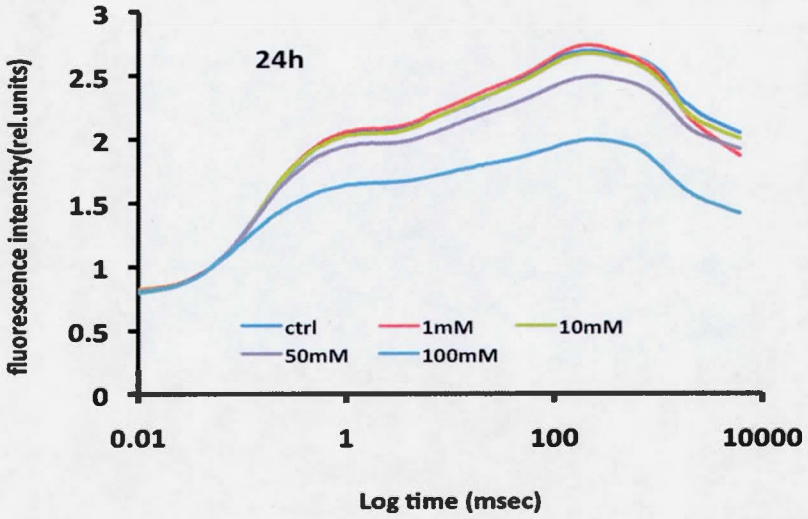

Figure 3.4 Fast rise of Chl fluorescence in alga *C. vulgaris* after 24 h (A) and 48 h (B) of treatment. Curve dark blue: Control; Curve red: 1 mM of $ZnCl_2$; Curve green: 10 mM of $ZnCl_2$; Curve purpel: 50 mM of $ZnCl_2$; Curve light bleu: 100 mM of $ZnCl_2$. For statistical analysis, see Material and Methods section.

These results showed that the intensity of fluorescence emission at transients J, I and P decreased in relation to the concentration of $ZnCl_2$. At 48 h, the intensity of the fluorescence induction curve showed a stronger reduction than at 24 h, which was dependent to the exposed concentration of $ZnCl_2$ (Figure 3.4). At 24 h, the inhibitory effect of Zn on PSII activity was seen only for the highest concentration of $ZnCl_2$ (100 mM). At 48 h, the alteration of PSII electron transport capacity was significantly noticed on algal cells treated from 10 mM of $ZnCl_2$. This interpretation is in agreement with earlier studies concerning the effect of metals on photosynthetic electron transport in green algae. In a previous study, the effect of Al (100-740 μM) was investigated during 24 h on the photosynthetic processes of alga *Chlamydomonas acidophila* (Perreault *et al.*, 2009). Results of this study showed that Al inhibited the PSII electron transport activity as indicated by the change of chlorophyll fluorescence emission, which decreased in relation to the increase exposure concentration of Al. Here, measurements of the rapid polyphasic kinetics of Chl *a* fluorescence with the Handy-PEA permitted a rapid and efficient analysis of the photosynthetic electron transport activity, which can be used as an indication of the toxic effects of metals on the plant physiological state and cell division. Indeed, the inhibition in photosynthetic activity may affect the synthesis of ATP and NADPH resulting in the alteration of the whole photosynthetic process and consequently cellular growth (see the introduction section). Therefore, we were able to use fluorescence measurements in order to determine the bioaccumulation effect of Zn on the PSII photochemistry and electron transport activity which provided an evaluation on the physiological state of algal cells.

3.3.2. Change in chlorophyll fluorescence parameters

From the rapid induction of chlorophyll fluorescence emission, it was possible to calculate several photosynthetic parameters indicative of the activity of PSII photochemical reactions (for more details, see "Material and Methods" section). The following fluorescence parameters were used: The relative variable fluorescence at J transient (V_J), the performance index of PSII activity (PI), the light absorption capacity of antenna complex per PSII reaction center (ABS/RC) and the maximal PSII quantum yield (F_V/F_M). These fluorescence parameters were monitored when algal cells were exposed during 48 h to different

concentrations of $ZnCl_2$. Obtained results permitted to determine the toxicity impact of bioaccumulated Zn on algal cell physiology, since these parameters were sensitive indicators of the changes in PSII activity (Dewez *et al.*, 2007).

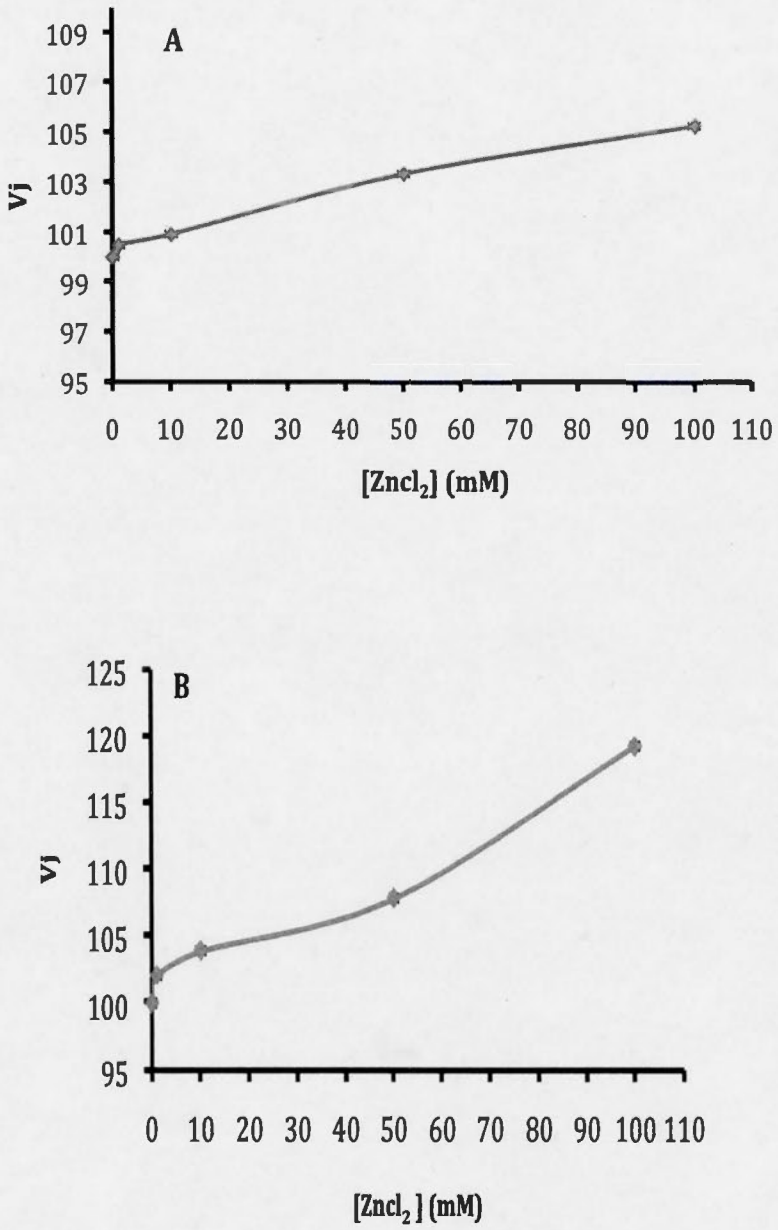

Figure 3.5 Change of fluorescence parameter V_J when algal cells of *C. vulgaris* were exposed to different concentrations of $ZnCl_2$ (1, 10, 50 and 100 mM). 24 h (A) and 48 h (B). Vertical bars show standard errors, n = 3. For statistical analysis, see Material and Methods section.

The relative value of variable fluorescence at J transient, V_J is known to indicate the accumulation of reduced PSII primary electron acceptor Q_A (induced by a saturating flash), and it was determined by $V_J = [(F_{2ms} - F_O) / (F_M - F_O)]$. The Figure 3.5 is showing the change of V_J value in relation to the exposure concentration of $ZnCl_2$ for both 24 and 48 h. At 24 h, there was not a significant difference between the control and treated algal cells. But at 48 h, we observed a strong significant increase of V_J value, especially for the highest concentration of $ZnCl_2$ (100 mM) in comparison to the control. This increase in V_J value indicated an inhibition in the reoxidation capacity of the PSII primary electron acceptor Q_A, affecting the electron transport flux beyond Q_A. Thus, the bioaccumulated Zn inside the cell did alter the electron transfer from Q_A to Q_B in the PSII system. In a previous study, the inhibitory effect of Okadaic acid was investigated on the physiology of alga *Dunaliella tertiolecta*. It was shown that V_J parameter value increased under the exposure of 0.5 µM of Okadaic acid during 72 h, and this change was related to an inhibition of the PSII electron transport activity (Perreault *et al.*, 2012). Therefore, our results indicated that the increase in V_J value is associated to the inhibitory effect of Zn on PSII electron transport activity.

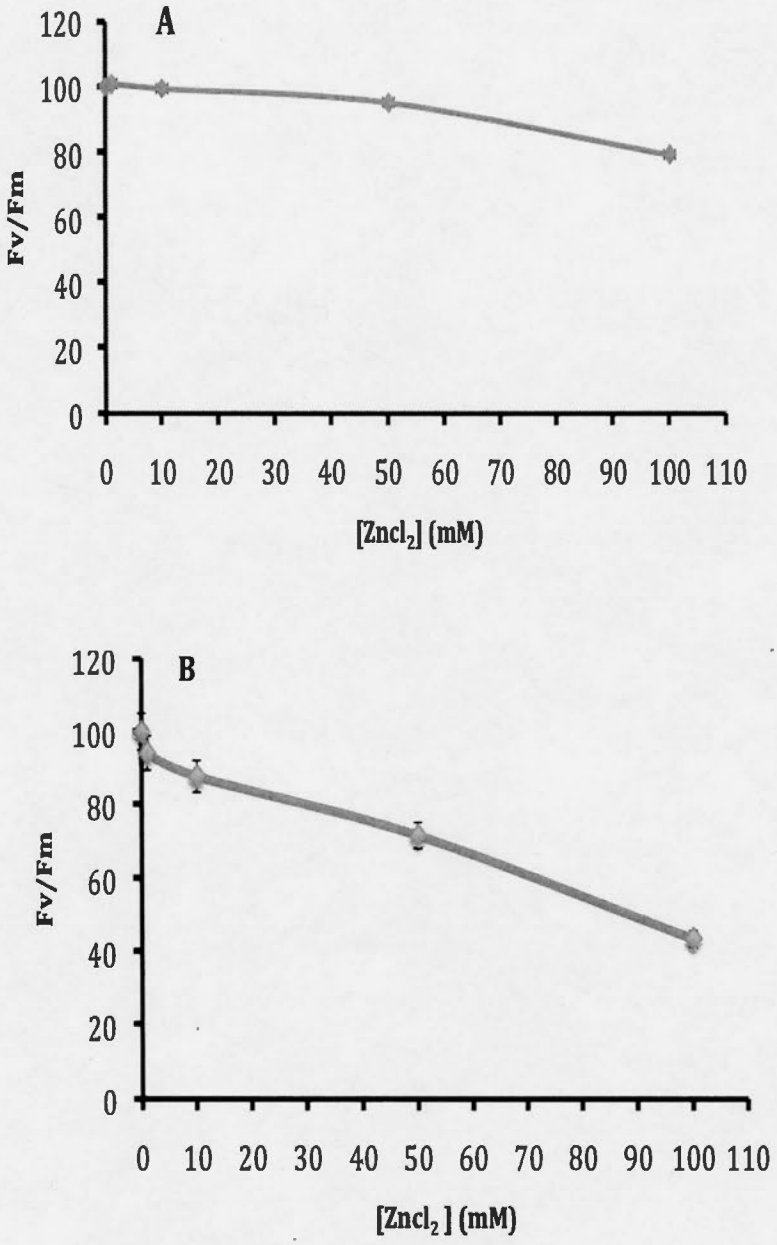

Figure 3.6 Change of the fluorescence parameter Fv/Fm when algal cells of *C. vulgaris* were exposed to different concentrations of $ZnCl_2$. 24 h (A) and 48 h (B). Vertical bars show standard errors, n=3. For statistical analysis, see Material and Methods section.

The effect of $ZnCl_2$ on the photochemical reactions of PSII was also monitored by the change of the maximum quantum yield of primary photochemistry, Fv/Fm. These results are shown in Figure 3.6, and we found that Fv/Fm value gradually decreased significantly especially when alga *C. vulgaris* was exposed for 48 h to $ZnCl_2$ from a concentration range of 10-100 mM. At 24 h of exposure, the Fv/Fm value decreased by 20 % in comparison to the control, only under 100 mM of $ZnCl_2$. This indicated a small toxicity effect of Zn on the capacity of PSII reaction center to convert absorbed light energy into charge separation and electron transport activity under this treatment condition. At 48 h, the inhibition of the photochemical efficiency of PSII (Fv/Fm value) was dependent to the exposure time and concentration of $ZnCl_2$. Under 100 mM of $ZnCl_2$, the decrease of Fv/Fm value was almost by 60 % compared to the control. Indeed, it was previously shown that metal toxicity can alter directly PSII functions, which can be identified by the change of the maximum PSII quantum yield, Fv/Fm. Also, it has been shown that the inhibition of the oxygen evolving complex by metals may lead to the loss of the photochemical activity of the reaction center of the PSII (Martin and Ort, 1982). Therefore, by causing structural and functional damages to the photosynthetic apparatus, metals effect may result in the inhibition of growth and cell death (Plenkhanov and Chemeris, 2003; Miao, Wang and Juneau, 2005). In a previous study, the effect of copper (0, 1, 5, 10 and 20 µM) was investigated during 24 h on alga *C. vulgaris*, and several fluorescence parameters related to PSII photochemistry were used (Oukarroum, Perreault and Popovic, 2012). It was shown that F_V/F_M was affected by Cu^{2+}, and the value of F_V/F_M decreased by 49 % compared to control under the exposure of 20 µM of Cu^{2+}, indicating a decrease in photochemical efficiency of photosynthetic electron transport. Therefore, our results indicat that the decrease in Fv/Fm value is due to the functional alteration of reaction centers of PSII caused by bioaccumulated Zn.

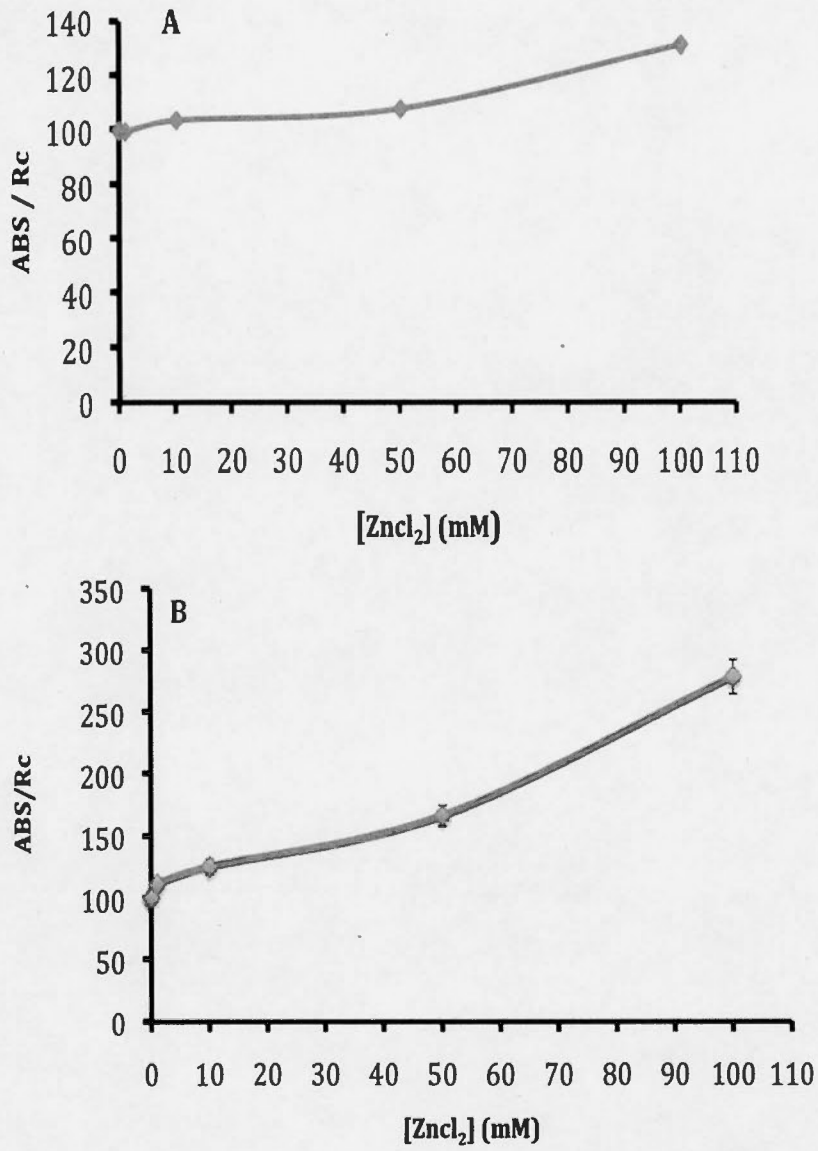

Figure 3.7 Change of fluorescence parameter ABS/RC when algal cells of *C. vulgaris* were exposed to different concentrations of $ZnCl_2$. 24 h (A) and 48 h (B). Vertical bars show standard errors, n=3. For statistical analysis, see Material and Methods section.

The efficiency of energy transfer by the light harvesting antenna complexes through reaction centers of PSII (ABS/RC) was also evaluated under similar experimental conditions.

The ABS/RC parameter provides an estimate of the number of photons absorbed by light harvesting antenna complexes (LHCII) relative to the functional amount of PSII reaction centers. The change in light-energy absorption of antenna complex per reaction center (ABS/RC) was investigated when algal cells of *C. vulgaris* were exposed during 48 h to different concentrations of $ZnCl_2$ (see Figure 3.7). Our results indicated that the $ZnCl_2$ has an inhibitory effect on the effective energy transfer from antenna complex to the RC of PSII which is related to the ratio of active/inactive RCs. Indeed, it has been shown that the changing of ABS/RC parameter value was indicative of the inactivation of PSII reaction centers, while the relative size of antenna complex remains unchanged. Therefore, the light harvesting antenna complex is transferring excitation energy to a smaller number of active PSII (Lecci and Lavergne, 1993; Strasser and Tsimilli-Michael, 1998).

When *C. vulgaris* was exposed to $ZnCl_2$ for 24 h, the ABS/RC parameter value increased gradually in relation to the exposure concentration of $ZnCl_2$ (Figure 3.7). At 24 h under high concentration of exposure (100 mM), this parameter value increased significantly by 35 % compared to the control. At 48 h of exposure, we noticed a strong significant increase in the ABS/RC value for the highest concentrations of $ZnCl_2$, 50 and 100 mM. Indeed, the ABS/RC value increased by 185 % compared to control under the exposure of 100 mM of $ZnCl_2$ at 48 h. Therefore, our results indicat that the bioaccumulated Zn inactivated PSII reaction centers in algal cells of *C. vulgaris*. In a previous work, the effect of Al (at 100, 188 and 740 µM) was investigated on the physiological processes of alga *C. acidophila* for 24 h, and results showed a direct relation between the change in ABS/RC value and the increase in Al exposure concentration. It was demonstrated that Al induced the inactivation of some active PSII reaction centers, causing an increase in thermal energy dissipation. This increase indicated lower capacity for PSII reaction centers to dissipate energy into variable fluorescence emission and photochemistry (Perreault *et al.*, 2010). Therefore, when the relative size of antenna complex increases per RC, it indicated an increase in the proportion of inactive PSII reaction centers.

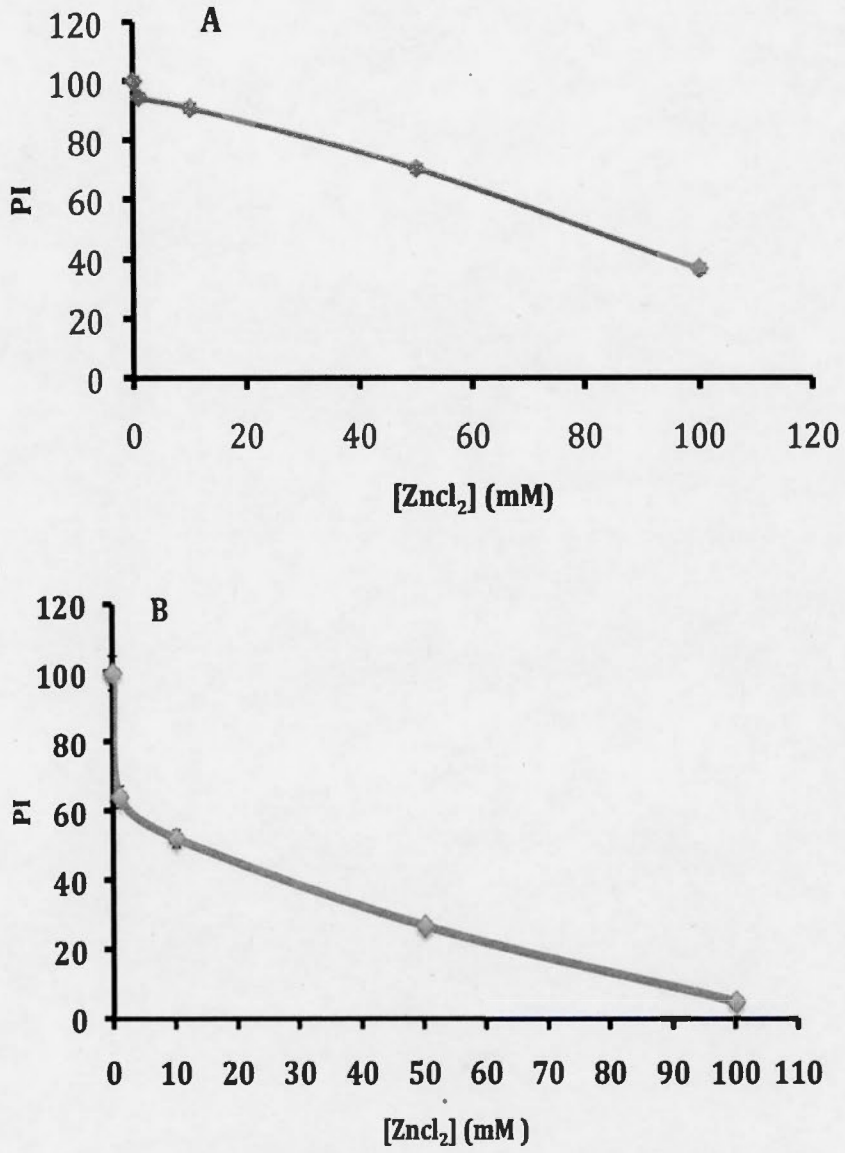

Figure 3.8 Change of fluorescence parameter PI when algal cells of *C. vulgaris* were exposed for 48 h to different concentrations of $ZnCl_2$. 24 h (A) and 48 h (B). Vertical bars show standard errors, n = 3. For statistical analysis, see Material and Methods section.

The Performance Index of PSII activity (PI) is one of the most powerful and important parameter in chlorophyll fluorescence measurements for plant cell physiological analysis. Indeed, it is a multiparameter integrating all PSII photochemical processes such as

the absorption, the trapping of excitation energy and the electron transport process (for more details, see material and methods). In this study, we monitored the change of fluorescence parameter PI value when algal cells of *C. vulgaris* were exposed during 48 h to different concentrations of $ZnCl_2$ (Figure 3.8). At 24 h, obtained results showed that, under the highest exposure concentration of $ZnCl_2$ (100 mM), the PI value decreased significantly by 60 % in comparison to control, while at 48 h, it decreased significantly by 90 % compared to control. Thus, this indicator of toxicity based on PSII photochemical reactions represented here a sensitive biomarker of Zn bioaccumulation effect on photosynthetic activity and the physiological state of algal cell. In an earlier study, the effect of chromate (50 µM) was investigated during ten days on the photosynthetic activity of alga *Spirodela polyrhiza.* The performance index of PSII activity (PI) was monitored which showed a decrease every day in relation to the chromate treatment concentration. The PI value decreased by 83 % compared to control for a treatment of 50 µM of chromate during 10 days, and it was demonstrated to be related to a strong inhibitory effect on PSII primary photochemistry and electron transport activity (Appenroth *et al.*, 2001).

3.3.3 Change in pigments content

The change of the total chlorophyll content was investigated when algal cells of *C. vulgaris* were exposed during 48 h to different concentrations of $ZnCl_2$. The results are shown in Figure 3.9, and presented significant differences in the total chlorophyll content (chlorophyll *a* and *b*) at both 24 and 48 h between control and treated samples from 10 mM of $ZnCl_2$. Especially at 48 h, we observed a decrease of the total chlorophyll content for treated samples compared to the control: 20 % for 1 mM, 40 % for 10 mM, 80 % for 50 mM and 90 % for the highest concentration of $ZnCl_2$ (100 mM). These results indicated an inhibitory effect of bioaccumulated Zn on chlorophyll synthesis which did cause indirectly an alteration of photosynthetic activity. This may explain in part the results obtained concerning the effect of $ZnCl_2$ on the PSII photochemical reactions, as indicated by the change of the fluorescence parameters such as Fv/Fm during 48 h.

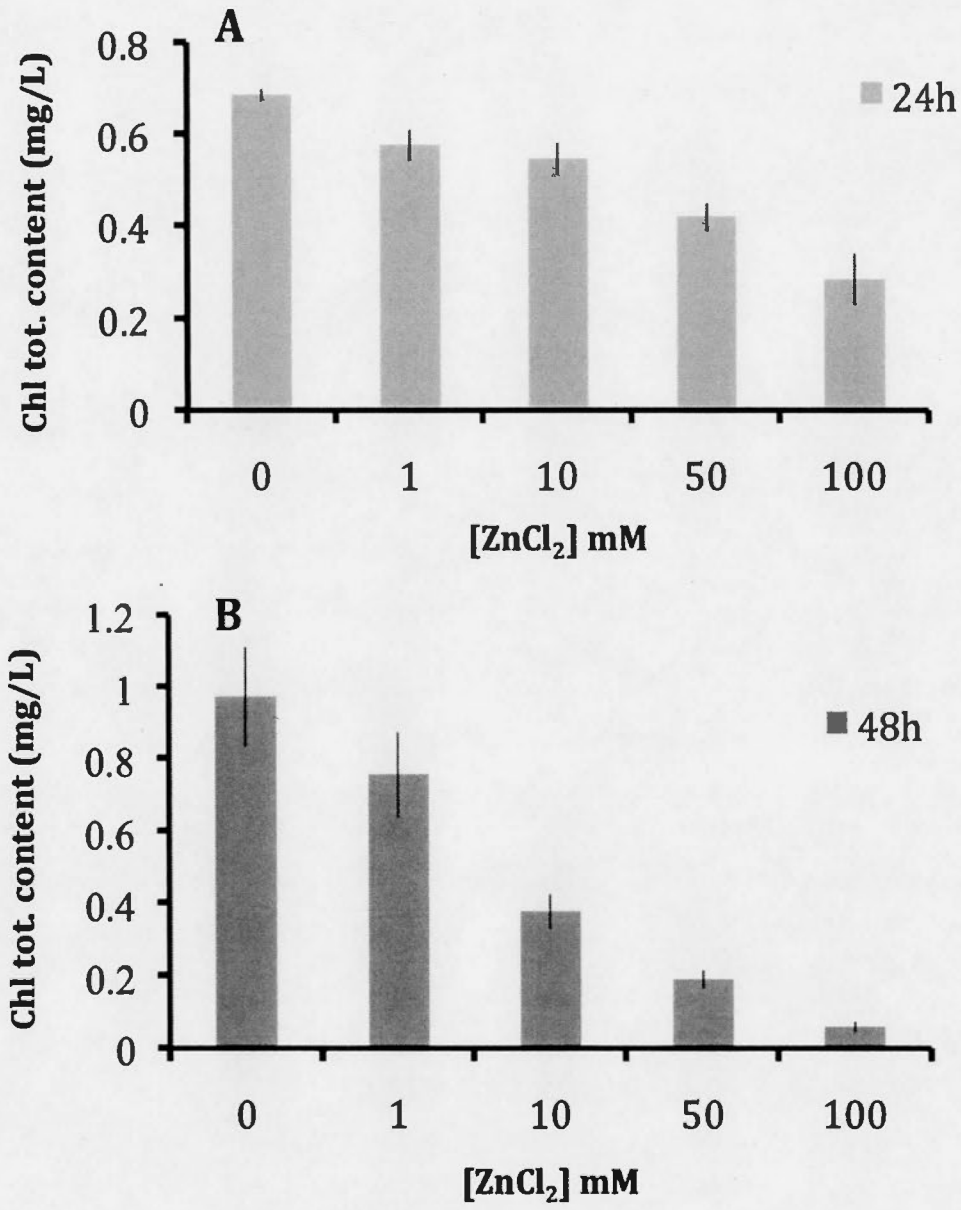

Figure 3.9 Change of the total chlorophyll content when algal cells of *C. vulgaris* were exposed for 48 h to different concentrations of $ZnCl_2$. 24 h (A) and 48 h (B). Vertical bars show standard errors, n = 3. For statistical analysis, see Material and Methods section.

In a previous study, it was reported that the exposure to low concentration of $ZnCl_2$ induced an increase on the total chlorophyll content in *Asterionella japonica* (Fisher and

Jones, 1981). However, under the exposure to high concentrations of this metal, the inhibition on the enzymatic activities involved in chlorophyll synthesis was noticed (Prassad and Prassad, 1987). Indeed, it was reported that the toxicity mechanism of $ZnCl_2$ was related to its binding to SH group affecting the structure of enzyme (De Filppis and Pallaghy, 1994; Afkar, Ababna and Fathi, 2010). In addition, Zn is known to not induce the formation of reactive oxygen species due to its redox inertness (Tripathi and Gaur, 2006). Therefore, the direct effect of Zn caused a decrease in total chlorophyll content dependent to the increasing concentration and exposure of $ZnCl_2$. This indicated that the bioaccumulation of Zn inhibited the biosynthesis of chlorophylls which affected the function of the photosystems light-harvesting energy transfer.

3.4. Synthesis of thiolated peptides

Thiolated peptides are intracellular metal ligands produced by algal cells in response to the bioaccumulation of metals upon the exposure to high metal concentration in freshwater ecosystems (Cobbett, 2000). For example, the GSH is produced in algal cells since this compound participates in the sequestration and trafficking of metals in order to maintain cellular homeostasis (see the introduction section). In this study, we investigated the induction of thiolated peptides in relation to the exposure to different concentrations of $ZnCl_2$. Cystein (Cys), gamma-glutamylcystein (γ-GluCys), glutathione (GSH) and phytochelatins (PC_n) contents were determined in algal biomass of *C. vulgaris* when exposed during 24 and 48 h to low (10 mM) and high (100 mM) concentrations of $ZnCl_2$. Indeed, the production of GSH and PCs has been investigated for many phytoplankton species exposed to metals under laboratory culturing conditions (Ahner and Morel, 1995; Ahner, Muffett and Morel, 1997; Le Faucheur, Behra and Sigg, 2004; Lavoie *et al.*, 2009). These previous studies showed that the induction of PCs could be used as a sensitive tool to indicate the level of stress effect caused by the exposure to metals.

In Figure 3.10, the change in glutathione (GSH) content in algal biomass is shown for *C. vulgaris* exposed during 24 h to low (10 mM) and high (100 mM) concentrations of $ZnCl_2$. In comparison to control, the GSH content increased significantly for both treatment

conditions, but it was dependent on the concentration of $ZnCl_2$. Under high treatment concentration (100 mM of $ZnCl_2$), a maximum induction of GSH was reached at 24 h, which was related to the highest bioaccumulation efficiency of Zn. However at 48 h, there were no significant differences between treated samples (10 mM of $ZnCl_2$) and control, and there was a decrease in glutathione content for samples treated to 100 mM of $ZnCl_2$. In a previous study, the glutathione content was investigated on *Scenedesmus vacuolatus* exposed to $ZnCl_2$ (1×10^{-9} to 1×10^{-7} M). The increase of free Zn^{+2} concentrations from 6×10^{-12} M in the control to 1×10^{-7} M in the Zn treated media caused a significant increase in GSH content in comparison to control. It was demonstrated that GSH was involved in different cellular processes such as the synthesis of proteins and nucleic acids and also the modulation of enzymatic activities (Le Faucheur *et al.*, 2006). Therefore, the production of thiols content depends on many factors such as the species of algae and the experimental condition used.

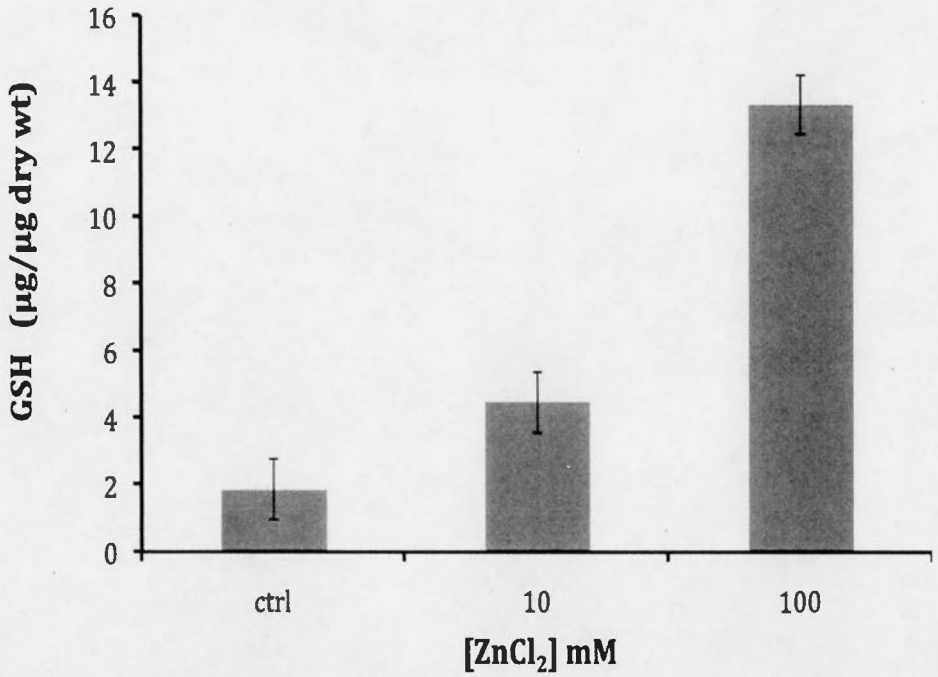

Figure 3.10 Glutathione (GSH) content in algal biomass of *C. vulgaris* exposed 24 h to low (10 mM) and high (100 mM) concentrations of $ZnCl_2$. Vertical bars indicated standards error, n = 3. For statistical analysis, see Material and Methods section.

Moreover, it is well known that the concentrations of precursors of GSH (γ-GluCys and Cys) usually increased significantly in algal cultures in response to metal exposure in order to regulate the synthesis of GSH and PCs (Wei *et al.*, 2003; Kawakani, Martha and Achterberg, 2006). The Figure 3.11 show the contents of γ-glutamylcystein (γ-GluCys) and cystein (Cys) in algal biomass of *C. vulgaris* when exposed during 24 h to low (10 mM) and high (100 mM) concentration of $ZnCl_2$. The content of γ-GluCys determined in control was of 0.05 mg/mg of dry weight, and it increased significantly only under high treatment concentration of $ZnCl_2$ (100 mM) to 0.39 mg/mg of dry weight of biomass. It is interesting to notice that the change in GSH and γ-GluCys content did not respond similarly to the exposure of $ZnCl_2$ (compare Figure 3.10 and 3.11). Moreover, the content of cystein in algal biomass was determined for *C. vulgaris* exposed during 24 h to low (10 mM) and high (100 mM) concentrations of $ZnCl_2$. (Figure 4.11). However, statistical analysis of these results indicated that the content of cystein did not increase significantly in comparison to the

control under these treatment conditions. Therefore, only the content of γ-GluCys increased significantly under the highest concentration of $ZnCl_2$ (100 mM), which was related to the maximum bioaccumulation efficiency of algal cells.

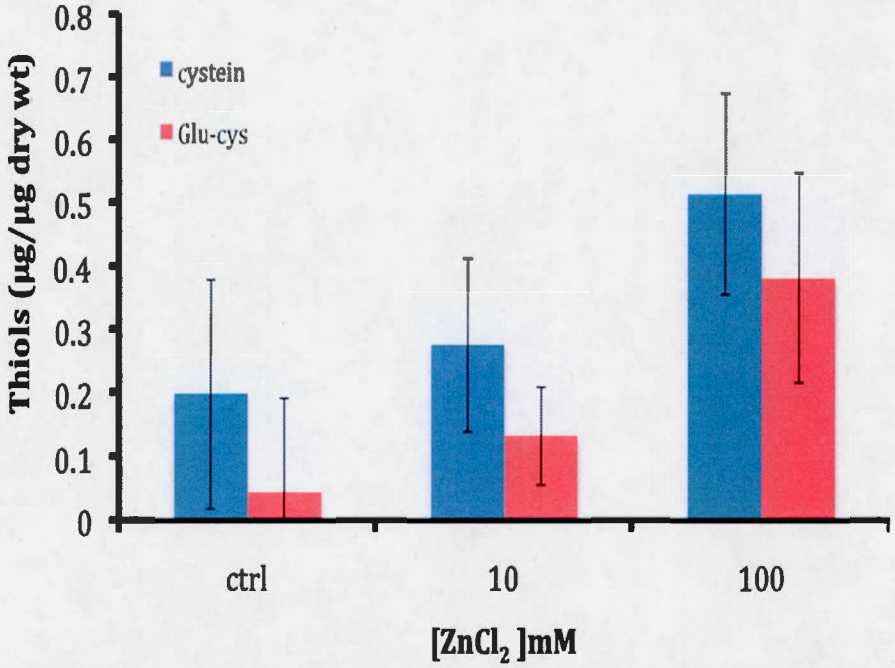

Figure 3.11 Contents of y-glutamylcystein (γ-GluCys) and cystein (Cys) in algal biomass of *C. vulgaris* exposed 24 h to low (10 mM) and high (100 mM) concentrations of $ZnCl_2$. Vertical bars indicated standards error, n = 3. For statistical analysis, see Material and Methods section.

Previously, Rijstenbil and Wijnholds (1996) reported a strong increase of the cysteine pool in alga *Thalassiosira pseudonana* after copper exposure. In a more recent study, the induction of thiols group was investigated on alga *D. tertiolecta* exposed during 24 h to 0-600 µM of $ZnCl_2$, and results showed that the level of GSH did not change in response to Zn exposure while phytochelatins did increased. Indeed, contents of Cys and γ-GluCys in treated samples were lower than those in control sample (Naoki Tsuji *et al.*, 2002). However,

we observed different responses in the contents of GSH, y-GluCys and Cys to the exposure of $ZnCl_2$, indicating that the change in these thiols group contents was dependent to the algal species and the experimental conditions.

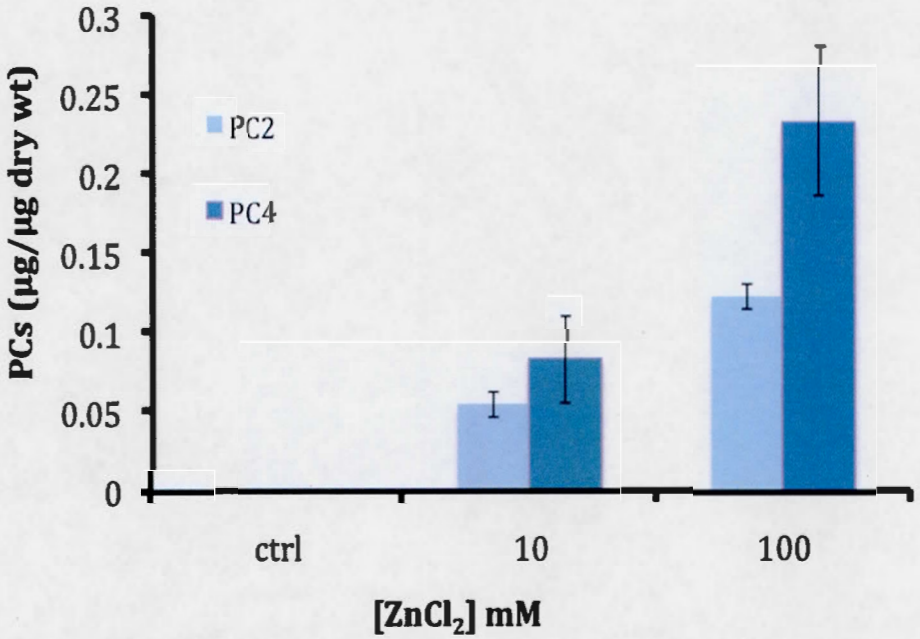

Figure 3.12 Phytochelatins (PC_2 and PC_4) content in algal biomass of *C. vulgaris* exposed 24 h to low (10 mM) and high (100 mM) concentrations of $ZnCl_2$. Vertical bars indicated standards error, n = 3. For statistical analysis, see Material and Methods section.

According to our results, the induction of GSH reached the highest content under the highest treatment concentration of $ZnCl_2$ (100 mM) at 24 h of exposure permitting the most efficient bioaccumulation of Zn by algal cells. Therefore, under this condition, it is evident that GSH was involved into the synthesis of PCs in response to bioaccumulated Zn inside the cells. Therefore, the content of phytochelatins, PC_2 and PC_4, in algal biomass was determined on *C. vulgaris* exposed during 24 h to low (10 mM) and high (100 mM) concentrations of $ZnCl_2$ (Figure 3.12). No PCs were found in the control sample, indicating that the concentration of Zn inside the cell was insufficient to induce the synthesis of PC. Obtained

results indicated that the content of phytochelatins increased with the increasing exposure concentration of $ZnCl_2$. The content of PC_2 under the highest exposure concentration of $ZnCl_2$ (100 mM) increased significantly by 2 fold compared to the lowest concentration (10 mM). The same result was observed for PC_4 content under the highest exposure concentration of $ZnCl_2$, which increased significantly by 3 fold (from 0.24 to 0.08 μg/μg of dry weight) in comparison to the lowest exposure concentration. Therefore, these results showed that 100 mM of $ZnCl_2$ induced a high level of PC synthesis which was related to the high induction of GSH synthesis.

In an earlier study, the unicellular green alga *Scenedesmus vacuolatus* was exposed to several trace metals and its content in thiols (gamma-glutamylcystein, glutathione and phytochelatins) was examined (Le Faucheur *et al.*, 2006). The glutathione content decreased upon the exposure to Zn (1×10^{-9} and 1×10^{-7} M) in comparison to Pb and Ni (6×10^{-8} and 2×10^{-10} M), respectively. However, the exposure to Zn or Ni did not induce any production of phytochelatins over the test concentration range. It was suggested that the dynamic in thiol content indicated its involvement in many cellular processes, including redox buffering, synthesis of proteins and nucleic acids and the modulation of enzymatic activities (Fathey *et al.*, 1987; Renneberg and Lamoureux, 1990). It was shown that the GSH synthesis was produced by different cellular demands and its homeostasis is controlled at many levels (Xing and Oliver, 1998). It was shown that the exposure to $ZnCl_2$ decreased the GSH content in green algal cells (Le Faucheur *et al.*, 2005), whereas it was found an increase in another species of marine algae (Stauber and Florence, 1990; Malea *et al.*, 2006). In a previous study, it was shown that the phytochelatin synthesis was induced by Zn^{+2} and Cd^{+2} in alga *D. tertiolecta*, which was exposed to a concentration range of 0-600 μM. The PC synthesis was strongly induced by Cd^{+2} at 24 h and this metal was the most effective activator of PC synthesis in comparison to Zn (Naoki Tsuji *et al.*, 2002). Therefore, by comparing our results with previous works, it is most likely that the induction of PCs synthesis in response to metals exposure will depend on the metal species, the algal specie and the exposure conditions.

3.5 Discussion

The main objective of this Master work was to investigate the bioaccumulation effects of Zn on the algal cell physiology of *C. vulgaris* by monitoring several cellular parameters, such as the cell division, the bioaccumulation efficiency of Zn in algal biomass, the photochemical efficiency of photosynthesis (by the activity of PSII photochemistry) and also the synthesis of glutathione and phytochelatins as protective molecular mechanisms against cellular metal toxicity. The synthesis of our obtained results concerning the change of these cellular parameters is summarized and presented in the table 3.1. The variation of these parameters permitted to explain the highest efficiency of Zn bioaccumulation and to compare treatment conditions at 24 h and 48 h. Based on our obtained results, we found in this study that the highest efficiency of Zn bioaccumulation by algal cells of *C. vulgaris* was under the treatment condition of 100 mM of $ZnCl_2$ during 24 h (Table 3.1). Under this experimental condition, the bioaccumulation of Zn was five times more compared to control. The cell division decreased only by 25 % compared control. The inhibition of photosynthesis, related to PSII primary photochemistry and electron transport activity, decreased only by 60 % compared to control. Moreover, the induction of GSH content in algal cells was 6 times more compared to control. The synthesis of phytochelatins was highly induced where the PC_4 increased twice more in comparison to PC_2. Under this condition, the synthesis of PCs, produced from GSH, was important for the cellular detoxification of Zn in algal cells. Particularly, the formation of high molecular weight phytochelatin-Zn complexes permitted the efficient sequestration of Zn into the cellular vacuole. Therefore, the induction in the synthesis of glutathione and phytochelatins was able to limit the toxicity impact of Zn into the algal cellular system, as indicated by the change of the cellular division and the PSII photochemical activity. These results explain the level of tolerance of *C. vulgaris* against the bioaccumulation effect of Zn at 24 h.

However, results concerning the variation of these cellular parameters were different at 48 h in comparison to 24 h under the treatment condition of 100 mM of $ZnCl_2$. At 48 h of exposure, the efficiency was lower concerning the bioaccumulation capacity of Zn in algal biomass, 1.5 times less in comparison to 24 h (Table 3.1). Indeed, the inhibitory effects on cell division was stronger (a decrease of 50 % compared to control) and the photochemical

activity of photosynthesis was inhibited by 90 % compared to control which was due to the toxicity impact of bioaccumulated Zn. Under this condition, the induction of GSH decreased by 1.2 % compared to control, and we did not detect any PCs. This may indicate that the protective molecular mechanisms involving GSH and PCs were not induced against cellular Zn toxicity. Moreover, it is most likely that the Zn toxicity on alga cell physiology caused severe damages which may affect cellular metabolism and the synthesis of phytochelatins. Therefore, these results demonstrated that bioaccumulated Zn induced a much stronger toxicity impact in algal cells at 48 h in comparison to 24 h of exposure. This cellular toxicity impact can explain the increasing number of dead cells, a stronger inhibitory effect on photosynthesis, a lower uptake and sequestration capacity of Zn by algal cells at 48 h of exposure. Therefore, the change of used cellular parameters during 48 h was related to the efficiency of the bioaccumulation of Zn in algal biomass, which reached the highest level at 24 h of exposure and decreased from at 48 h.

Since it is well known that algal cells are able to bioaccumulate metals by controlling the uptake, the transport and the sequestration of metal inside the cell (Yun and Wang, 2012), it was of high interest to determine the bioaccumulation efficiency of *C. vulgaris* as a phycoremediation approach permitting the removal of Zn from wastewater. In this work, the growth and physiology of *C. vulgaris* was inhibited at higher exposure levels of $ZnCl_2$, affecting the bioaccumulation efficiency of algal cells. Since bioaccumulated Zn was not toxic at low level, high amount was needed to cause an inhibitory effect on algal physiology. The experimental exposure of algal cells over a wide range of $ZnCl_2$ concentrations (1-100 mM) permitted to indicate the tolerance and sensitivity of alga *C. vulgaris* to $ZnCl_2$. Therefore, the inhibitory effect of bioaccumulated Zn on cellular division and photochemical activity of PSII was highly dependent to the exposure metal concentration. Our results are in accordance with previous studies concerning the Zn effects on algal physiology (Anderson and Morel, 1978; Buitenhuis, Timmermans and de Baar, 2003). While the interaction of metals are well known due to additive, synergistic or antagonistic effects on their toxicity mechanisms (Norwood *et al.*, 2003), it is less known how biological factors may affect their toxicity, such as the induction of phytochelatins (Wei *et al.*, 2003), transcriptional responses (Hutchins *et al.*, 2010) and the induction of cellular oxidative stress (Israr *et al.*, 2011). Moreover, our results are in accordance with previous studies concerning the induction of

phytochelatins on algal cells of *C. vulgaris* (Hirata *et al.*, 2001). In contrast to the results obtained by Hassler, Behra and Wilkinson (2005), the induction of PCs on algal cells of *C. reinhardtii* was not observed following short term exposures to Pb. Therefore, the amounts of PCs that are synthesized depend on the algal species, the metal and the experimental conditions (Rijstenbil and Wijnholds, 1996; Anher and Morel, 1995).

Table 3.1 Change of investigated cellular parameters when *C. vulgaris* was exposed at 24 and 48 h to 100 mM of $ZnCl_2$.

Cellular parameters	**100 mM of $ZnCl_2$ 24 h**	**100 mM of $ZnCl_2$ 48 h**
Bioaccumulation efficiency	↗ 5× compared to control	↗ 3.5 × compared to control
Division of cells (cells/ml)	↘ 25 % compared to control	↘ 50 % compared to control
PSII photochemistry of photosynthesis	↘ 60% compared to control	↘ 90 % compared to control
Induction of GSH	↗ 6 × compared to control	↘ 1.2 × compared to control
Induction of PC_2 and PC_4	2 × PC_4 compared to PC_2	No PCs

3.6 Conclusion

In order to determine the maximum capacity of algal cells of *C. vulgaris* to accumulate Zn, the bioaccumulation efficiency was investigated by estimating the content of Zn in algal biomass when *C. vulgaris* was exposed during 72 h to different concentrations of $ZnCl_2$. Furthermore, the characterization of the cellular toxicity impact induced by the bioaccumulation effect of Zn was determined by monitoring several cell physiological parameters which permitted to better explain the bioaccumulation efficiency of Zn in algal cells. According to our obtained results, the highest efficiency of bioaccumulation was reached for the highest concentration of $ZnCl_2$ (100 mM) at 24 h of exposure time. The use of growth and photosynthesis parameters were indicative of the alteration of the physiological processes of algal cells affected by $ZnCl_2$ exposure. Thus, these parameters were reliable indicators of this metal toxicity impact inside the cell. Moreover, our results showed that, to maintain optimal bioaccumulation efficiency, the induction of thiols group (glutathione and phytochelatins) was important for algal cells in order to avoid the inhibitory effects of Zn. In conclusion, results of this study permitted to determine the bioaccumulation efficiency of *C. vulgaris* for the aqueous removal of Zn. The condition limit for using this algal species for removing Zn is under the exposure up to 100 mM of Zn during 24 h. However, future works need to focus on the determination of the bioaccumulation efficiency for others metals (Cd, Pb) and algal species. Since laboratory scale showed some limitations, more phycoremediation studies must be performed at the industrial level for wastewater treatment at larger scales.

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