UNIVERSITÉ DU QUÉBEC À MONTRÉAL

BIOACCUMULATION EFFECT OF MERCURY ON THE GREEN ALGA *CHLORELLA VULGARIS*

THESIS

PRESENTED

AS PARTIAL REQUIREMENT

FOR THE MASTER IN CHEMISTRY

BY

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JANUARY 2015

UNIVERSITÉ DU QUÉBEC À MONTRÉAL

L'EFFET DE LA BIOACCUMULATION DU MERCURE CHEZ L'ALGUE VERTE *CHLORELLA VULGARIS*

MÉMOIRE

PRÉSENTÉ

COMME EXIGENCE PARTIELLE

DE LA MAÎTRISE EN CHIMIE

PAR

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JANVIER 2015

ACKNOWLEDGMENTS

During this Master, I gained experience which gave me scientific professional knowledge and I would like to thank many people who supported me throughout my study.

I greatly thank my Supervisor D. Dewez for his support and advices which permitted the realization of my research project. His international expertise in the domain of Environmental toxicology and chemistry related to green algae allowed me to achieve my Master's studies.

I would like to thank my colleagues of the laboratory, and a big thank to Mrs Sonia Lachance for her assistance.

Finally, I would like to thank all my family, who encouraged me until the end of my studies, and finally I thank all my friends.

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LIST OF ABREVIATIONS

A_o	A special molecule of chlorophyll *a*
AAS	Atomic Absorption Spectrometry
ABS/RC	Absorption of light energy by antenna complex per reaction center
DNA	Deoxyribonucleic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
COE	Complex of oxygen evolution
Chl_a	Chlorophyll *a*
Chl_b	Chlorophyll *b*
Chl^*	Chlorophyll in excited state
Cys	Cysteine
EDTA	Ethylene diamine tetraacetic acid
ROS	Reactive oxygen species
F_d	Ferredoxin
Glu	Glutamine
Gly	Glycine
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GS	Glutathione Synthase
HMW	High Molecular Weight complex
HSM	High Salt Medium
KDa	Kilodalton

LED	Light Emitting Diode
LHC	Light Harvesting Complex
LMW	Low Molecular Weight complex
NADP	Nicotinamide adenine dinucleotide phosphate in oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate in reduced form
P680	Reaction center of PSII
PC	Phytochelatin
PEA	Plant efficiency analyzer fluorimeter
PI	Performance index of PSII
PQ	Plastoquinone in the oxidized state
PQH_2	Plastoquinone in the reduced state (Plastoquinol)
PSI	Photosystem I
PSII	Photosystem II
Q_A	Quinone A, primary acceptor of PSII electron
Q_B	Quinone B, secondary acceptor of PSII electron
RC	Reaction Center
SH	Thiol Group
TEM	Transmission Electron Microscopy

RÉSUMÉ

Le mercure (Hg) représente un dangereux polluant aquatique en raison de sa libération importante dans les eaux usées industrielles. En milieu aquatique, le Hg^{2+} sera converti en méthylmercure (MeHg) par le biais de l'activité des micro-organismes. Ensuite, la bioaccumulation de MeHg se produira dans les organismes aquatiques par l'intermédiaire de la chaîne alimentaire, et enfin, il peut être transféré à l'humain causant des problèmes de santé graves. La technologie de phycoremediation est une nouvelle méthode en développement utilisant les microalgues pour l'assainissement des eaux usées contenant du mercure. L'objectif de ce projet est de déterminer la capacité de bioaccumulation du mercure dans la biomasse algale de *Chlorella vulgaris* exposée aux différentes concentrations de $HgCl_2$ pendant 72 h. Les effets de la bioaccumulation du mercure sur la division cellulaire et l'activité du photosystème II (PSII) ont aussi été examinées en ce qui concerne l'induction du glutathion (GSH) et des phytochélatines (PCs). Les méthodes d'analyse utilisées dans cette étude étaient la spectrométrie d'absorption atomique pour déterminer l'accumulation de mercure dans la biomasse algale, le compteur de cellules pour évaluer l'inhibition de la division cellulaire, l'émission de fluorescence de la chlorophylle pour analyser l'activité du PSII et enfin la chromatographie liquide de haute performance (HPLC) pour déterminer l'induction du GSH et des PCs. Nos résultats indiquent que la plus grande efficacité de bioaccumulation a été atteinte lorsque les cellules d'algues ont été exposées pendant 24 h à 100 µM de $HgCl_2$. Sous cette condition, la division cellulaire et l'activité du PSII ont diminué respectivement de 32 et 30 % par rapport au témoin. En effet, l'effet inhibiteur du mercure sur la photosynthèse peut expliquer la diminution de la division cellulaire. Dans les mêmes conditions de traitement, l'induction du GSH et des PCs augmente aussi considérablement à 24 h. Cela explique la tolérance des cellules algales contre les effets de la bioaccumulation du mercure montrant l'importance de ce mécanisme de détoxication moléculaire. De plus, nos résultats démontrent que le $HgCl_2$ induit un impact plus fort de toxicité dans les cellules d'algues à 48 h par rapport à 24 h d'exposition, comme en témoigne le nombre croissant de cellules endommagées ou mortes, expliquant l'absorption inférieure de mercure par les cellules d'algues après 24 h d'exposition. En effet, après 48 h d'exposition, l'induction de PC_2 et PC_4 n'a pas

été détectée en raison d'un plus fort effet toxique de mercure provoquant des dommages sur le système cellulaire plus importants affectant les activités enzymatiques. Par conséquent, nos résultats indiquent que l'activité du PSII a été utilisée comme un biomarqueur fiable des effets de la bioaccumulation du mercure au niveau cellulaire, qui était indicateur de l'efficacité de la bioaccumulation du mercure dans la biomasse algale. En conclusion, selon les résultats obtenus dans cette étude, il est démontré que cette espèce d'algue verte, *C. vulgaris* a été assez résistante à l'effet du $HgCl_2$ jusqu'à 100 µM pendant 24 h d'exposition tout en maintenant son efficacité maximale de bioaccumulation.

ABSTRACT

Mercury (Hg) represents a dangerous aquatic pollutant due to its important release from industrial wastewater. In aquatic environment, inorganic Hg^{2+} will be converted into methylmercury (MeHg) through the activity of microorganisms. Then, the bioaccumulation of MeHg will occur in aquatic organisms through the food chain, and finally it can be transferred to human causing serious health problems. A new developing method using microalgae permitting the remediation of wastewater containing mercury is the technology of phycoremediation. The objective of this project is to determine the bioaccumulation capacity of Hg in algal biomass of *Chlorella vulgaris* exposed to different concentrations of $HgCl_2$ during 72 h. the bioaccumulation effects of Hg on cellular division and Photosystem II (PSII) activity were also investigated in relation to the induction of glutathione (GSH) and phytochelatins (PCs). Analytical methods used in this study were Atomic Absorption Spectrometry to determine mercury accumulation in algal biomass, cells counter to evaluate the inhibition of cellular division, chlorophyll fluorescence emission to investigate PSII activity and High performance liquid chromatograpy (HPLC) to determine the induction of glutathione and phytochelatins. Our results indicated that the highest bioaccumulation efficiency was reached when algal cells were exposed during 24 h to 100 µM of $HgCl_2$. Under this condition, cellular division and PSII activity decreased respectively by 32 and 30 % in comparison to control. Indeed, the inhibitory effect of mercury on photosynthesis may explained the decrease in cellular division. Under the same treatment condition, the induction of GSH and PCs increased also significantly at 24 h. This explained the algal cell tolerance against the bioaccumulation effect of mercury, determining the importance of this molecular detoxification mechanism. Moreover, our results demonstrated that $HgCl_2$ induced a stronger toxicity impact in algal cells at 48 h in comparison to 24 h of exposure as indicated by the increasing number of injured or dead cells, explaining the lower uptake and sequestration capacity of mercury by algal cells after 24 h of exposure. Indeed, at 48 h of exposure, the induction of PC_2 and PC_4 was not detected due to the strong toxic effect of mercury causing damages on the cellular system and affecting enzymatic activities. Therefore, our results indicated that the PSII activity was used as a reliable biomarker of the bioaccumulation effects of mercury at cellular level

which was highly related to the efficiency of the bioaccumulation of mercury in algal biomass. In conclusion, according to obtained results in this study, it is most likely that this species of green algae, *C. vulgaris* was enough resistant to the effect of $HgCl_2$ up to 100 μM and until 24 h of exposure in order to maintain its maximum bioaccumulation efficiency.

GENERAL INTRODUCTION

Human health is at risk to toxic metals through different ways of exposure, such as the consumption of contaminated food and drinking water (Adriano *et al.*, 2005). For example, it has been shown that metals as cadmium and mercury can cause serious damages to the nervous system and kidneys (Bernard, 2011). These metals can contaminate the environment from both natural and anthropogenic sources, representing a threat to the quality of aquatic ecosystems (Adriano *et al.*, 2005; Gadd, 2009). In particular, wastewaters rejected from industrial effluents contribute to the pollution of aquatic ecosystems by their high level in metals. Therefore, the decontamination of polluted water with metals is one of the most important human needs for preserving environmental quality. Presently, it exists several conventional methods for the removal of metals having advantages and disadvantages that are defined according to their effectiveness, necessity and quantity of chemical reagents. For example, biological methods are interesting because microorganisms are capable of accumulating both organic and inorganic pollutants (Eccles, 1999). Furthermore, the use of microalgae for wastewater treatment is representing a recent environmental biotechnology in development, which is defined as "phycoremediation" of polluted water. Indeed, this technology uses the ability of microalgae to absorb and sequester large quantities of contaminants into their cells (Baumann *et al.*, 2009; Lavoie *et al.*, 2009; Nishikawa *et al.*, 2006). In addition, several studies had shown the ability of different species of microalgae to remove toxic metals and organic compounds from water (Wang *et al.*, 2007; Zhang *et al.*, 2011). This new approach possesses a strong advantage since it does not lead to secondary pollution such as usual methods for wastewater treatment, since microalgae use inorganic nitrogen and phosphorus for their growth (Abdel-Raouf *et al.*, 2012). Also, the biomass can be used for different

applications including the production of biogas and energy through the digestion of biomass (absence of oxygen) into CH_4 and CO_2 (Muñoz and Guieysse, 2006).

In the development of the phycoremediation technology, the main problem is to select the most appropriate algal species able to bioaccumulate efficiently contaminants in order to apply them for the remediation of wastewater. Therefore, it is important to consider both the efficiency of algal cells to bioaccumulate metals and the tolerance against the cellular toxicity of accumulated metals in relation to specific environmental conditions such as the pH, light irradiance and temperature. My study contributed to the use of microalgae in the treatment of wastewater contaminated with metals, by determining the efficiency of algal cells to accumulate mercury at different concentration and time of exposure. In this study, the highest capacity of mercury bioaccumulation was evaluated by using the green alga model *Chlorella vulgaris*.

Therefore, this work is based on these following specific objectives:

1- To determine the bioaccumulation capacity of mercury in algal biomass;

2- To investigate the toxic effects of mercury on cellular division and on Photosystem II photochemistry activity;

3- To investigate the induction of glutathione and phytochelatins in response to metal stress effect.

The realization of our specific objectives is based on two main working hypothesis:

1- The bioaccumulation efficiency of algal cells is dependent to the exposed mercury concentration and the time of exposure.

2- The induction of molecular detoxification mechanisms, especially the synthesis of glutathione and phytochelatins, is related to the cellular bioaccumulation of mercury.

This Master thesis is organized into four main chapters. In the first chapter, the environmental context is presented, including the selection of mercury, the management of environmental pollution sanitation and the use of remediation water technologies. In this chapter, the biochemistry of green algae is also presented including photosynthesis and related physiological processes, ability of plant cells to respond to metal contamination, criteria for the selection of green algae for wastewater treatment, characteristics of the algal model *C. vulgaris* and finally mechanism of mercury toxic effects at cellular level. Moreover, cellular and biochemical indicators related to the mechanism of stress response induced by mercury are presented. In the second chapter, methodological approaches used in this study are explained in details including the use of chlorophyll fluorescence emission as a biomarker of metal toxicity and the analysis of the induction of glutathione and phytochelatins in response to metal uptake. Finally, the results obtained in this study are presented and discussed in the third chapter.

CHAPTER I

ENVIRONMENTAL CONTEXT

1.1 Contamination of water by metals

Metals are present in all environmental compartments such as atmosphere, soil and water at even low concentrations which can bioaccumulate through the food chain (di Toppi and Gabbrielli, 1999). Several metals such as Fe, Cu, Zn and Mn are considered essential for biological functions and they are needed in small quantities in aquatic ecosystem, while others like Cd and Hg are toxic at very low concentrations (Adriano *et al.*, 2005). Metal sources of input come from anthropogenic activities such as agriculture, metallurgy, power generation, mineral extraction, nuclear processes and industrial effluents (Adriano *et al.*, 2005; Gadd, 2009). Indeed, there are a lot of rivers especially in developing countries that are contaminated by the release of metals from anthropogenic activities (Macklin *et al.*, 1994; Swennen *et al.*, 1994. Miller *et al.*, 2002). The risk of toxicity of metals is determined by their bioavailability, which is defined by the capacity of living organisms to absorb chemicals that are involved in their metabolism (Adriano *et al.*, 2005). It is well known that excess consumption of metals such as Cd and Pb can result in neurological, bone and cardiovascular diseases, renal dysfunction, and various cancers, even at relatively low levels (Calderon, 2000; Watt *et al.*, 2000; Jarup, 2002). Moreover, mercury can deteriorate the nervous system, impairs hearing, speech, vision and gait, difficulties on chewing and swallowing, and it can also causes involuntary muscle movements, corrodes skin and mucous membranes. In Canada, almost 150 billion liters of untreated and undertreated wastewater (sewage) are released into our waterways every year (Environment Canada, 2011). Particularly,

in 2011, the amount of Hg released into water was about 351 kg. More details are presented in the Table below (1.1), the sources of mercury releases into water in Canada are presented. Therefore, it is well known that the release of metals into water represent a current environmental problem for the St. Lawrence River. According to studies by Environment Canada (2011), the surface sediments in the river are generally less contaminated in comparison to sediments 20 years ago which resulted from effluent treatment, but concentrations of some substances have remained unchanged or increased.

Table 1.1 Sources of releases in mercury into water in Canada (Environment Canada, 2011).

Sources of releases in mercury into water	Kg	%
Water, sewage and other systems	174	49
Pulp, paper and paperboard mills	77	22
Metal ore mining	77	22
Non-ferrous metal (except aluminum) production and processing	9	3
Iron and steel mills and Ferro-alloy manufacturing	4	1
Alumina and Aluminum production and processing	3	1
Electric power generation, transmission and distribution	3	1
Other sources	1	<1

1.2 Studied metal, mercury

Mercury is not degradable because it is a metallic element, so it can just converts into various species forms, and it is a naturally occurring element known as "heavy metal" which can be toxic for organisms at low concentrations (Jackson, 1998; Selin, 2009). In nature, mercury can have 3 possible conditions of valence state (Morel *et al.*, 1998; Leermakers *et al.*, 2005; Leopold *et al.*, 2010): 1- Elemental mercury giving a volatile liquid colourless and odourless vapour at room temperature; 2- Inorganic mercury when combined with sulphur, chlorine or oxygen; 3- Organic mercury such as methyl mercury or dimethyl mercury.

As a natural element, mercury is ubiquitous in the environment (Figure 1.1), almost 10,000 tons produced from degassing of earth's crust, and approximately 20,000 tons/year is added by anthropogenic activity (Hansen and Dasher, 1997; Zahir *et al.*, 2005). One of the main sources of anthropogenic discharge and mercury pollution in atmosphere is the emission of mercury from the coal smoke and also is estimated that it will increase at a rate of 5% a year (Zhang *et al.*, 2002). Mercury in air eventually passes into rivers, lakes and oceans. With mercury contaminating rain (Domagalski *et al.*, 2004; Levine, 2004), ground and seawater (Beldowski and Pempkowiak, 2003), no one is safe. When atmospheric mercury falls to earth, it can be changed by bacterial or chemical action into an organic form known as methylmercury that has the ability to migrate through cell membranes and "bioaccumulate" in living tissue. Bioaccumulation is the process by substances that builds up in a living organism from air or water, or through the contaminated food (Jackson, 1998; Zahir *et al.*, 2005; Selin, 2009).

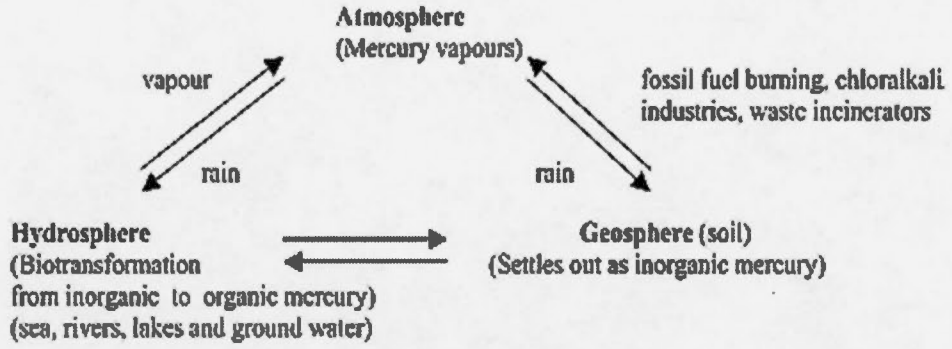


Figure 1.1 Mercury distribution in the environment (Zahir *et al.*, 2005).

As it is shown in figure 1.2, in aquatic environments, inorganic mercury can be microbiologically transformed into lipophilic organic compound, methylmercury (the accumulated methylmercury is indicated by the red dots) (Environment Canada, 2011). Therefore, methylmercury is easily accumulated into phytoplankton, and then can be transfer through the food chain to zooplankton, fishes, mammals, birds and finally to humans *via* consumption. Indeed, this process known as "biomagnification" is an environmental concern since it inflicts increasing toxicity to species of higher trophic levels (Environment Canada, 2011).

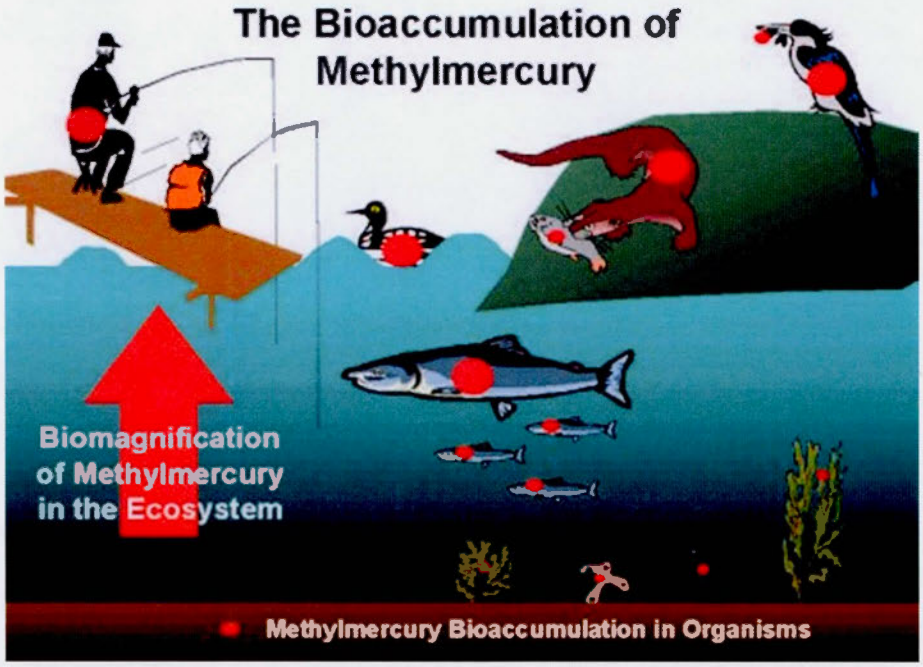


Figure 1.2 Methylmercury bioaccumulation in aquatic organisms. If the concentration of methylmercury in lake water is considered to have an absolute value of 1, then approximate bioaccumulation factors for phytoplankton are 10^5; for zooplankton are 10^6 and for fish, birds and humans are 10^7 (Environment Canada, 2011).

1.2.1 Mercury in pharmaceuticals and utility products

In developed country, skin whitening creams and soaps were recognized as a source of chronic mercury poisoning (Harada *et al.*, 1999, 2001). Also, it was demonstrated that mercury develops acrodynia and kawasaki disease in young children who used mercuric chloride in teething powders (Kazantzis, 2002). The use of mercury in vaccines had previously made some concern about death of infants (Westphal and Hallier, 2003). Moreover, there are some concerns about mercury since it has been widely used in pharmaceuticals products such as preservative in

Hepatitis B, Diphtheria, Pertussis, Acellular pertussis and Tetanus vaccines (Zahir *et al.*, 2005).

1.2.2 Mercury toxicity and human health

It has been demonstrated that low dose of mercury had some effects on the nervous system of foetus, children and adults. Such developmental neurotoxicity can have an impact on the entire population and the life span of humans (Rice and Barone, 2000; Zahir *et al.*, 2005). Moreover, mercury toxicity can also affect cardiovascular system. Indeed, recent evidence suggested that mercury content in fish may reduce the cardioprotective effect of fish intake (Chan and Egeland, 2004; Zahir *et al.*, 2005). In Table 1.2, it is shown a shortlist of toxic effects of mercury on various organ systems.

Table 1.2 Toxicity effect of low dose of mercury in various organ systems (Zahir *et al.*, 2005).

Nervous system (Adults)	Memory loss, including Alzheimer like dementia, deficit in attention, hypoesthesia, ataxia, dysarthrea, subclinical finger tremor impairment of hearing and vision, sensory disturbances, increased fatigue.
Children/infants	Deficit in language (late talking) and memory deficit in attention, Autism.
Motor system Adults	Disruption of fine motor function, decreased muscular strength, increased tiredness.
Children/infants	Late walking.
Renal system	Increases plasma creatinine level.

Cardiovascular system	Alters normal cardiovascular homoeostasis.
Immune system	exacerbates lupus like autoimmunity, multiple sclerosis, autoimmune thyroiditis or atopic eczema.
Reproductive system	Decreases rate of fertility in both males and females, birth of abnormal offsprings.

1.3 Clean water technologies

The management of wastewater pollution has been an important environmental topic, especially during the last two centuries (Eccles, 1999): The elimination of biochemical oxygen demand (BOD), N and P, mater in suspension, coliforms and bacteria was the main objective for the remediation of wastewater. The wastewater treatment consists in several steps (Abdel-Raouf *et al.*, 2012): The first step of is the elimination of bulk materials such as wood and heavy grain. The reduction of BOD by removing organic matter is the secondary treatment of wastewater. Tertiary wastewater treatment involves the elimination of metal ions of ammonium, nitrate, phosphate and of toxic organic compounds. Finally, the destruction of pathogens will be carried out by the stage of disinfection of wastewater.

Several conventional methods showed the removal of metals from aqueous solutions such as ion exchange, electrolysis, precipitation, filtration and evaporation (Fabiani, 1992; Aderhold *et al.*, 1996). As a result, the conventional separation of metals from aqueous solutions as chemical precipitation and reverse osmosis requires large quantities of reagents and energy, which restricts the practice of these methods with low concentrations of metals (Ozdemir *et al.*, 2005; Pehlivan and Altun, 2006).

1.4 Use of microalgae in water remediation

The contamination of wastewater by nitrogen, phosphorus, metals and organics which are not processed completely by conventional treatment will cause environmental problems in the watercourse. The use of algae for the treatment of wastewater has been recently applied since some strains can be produced in large quantities such as *Chlorella* and *Dunaliella* using nitrogen and inorganic phosphorus for their growth (Abdel-Raouf *et al.*, 2012). In addition, studies have shown the capacity of algae to remove pollutants: For example, the capacity of *C. reinhardtii* has been proposed as a biotechnological approach interesting for the decontamination of organic pollutants (Wang *et al.*, 2007; Zhang *et al.*, 2011). However, the use of algal biomass from the industrial wastewater treatment is rarely appropriate for the production of food or chemicals such as fertilizer because of the existence of toxic metals and organic contaminants (Muñoz and Guieysse, 2006). Therefore, the utilization of wastewaters as nutrient sources can replace growth media and reduce the cultivation costs for algal growth economics. For example, it had been shown that *C. vulgaris* could be cultured in wastewater for biomass production while nitrogen and phosphorus are reduced during the tertiary treatment of wastewater (Wang *et al.*, 2010; Ruiz *et al.*, 2014; Ponnuswamy *et al.*, 2013). Another study had shown that the cultivation of alga *C. vulgaris* on wastewater had two positive effects, first the removal of inorganic elements from wastewater and second the produced biomass used as a source for biofuel or byproduct (Fathi *et al.*, 2013).

Moreover, photobioreactors systems (open or closed) for the treatment of wastewater using algae need a high efficiency for the use of light, a good mix, low hydrodynamic stress on photosynthetic algal cells, a low risk of contamination, a minimum of space, ease of cleaning, an economical energy consumption and ease to control the temperature (Pulz, 2001; Ugwu *et al.*, 2008; Xu *et al.*, 2009). For

example, Cyanotech Corp in Hawaii and Earthrise Farms in California are two companies that grow algae on a large scale (Richmond, 2004; Muñoz and Guieysse, 2006; Spolaore *et al.*, 2006). However, the loss of water by evaporation and contamination, large space, predation risk, no control over volatilization of pollutants and on growing conditions are disadvantages concerning open compared with closed photobioreactors systems (Borowitzka, 1999; Pulz, 2001; Muñoz and Guieysse, 2006).

At commercial level, the biomass production of microalgae has been found to be useful for many applications in various branches of industry such as the production of dyes, food additives, antibiotics and biofuel energy (Liang *et al.*, 2009; Görs *et al.*, 2010; Priya, 2012; Mallick *et al.*, 2012). These studies showed that the industrial potential of green microalgae relies on their rapid growth of biomass in photobioreactors, since they need a relatively small area required for cultivation in comparison to crop plants such as maize. Moreover, optimizing the growth media is a critical step for algal biomass production at large scale, since the cost of growth media nutrients required in large quantities can be significant (Sharma *et al.*, 2011; Blair *et al.*, 2014).

1.5 Biochemistry of green algae *Chlorella vulgaris* as a model of study

Green microalgae are a large group of microorganism eukaryotes using the solar energy to perform photosynthesis to create biomass with water, carbon dioxide and other nutrients. As the major primary producers in freshwater ecosystems, microalgae play a primordial role in the functioning of aquatic ecosystem, and can be indicative of the environmental quality of freshwater for aquatic organisms of higher tropic levels (Buchanan *et al.*, 2000).

The species *C. vulgaris* presents several morphological and physiological advantages, belonging to the phylum of *Chlorophyta* (Vymazal, 1995). It is single-cell and spherical in shape, about 2 to 10 μm in diameter, without flagella (Figure 1.3). Since this algal species can bioaccumulate easily aquatic pollutants, it had been used as a sensitive biosensor of environmental quality for freshwater, permitting the assessment of the risk of toxicity in the development of environmental regulations (Rioboo *et al.*, 2002; González-Barreiro *et al.*, 2006; Afkar *et al.*, 2010; Ou-Yang *et al.*, 2013). For example, it has been shown an inhibition of growth during 96 h on *C. vulgaris* for two herbicides used wide-spread, isoproturon (class of phenylurea) and terbutryn (class of triazine) (Rioboo *et al.*, 2002): These herbicides classes act on the photosynthesis process by displacing a plastoquinone from its binding site in the photosystem II. However, the possibility of making a bioremediation system for the removal of herbicides in water with this species has been shown for short time of exposure only. Indeed, it was shown recently that, during 24 hours of treatment, algal cells of *C. vulgaris* were able to bioconcentrate efficiently the triazine herbicides atrazine and terbutryn (González-Barreiro *et al.*, 2006). Moreover, the change in growth rate of *C. vulgaris* has been used as an indicator of the inhibitory and stimulatory effects of several metals (zinc, cobalt and copper) depending to their concentration in the aqueous solution (Afkar *et al.*, 2010). In a recent study, the toxic effects of cadmium on *C. vulgaris* were investigated during 96 h which resulted in the alteration of cellular functions as indicated by the response of several biomarkers such as cell growth, photochemical efficiency of photosynthesis by chlorophyll *a* fluorescence and cell viability (Ou-Yang *et al.*, 2013).

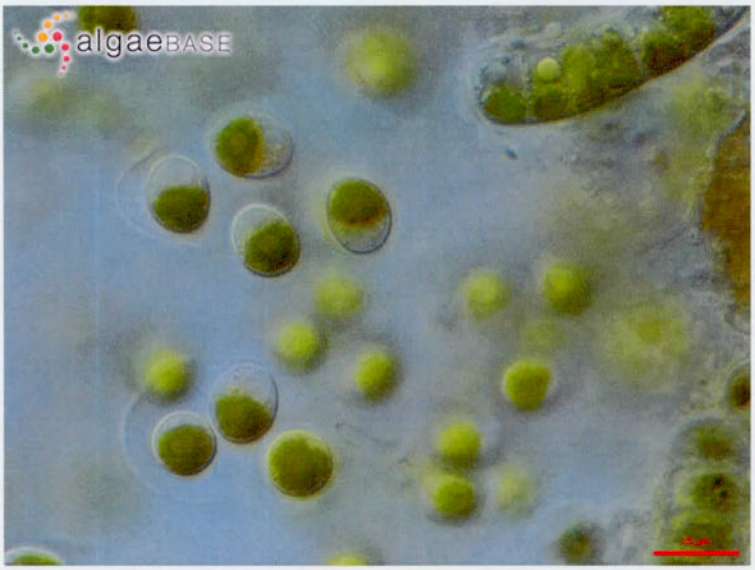


Figure 1.3 Photo of algal cells of *Chlorella vulgaris*. Seattle, Washington, USA, cement wall, 1000 ×, DIC. 01 Nov 2008, taken by Karl Bruun© (skogenman@earthlink.net).

1.6 Photosynthesis

Photosynthesis is the only source of solar energy storage on Earth permitting the production of biomass by plants and algae which use directly the light energy by the process of photosynthesis permitting the synthesis of organic compounds (Ort and Whitmarsh, 2001): The oxygenic photosynthesis is a redox reaction able to perform the photolysis of water providing the electrons for the reduction of carbon dioxide (CO_2) and the synthesis of carbohydrates. Therefore, photosynthesis includes absorption of light, the transformation of energy, electron transport and enzymatic pathways. The photosynthesis takes place in a cellular organelle called chloroplast which is surrounded by a lipid bilayer and an internal membrane system called thylakoid (Taiz and Zeiger, 2010) (Figure 1.4) : The thylakoids membranes are in an

aqueous matrix called stroma, and there is a regional distinction by granum and stromal lamellae (a granum is made up of 10 to 100 discs).

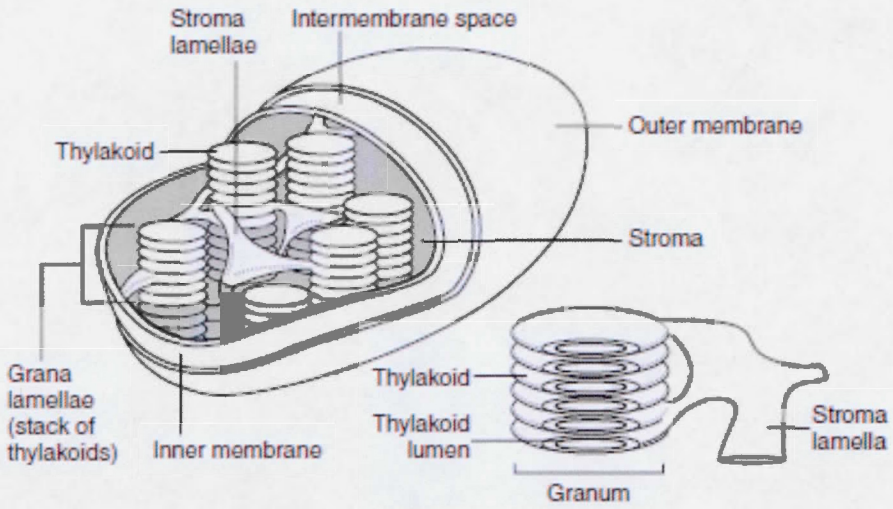


Figure 1.4 (A) Schematic diagram of a chloroplast including thylakoid membranes (Taiz and Zeiger, 2010).

The photosynthetic process can be divided into two phases (see Figure 1.5) according to Malkin and Niyogi (2000): During the light reaction, O_2, ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate) are synthesized, and ATP and NADPH produced are used by stromal enzymes for the synthesis of carbohydrate by fixing CO_2 in the cycle of Calvin-Benson.

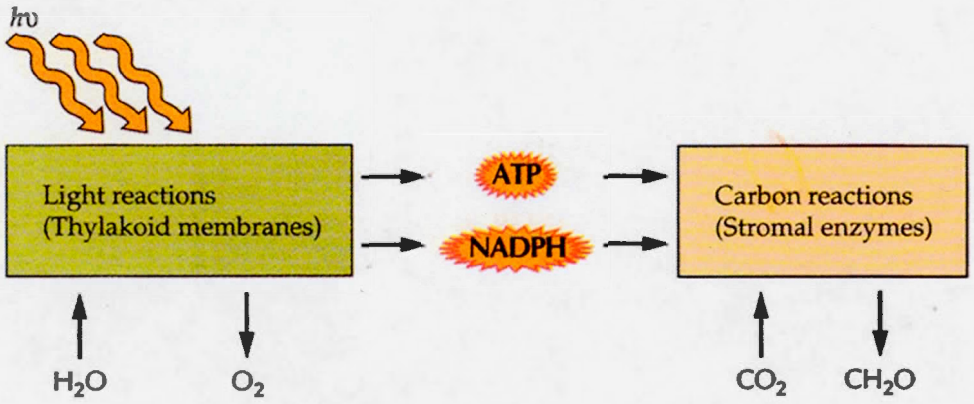


Figure 1.5 Light and dark reactions of photosynthesis (Malkin and Niyogi, 2000).

1.6.1 Photosynthetic apparatus

Oxygenic photosynthesis depends on two photosystem reaction center complexes, Photosystem II (PSII) and Photosystem I (PSI), that are linked by electron transport carriers (Malkin and Niyogi, 2000; Ort and Whitmarsh, 2001; Whitmarsh and Govindjee, 2002). In paticular, the PSII is composed of a core reaction center surrounded by a light-harvesting antenna system (Whitmarsh and Govindjee, 2002): The core center includes D1 and D2 polypeptides that bind the cofactors of the photochemical charge separation and electron transfer carriers that oxidize water and reduce plastoquinone pool. Moreover, the antenna system consists of protein complexes that contain light-absorbing molecules, as chlorophylls and others accessory pigments, which permit the capture of photons and the transfer of the excitation energy to reaction centers where primary charge separation occurs. Therefore, photosynthesis is driven by visible light that is absorbed by chlorophylls

and carotenoids bound to the light-harvesting proteins that surround the PSII and PSI reaction centers in the photosynthetic membrane.

The major light-absorbing pigment in many algae is chlorophyll, a cyclic tetrapyrrole in which the nitrogens of the pyrroles are coordinated to a central magnesium ion (Malkin and Niyogi, 2000; Ort and Whitmarsh, 2001; Whitmarsh and Govindjee, 2002), and it is a green pigment that strongly absorbs red and blue light since it absorbs wavelengths of 430 and 680 nm while the green light will be reflected (see Figures 1.6 and 1.7; Malkin and Niyogi, 2000). The light antenna collector of PSII possess between 200-250 molecules of chlorophylls and 60-70 molecules of carotenoids, enhancing the spectrum of visible light absorbed by the antenna system (Govindjee *et al.*, 2010) : The light is absorbed by chlorophylls and carotenoids which are linked to the light collector complexes in the membrane of thylakoids. In fact, chlorophyll molecule enters into the excited state and the excitation energy can be transferred to the reaction centers causing the primary photochemical act. Blue light has a higher energy than red light causing the passage of chlorophyll in the upper excited state. During the absorption of light by pigments, these are electrons π that interacts with visible light. In fact, the cycles of chlorophyll have conjugated double bonds and electron π is involved in responsible for the absorption of light. Excitation energy is transferred by resonance of a molecule of chlorophyll to another until the special pair of Chl reached the PSII reaction center, called P680, which will rise to an excited state P680*. The internal passages or relaxations take place during the return of the chlorophyll excited at a lower level than the energy of excitation energy dissipation in the form of heat or in the form of fluorescence (Ort and Whitmarsh, 2001; Whitmarsh and Govindjee, 2002; Govindjee *et al.*, 2010).

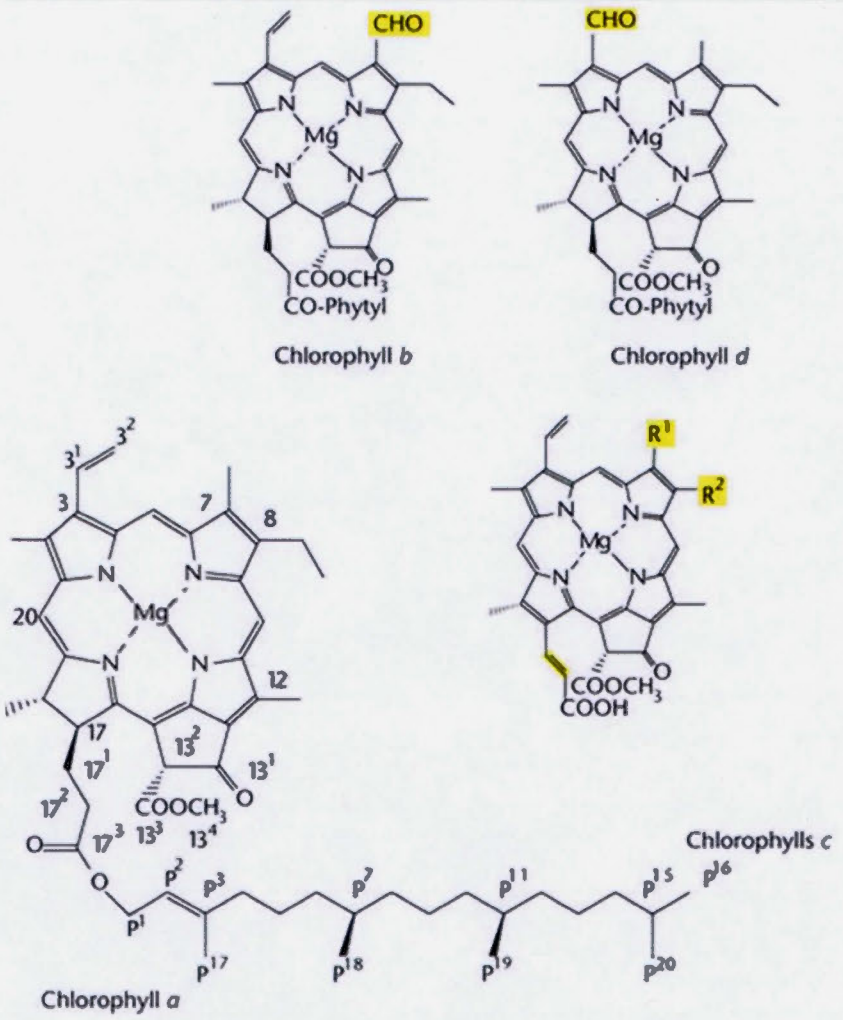


Figure 1.6 Chemical structures of chlorophylls (Fujita, 2005).

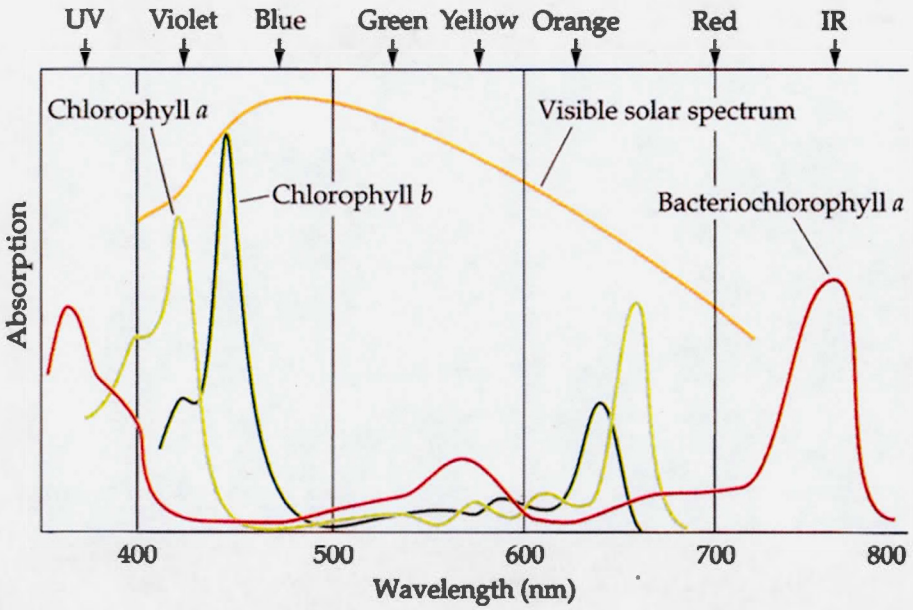


Figure 1.7 Absorption spectra of chlorophylls *a* and *b* in the visible region of the solar spectrum when dissolved in nonpolar solvents (Malkin and Niyogi, 2000).

In the thylakoid membrane, photosynthetic complexes as the photosystems I and II (PSI and PSII) are responsible for performing the photochemical reactions (Malkin and Niyogi, 2000; Ort and Whitmarsh, 2001; Whitmarsh and Govindjee, 2002; Govindjee *et al.*, 2010). Indeed, it is the PSII reaction center that triggers the primary photochemical act and the electron transport from the water splitting system (Figure 1.8) (Govindjee *et al.*, 2010): The light is absorbed by antenna systems of PSII and PSI providing energy for the transport of electrons from water in nicotinamide adenine dinucleotide phosphate ($NADP^+$). Indeed, the PSII and PSI electron transport provides the energy for the formation of the proton gradient across the membrane, and this proton gradient will be used by the ATP synthase to produce ATP (Figure 1.9) (Govindjee *et al.*, 2010).

These photochemical reactions consist on two stages, the photooxidation of water and the reduction of the plastoquinone pool (Lubitz *et al.*, 2008) : The photooxidation of water is the sum of four individual reactions in which two water molecules are split to obtain a molecule of O_2 and four protons are released into the lumen contributing to the formation of the proton gradient. The oxygen evolving complex participates in the photolysis of water, and it is composed of three extrinsic proteins next to the lumen: PsbO (33 kDa), PsbQ (17 kDa) and PsbP (23 kDa). In fact, the cluster of Mn_4O_xCa is surrounded by proteins D1 and D2, internal antenna proteins CP43 and CP47 and several extrinsic polypeptides which stabilize and optimize the photolysis of water complex and its activity (Rivas *et al.*, 2004).

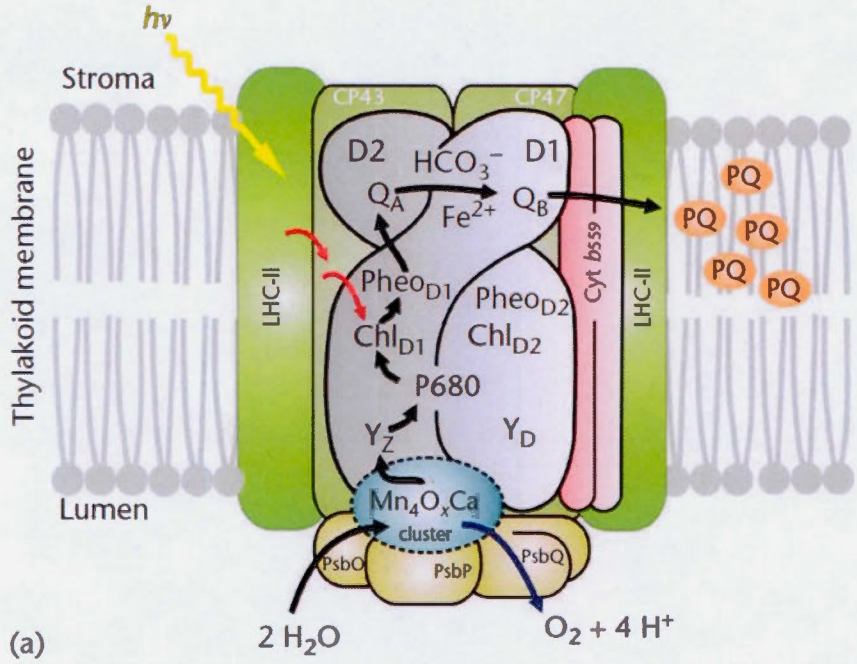


Figure 1.8 Structural model of PSII reaction center P680, showing proteins D1, D2, CP43 and CP47. The oxidation of water by the Mn cluster triggers the primary

photochemical act. Electrons are transferred from P680 to plastoquinone molecules Q_A and Q_B (Govindjee *et al.*, 2010).

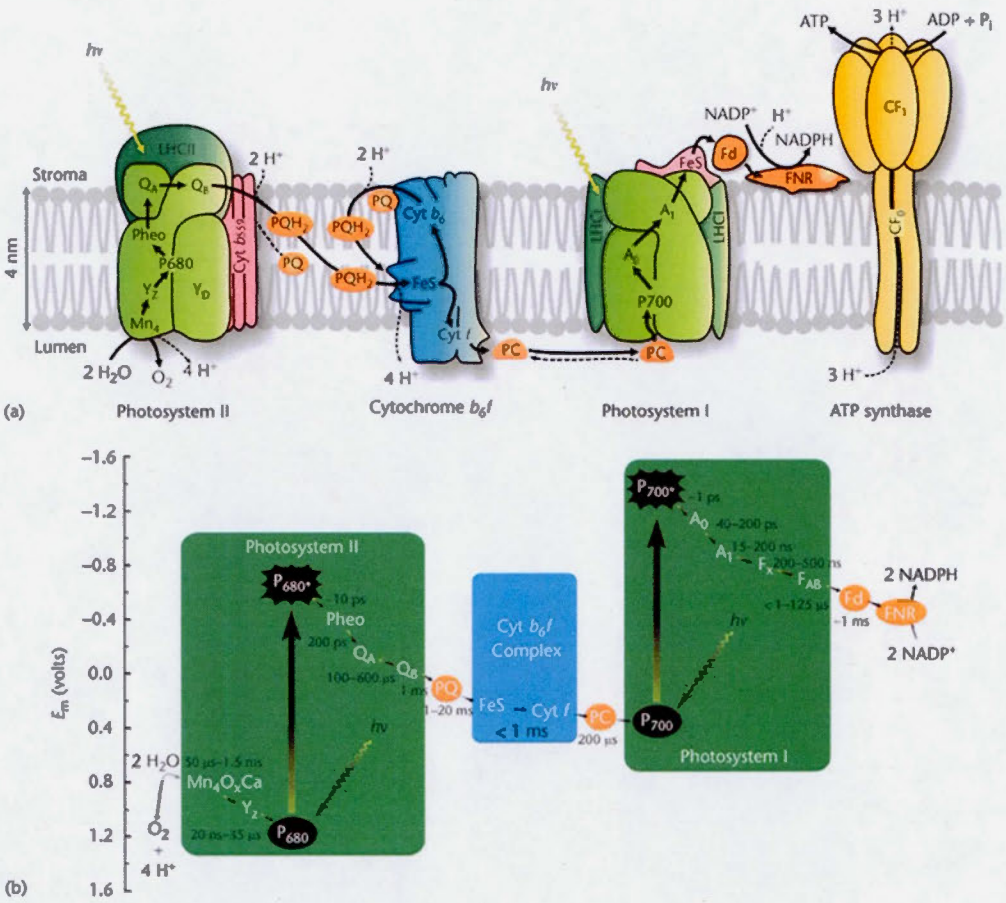


Figure 1.9 (a) Thylakoid membrane organization of photosystems and electron transport carriers in higher plants. (b) Z-scheme indicating the E_m values of photosynthetic electron transport carriers (Govindjee *et al.*, 2010).

1.6.2 Energy dissipation through fluorescence

Several research groups (Strasser and Strasser, 1995; Strasser *et al.*, 2004; Lazar, 2006; Baker, 2008; Govindjee *et al.*, 2010) participated to the development of a comprehensive model representing the photochemical reactions within the PSII in which the excitation energy can be used for photochemistry or dissipated in the forms of chlorophyll fluorescence and heat (Figure 1.10). Therefore, it was considered that the photochemistry of PSII was in competition with the processes of fluorescence emission and heat loss.

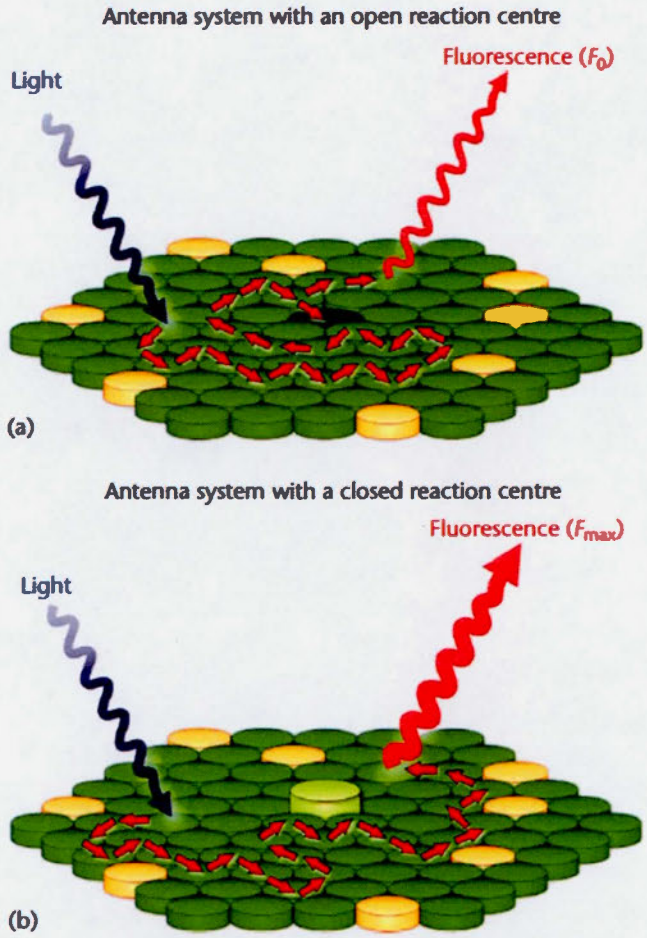


Figure 1.10 Schematic representation of the excitation energy transfer (small red arrows) between chlorophyll molecules in the light collector antenna system. Green discs represent chlorophylls *a* and *b*, and yellow discs represent carotenoids; the darker green disc represents an open reaction center and the lighter green disc represents a closed reaction center. (a) the reaction center is open and the energy is used for charge separation, while the fluorescence emission is minimal (labelled as F_O); (b) the reaction center is closed and the fluorescence emission is maximal (F_M) (Govindjee *et al.*, 2010).

The chlorophyll fluorescence emission comes from chlorophylls of the light collector antennas of PSII which can be used as a very sensitive, rapid and non-invasive method (Lazar, 2006; Govindjee *et al.*, 2010). Two main steps are observed in the fluorescence induction, the rapid increase (1 sec) of the fluorescence intensity from an initial level (F_O) to a maximum intensity level (F_M) under a saturating light (Lazar, 2006; Govindjee *et al.*, 2010): The minimum fluorescence level is named basal fluorescence (Fo) when Q_A is oxidized, and when the reaction center is closed, the chlorophyll fluorescence emission is maximum which is named maximum fluorescence level (F_M), when Q_A is completely reduced.

When the rapid kinetic of chlorophyll fluorescence was analyzed in a logarithmic time scale, different steps were identified as O-J-I-P, showing different states of redox carriers of electrons from PSII (Popovic *et al.*, 2003; Lazar, 2006; Govindjee *et al.*, 2010): The O-J transition represents the reduction of the primary acceptor of electron, Q_A; The emission of fluorescence to the transition I shows the first reduction of Q_B, the I-P transition represents an accumulation of Q_B completely reduced ($Q_A^- Q_B^{-2}$) and the maximum fluorescence level is reached when the PQ are completely reduced. Therefore, it was showed in ecotoxicological studies that any alteration of the electron transport by chemical inhibitors will be indicated by the

change of theses steps (Popovic *et al.*, 2003). The figure 1.11 is showing the change of the fluorescence transients of the fluorescence induction, which is affected differently depending on the electron transport inhibitor, DCMU or mercury, due to their different mode of action (Popovic *et al.*, 2003).

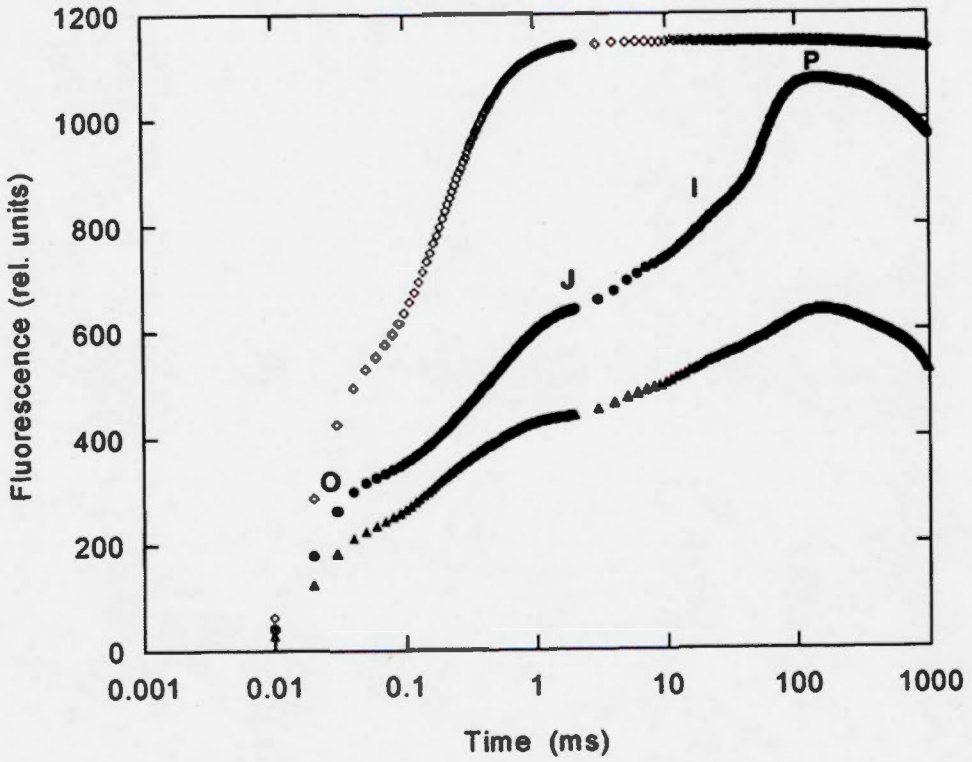


Figure 1.11 The rapid and polyphasic (OJIP) rise of Chl *a* fluorescence induction presented on a logarithmic time scale when *L. gibba* plants were exposed to a saturating light (9000 μmol of photons m^{-2} s^{-1}) after a dark adaptation of 20 min. Legend: Control, round; treated 5 h to 100 μg L^{-1} of Hg^{2+}, triangle; treated 5 h to 10 × 10^{-5} M of DCMU, square; (Popovic *et al.*, 2003).

1.7 Synthesis of glutathione and phytochelatins for metal detoxification

Phytochelatins (PCs) are composed of three amino acids: glutamine (Glu), cysteine (Cys) and glycine (Gly), and the general structure (ɤ-Glu-Cys)$_n$-Gly has been proposed for phytochelatin, where « n » can be between 2 and 11 (Crawford *et al.*, 2000; Pal and Rai, 2010) (see Figure 1.12). When algae or plants are exposed to toxic metals such as mercury, the synthesis of thiol-rich peptides as glutathione (GSH) and phytochelatins (PCs) are induced for their activities in the protection against oxidative stress and metal detoxification processes (Wu and Wang, 2012).

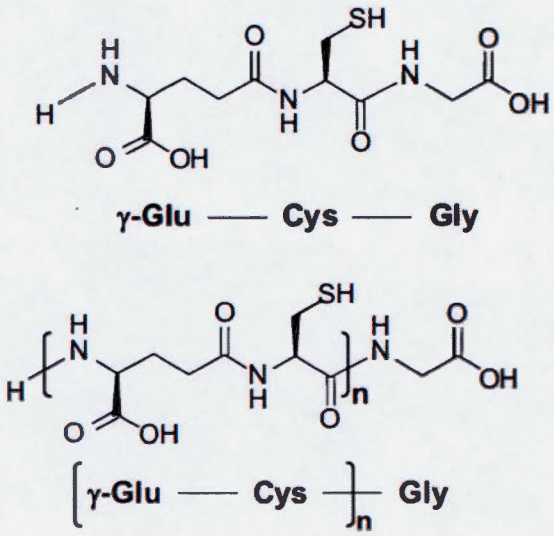


Figure 1.12 Chemical structures of glutathione and phytochelatin (Pal and Rai, 2010).

The glutathione (GSH) protects cells by reducing the reactive forms of oxygen (ROS) and also by binding to metals (Crawford *et al.*, 2000; Stoiber *et al.*, 2010): In the first stage, the synthesis of ɤ-glutamylcystein is done with L-glutamate and L-cysteine which is catalyzed by the enzyme ɤ-glutamylcystein synthetase; In the

second step, the addition of glycine is catalyzed by the enzyme glutathione synthetase to produce GSH. Finally, the GSH level remains high due to the intervention of the enzyme glutathione reductase in the reduction of oxidized glutathione which is essential for maintaining the redox of the cytoplasm. This interconversion between reduced and oxidized form of glutathione is illutrated in Figure 1.13.

It is well accepted that phytochelatins are formed from glutathione in the presence of metal ions, following the intervention of an enzyme, the ɤ-glutamyl-cysteine dipeptide transpeptidase, known as the phytochelatin synthase (Grill *et al.*, 1987; Grill *et al.*, 1989; Crawford *et al.*, 2000; Pal and Rai, 2010). In fact, Grill *et al.* (1987) first observed that the level of phytochelatins decreased in the presence of increasing concentrations of buthionine sulfoximine, which is an inhibitor of ɤ-glutamylcysteine synthetase enzyme involved in the synthesis of glutathione.

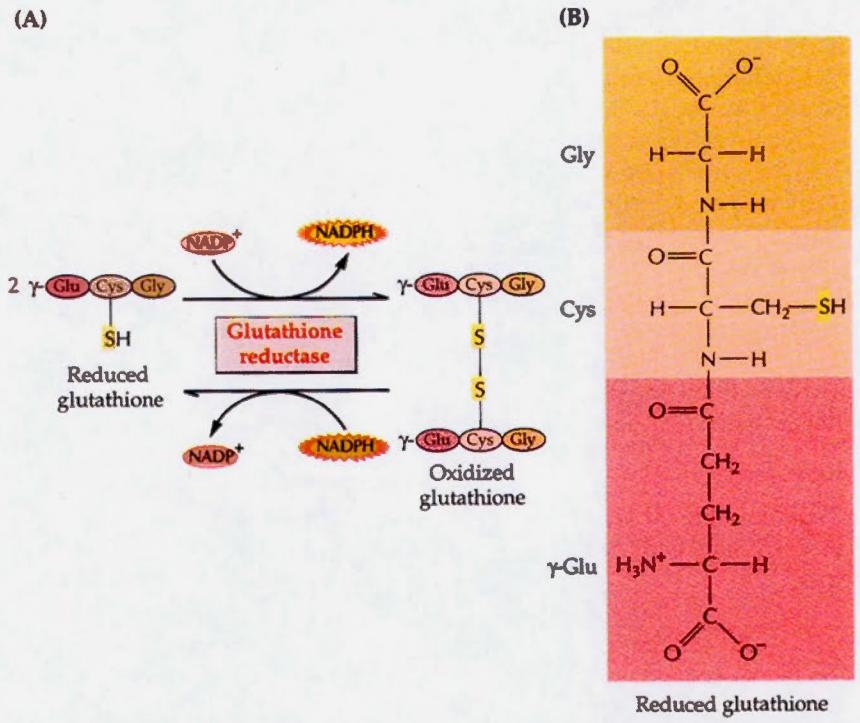


Figure 1.13 (A) Interconversion between reduced and oxidized form of glutathione; (B) Structure of reduced glutathione (Crawford *et al.*, 2000).

1.8 Sequestration of metals in the vacuole

Moreover, it has been reported that phytochelatins are induced in plant cells by several metals such as cadmium, zinc, copper, lead and mercury, since they are involved in the cellular distribution and vacuolar sequestration of metal ions (Crawford *et al.*, 2000). Indeed, it is because of the cysteine that phytochelatins are able to form complexes with metals preventing their excess in the cytosol by vacuolar sequestration (Kobayashi and Yoshimura, 2006; Pal and Rai, 2010).

Previously, Hu and colleagues (2001) reported the formation of two types of complex PC-Cd in *C. reinhardtii*, a complex of low molecular weight (LMW) which is formed quickly after exposure to Cd and a complex of high molecular weight (HMW) from the rapid conversion of HMW to LMW complex (Figures 1.14). In fact, they mentioned that the HMW complex was formed by the addition of sulfide in the LMW complex. Indeed, the complex of high molecular weight (HMW) is very stable and plays an essential role for Cd storage in the vacuole during the process of detoxification (Hu *et al.*, 2001). The formation of low and high molecular weight phytochelatin-Cd complexes is illustrated in Figure 1.15.

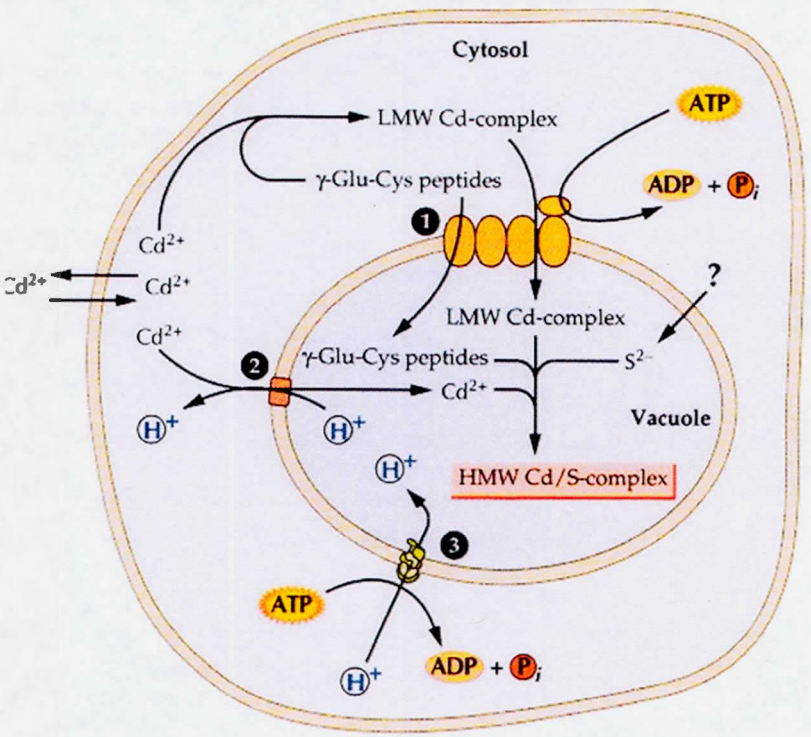


Figure 1.14 Mechanism of sequestration of cadmium in the cell by phytochelatins; LMW: low molecular weight phytochelatin Cd complex; HMW: high molecular weight phytochelatin Cd complex (Crawford *et al.*, 2000).

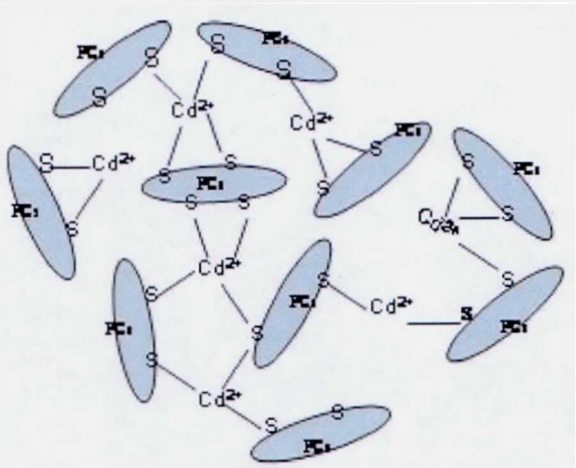


Figure 1.15 Formation of low or high molecular weight phytochelatin-Cd complexes. Interaction of PCs coordinately binding with sulfur atoms of Cys residues from either single or multiple PC molecules, forming different complexes (Pal and Rai, 2010).

CHAPTER II

MATERIAL AND METHODS

2.1 Algal culture

The green alga *C. vulgaris* was provided by the Culture Collection of Algae from the Canadian phycological culture center (University of Waterloo, ON). The alga *C. vulgaris* was grown in Bold's Basal Medium (BBM) under a constant temperature of 21 ± 1 °C and a continuous irradiance of 80 ± 10 µmol of photons m^{-2} s^{-1} provided by white fluorescent bulbs (Sylvania Grolux F36W, Drummondville, Canada). The BBM culture's composition is reported in Table 2.1 (Stein, 1973). This medium is highly enriched in nutriments and is used for many species of green algae. The culture medium was then sterilized by using the autoclave. All stock cultures were gently mechanically shaken (at 90 rpm). For all algal cultures, glasswares were cleaned by soaking them in 10 % HCl for at least 24 h, and then rinsed three times with de-ionized water.

Table 2.1 Bold's Basal Medium (BBM) modified from the original recipe (Stein, 1973).

Stock	Stock solutions		For 1L	concentration Final
1	KH_2PO_4	8.75 g/500 mL	10 mL	1.29×10^{-3}M
2	$CaCl_2.2H_2O$	1.25 g/500 mL	10 mL	1.70×10^{-4} M
3	$MgSO_4.7H_2O$	3.75 g/500 mL	10 mL	3.04×10^{-4} M
4	$NaNO_3$	12.5 g/500 mL	10 mL	2.94×10^{-3} M
5	K_2HPO_4	3.75 g/500 mL	10 mL	4.31×10^{-4} M
6	NaCl	1.25 g/500 mL	10 mL	4.28×10^{-4} M
7	Na_2EDTA KOH	10 g/L 6.2 g/L	1 mL	2.7×10^{-5} M 1.1×10^{-4} M
8	$FeSO_4.7H_2O$ H_2SO_4 (conc.)	4.98 g/L	1 mL	4.48×10^{-5} M
9	Trace metal solution	See below*	1 mL	See below*
10	H_3BO_3	5.75 g/500 mL	0.7 mL	1.3×10^{-4} M

***The composition of the trace metal solution:**

Substance	**g/L**	
H_3BO_3	2.86	$(4.62 \times 10^{-4}$ M)
$MnCl_2.4H_2O$	1.81	$(1.82 \times 10^{-5}$ M)
$ZnSO_4.7H_2O$	0.222	$(7.67 \times 10^{-5}$ M)
$NaMoO_4.5H_2O$	0.390	$(1.23 \times 10^{-5}$ M)
$CuSO_4.5H_2O$	0.079	$(1.57 \times 10^{-5}$ M)
$Co(NO_3)_2.6H_2O$	0.0494	$(4.21 \times 10^{-6}$ M)

The pH of the medium is then adjusted to 6.8 with NaOH or HCl before autoclave.

2.2 Determination of cell density

The change in the growth of algal cells was determined during 72 h of exposure to different concentrations of $HgCl_2$: 0, 1, 10, 25, 35, 50 and 100 µM. To investigate the effect of mercury on cellular division, the cell density was monitored every day. At t = 0, the initial cell density was of 10^6 cells/mL and all experiments were performed in triplicates. For the measurement of cell density, the Multisizer™ 3 Coulter Counter® (Beckman Coulter Inc, Fullerton, CA) was used for sizing and counting. This instrument is a particle sizing and counting analyzer available today using the Coulter Principle, also known as ESZ (Electrical Sensing Zone Method). In Figure 2.1, this instrument is illustrated.

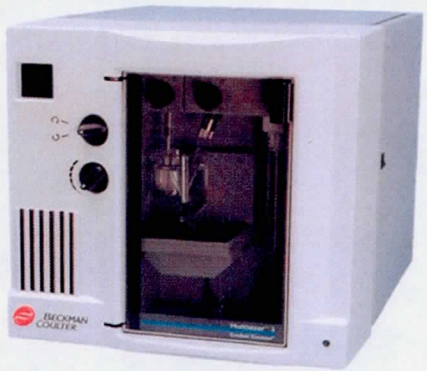


Figure 2.1. The Multisizer™ 3 Coulter Counter (www.beckmancoulter.com).

In order to determine the change of cell density during time with the change of absorbance, a standard curve was determined based on the relation between the change of absorbance at 750 nm and the change of cell density. Three initial cell densities of starting algal cultures were used: 10^6, 500000 and 250000 cells/mL. Cell density was evaluated by monitoring culture absorbance at 750 nm using a

spectrophotometer UV-Vis (Lambda 40, Perkin-Elmer, Woodbridge, Canada). Then, the change of cell density and absorbance at 750 nm were monitored during 72 h when algal cells of *C. vulgaris* were grown at 21 ± 1 °C in BBM under a constant illumination of 80 ± 10 µmol of photons m^{-2} s^{-1}. From this relation, a linear fitting curve of the data and its formula was determined (Figure 2.2).

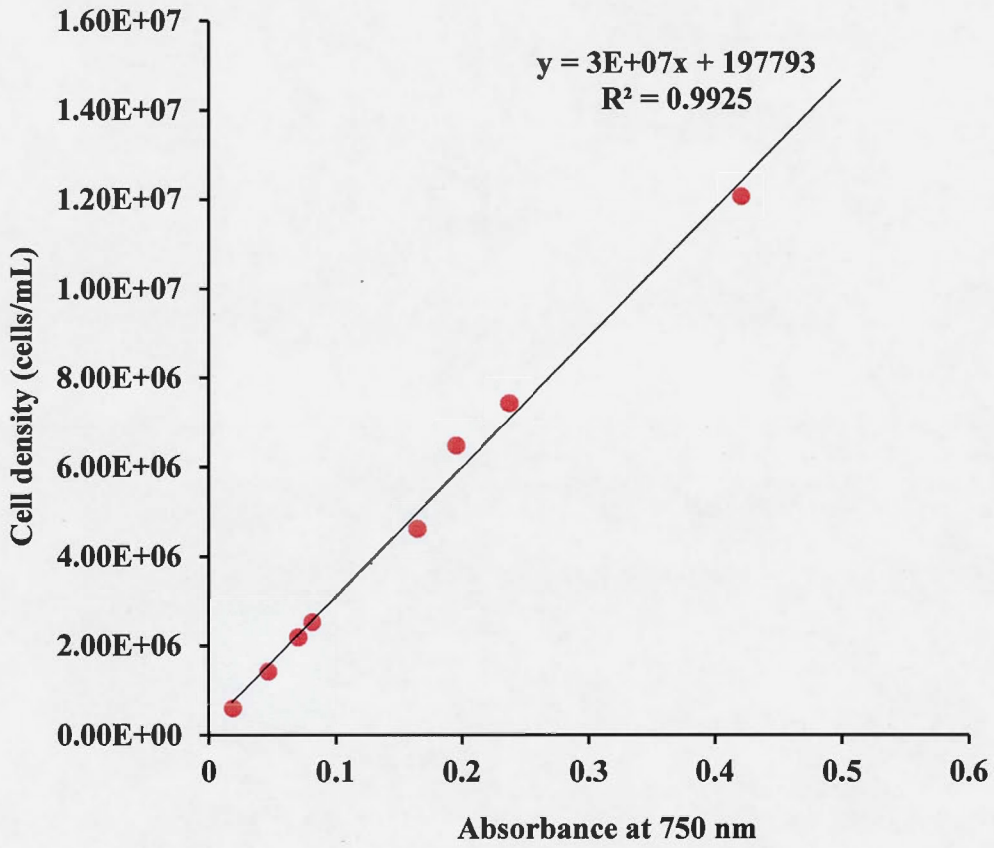


Figure 2.2 Standard curve indicating the relation between the change of cell density and the change of absorbance at 750 nm by using three initial algal cell densities of *C. vulgaris* grown during 72 h.

2.3 Analysis by Atomic Absorption Spectrometry

In this study, the bioaccumulation of mercury was quantified in algal biomass of *C. vulgaris* by using Atomic Absorption Spectrometry (AAS), which was determined as mg of Hg per mg of dry weight. The quantification of mercury was done using a Varian SpectrAA 220 FS system.

The configuration of the atomic absorption spectrometer possesses three basic required components that are shown in Figure 2.3 (Richard *et al.*, 1993):

(1) A light source,

(2) A sample cell,

(3) A means of specific light measurement.

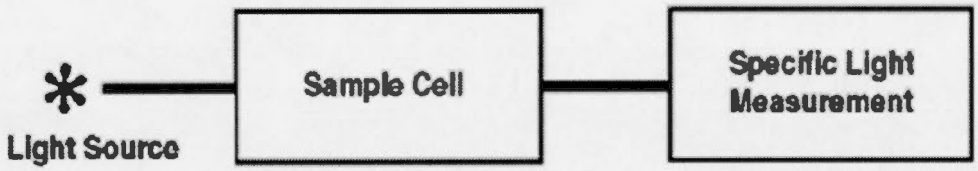


Figure 2.3 Technical requirements for absorption spectrometry (Richard *et al.*, 1993).

The components inside the atomic absorption system are illustrated in the Figure 2.3. There is a light source, a hollow cathode lamp that is designed to emit the atomic spectrum of a particular element, and specific lamps can be selected for analytical use depending on the element (Richard *et al.*, 1993).

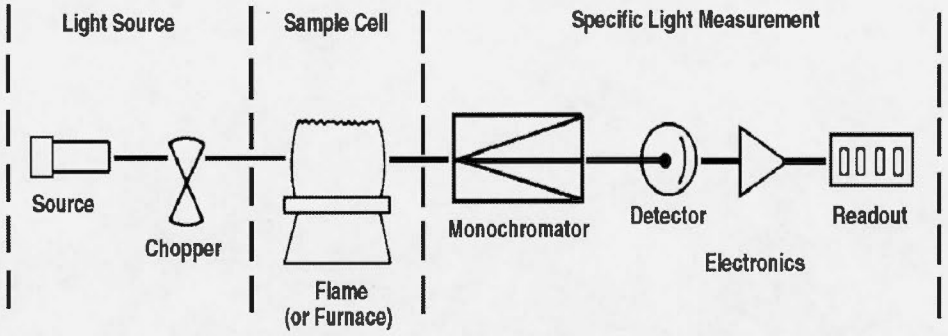


Figure 2.4 Basic representation of an atomic absorption spectrometer (Richard *et al.*, 1993).

2.4 Bioaccumulation of Hg in algal biomass

2.4.1 Standards used

The range of standard solutions was used according to the detection limit range of the atomic absorption spectrometry detector. All standard solutions were prepared in BBM with different concentrations of standard Hg(II): 200, 100, 50, 25, 10 and 0.5 mg/L. All solutions were stored at room temperature. The Figures 2.5 and 2.6 show the relation between the change of absorbance (Abs) at 253.652 ± 0.5 nm and the concentration of the standard solutions, for respectively 24, 48 and 72 h of experimental exposures.

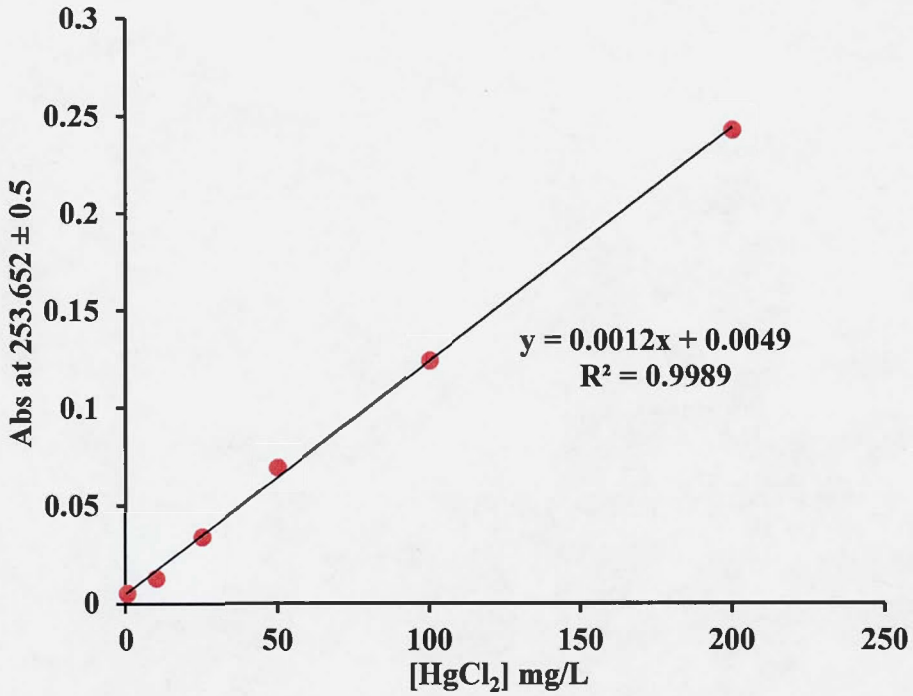


Figure 2.5 Standards curve of Hg(II) for 24 and 48 h of exposures.

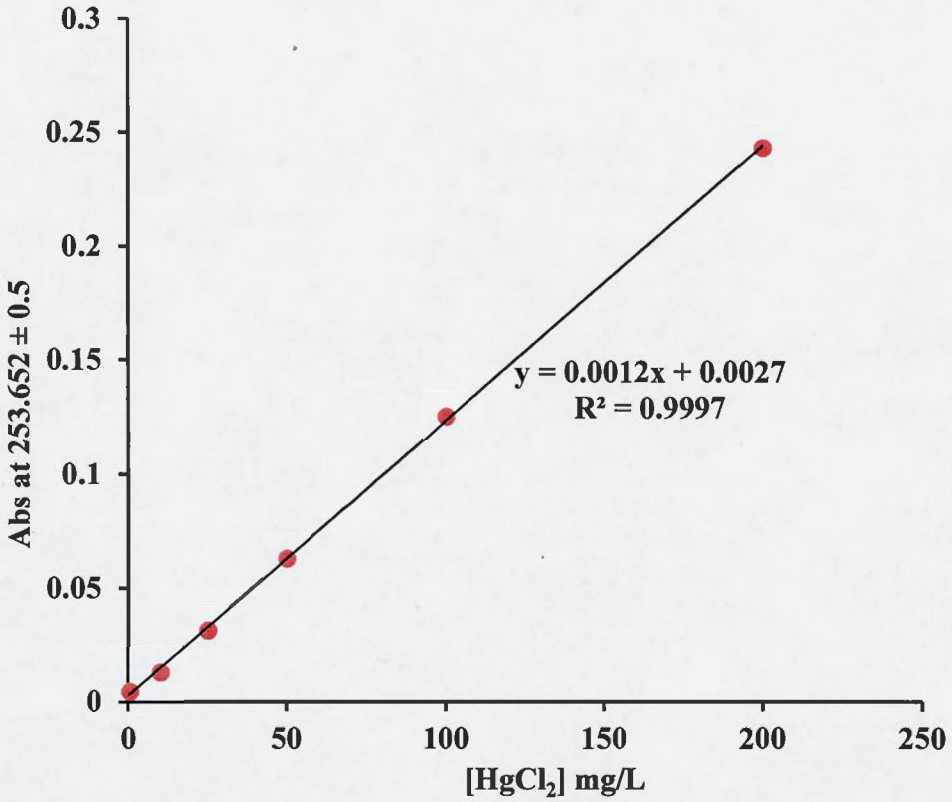


Figure 2.6 Standards curve of Hg(II) for 72 h of exposures.

2.4.2 Stock solutions of mercury

For experimental treatment conditions, stock solutions of $HgCl_2$ were prepared in the medium BBM having the following concentrations: 1, 10, 25, 35, 50 and 100 µM. All stocks solutions were stored at 4 °C.

2.5 Treatment conditions and preparation of samples

Before the preparation of experiments, all glassware and plastic bottles were cleaned by soaking with 10 % HCl for at least 24 h, and then rinsed three times with

de-ionised water and sterilized by autoclave. For all experiments, the initial cell density was 10^6 cells/mL in BBM, and algal samples were exposed to 0, 1, 10, 25, 35, 50 or 100 μM of $HgCl_2$ during 24, 48 and 72 h of exposure time. The total volume of BBM was always 20 mL, and all experiments were done in triplicates.

2.5.1 Determination of dry weight

Before measuring the dry weight, algal cells of *C. vulgaris* were exposed during 24-72 h to $HgCl_2$ as described previously in section 2.5: In the first step, the entire 20 mL of sample was filtered under gentle vacuum pressure (< 5 psi) to avoid breakage of cells and loss of biological material. Nitrocellulose paper filters of 47 mm in diameter and 0.8 – 1 μm pore size were employed for filtration and the recuperation of algal biomass. Before filtration, it was necessary to weight all filter papers. In the second step, all filter papers were put inside the oven for drying during 24 h at 100 °C, and then they were weight to determine the dry weight of algal biomass by subtracting the weight of the filter from this value.

2.6 Determination of phytochelatins and related thiol-peptides

For the analysis of the synthesis of thiol-rich peptides, algal cells of *C. vulgaris* were exposed 24-72 h to $HgCl_2$ as described previously in section 2.5. In this study, the synthesis of phytochelatins (PCs), cysteine (Cys), glutathione (GSH) and γ-glutamylcysteine (γ-EC) was determined in green alga *C. vulgaris* by HPLC separation coupled with a a diode array fluorescence detector.

2.7 High Performance Liquid Chromatography (HPLC) conditions

The separation of thiol-bimane derivatives was done using a reverse-phase C-18 column with 3-μm particle size, an injection valve with a 100-μL loop and the dectection was performed by fluorescence. For this seperation, chromatographic conditions were used according to Kawakami *et al.* (2006): Concerning solvents used, solvent A was composed of 0.1% trifluoroacetic acid (TFA) in water and solvent B of acetonitrile. The flow rate of the solvent pump was 1.0 mL/min. The gradient used was: 0–13 min, 10–21% B; 13–33 min, 21–35% B; 33–40 min, 35–100% B; 40–50 min, isocratic 100% B; 50–65 min, 100–10% B. These chromatographic conditions permitted the separation of derivatized GSH and PCs in a single run. In figure 2.7, we show the schematic representation of a HPLC (http://en.wikipedia.org, 2013). In this study, the HPLC system coupled with the fluorescence detector was from Agilent Technologies, model 1200 series.

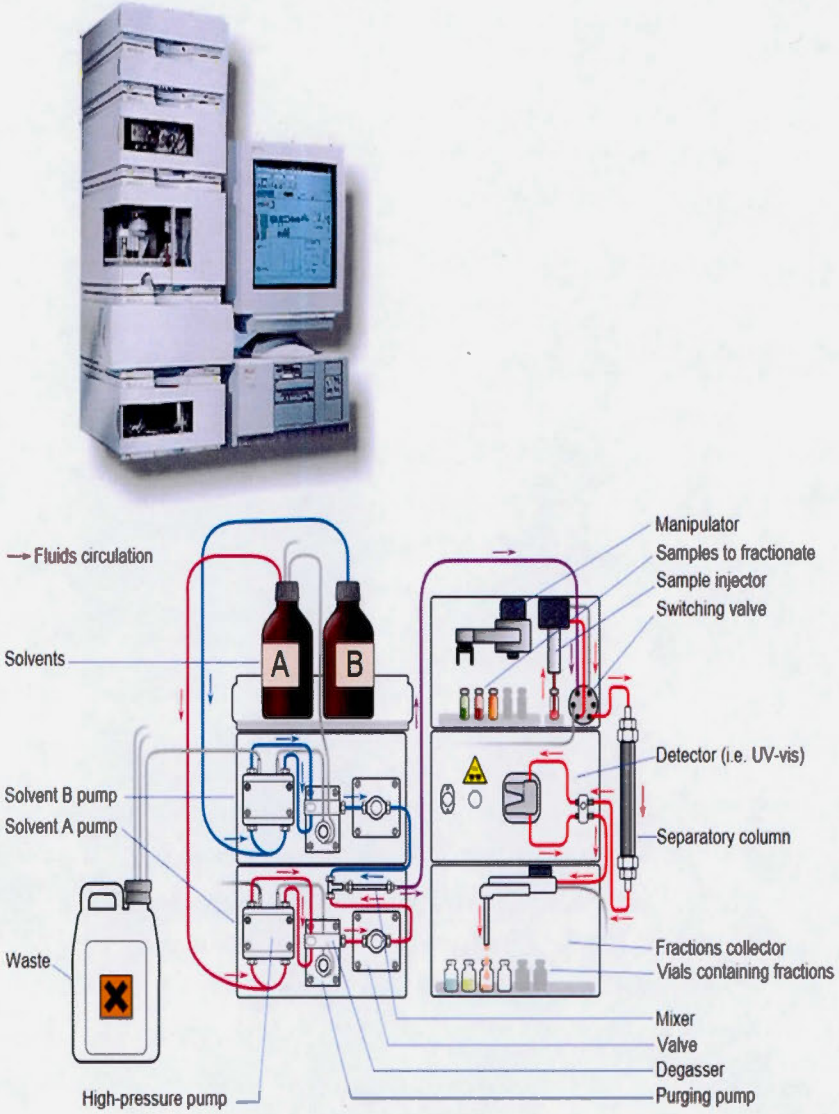


Figure 2.7 Schematic representation of the principle of HPLC (http://en.wikipedia.org, 2013).

Phytochelatin standards (PC_2, PC_3 and PC_4; having a purity > 95%) were obtained from Canpeptide Inc. (Montreal, Quebec, Canada). Others chemicals used in

this study were purchased from Sigma-Aldrich with the highest available purity for analytical grade. These chemicals were cysteine (Cys), glutathione (GSH), trifluoroacetic acid (TFA), ɣ-glutamylcysteine (ɣ-EC), trifluoroacetic acid (TFA), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), methanesulfonic acid (MSA), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), Diethylenetriamine-pentaacetic acid (DTPA), HPLC-grade acetonitrile (ACN) and monobromobimane (mBBr). The Hg(II) used in our experiments was as $HgCl_2$.

2.8 Samples collection, handling and preparation

For the determination of GSH and PCs, the preparation of the sample extracts was done according to this multi-step procedure: Sampling, sample filtration, extraction of thiols, thiol reduction, thiol derivatization, analysis of thiol derivatives by HPLC. The protocol of this approach for the analysis of GSH and PCs is summarised in Table 2.2 (Winters *et al.*, 1995; Rijstenbil and Wijnholds, 1996; Ahner *et al.*, 1997; Tang *et al.*, 2003; Kawakami *et al.*, 2006). According to these authors, more details are explained as follow: We used 20 mL of samples to ensure the collection of sufficient biomass exposed to $HgCl_2$ during 24 and 48 h, and then the biomass was collected by centrifugation at high speed (20 min, 13000 *g*, 4 °C). In the extraction of PCs and GSH, for avoiding the effects of enzymatic degradation of thiols and losses due to oxidation, HCl is added to promote the denaturation of enzymes which are capable of degrading thiol-peptides. To minimise the oxidation of the –SH groups of PCs and GSH caused by the effect of metals, DTPA was added. For the extraction of PCs and GSH from algal biomass, it was added 1.2 mL of 0.1 M HCl containing 5 mM of DTPA. Finally, algal cells were disrupted by ultrasonication (0 °C, 6 min). Cell extracts were centrifuged at high speed, 13000 *g* for 20 min at 4 °C, and the supernatant was collected. The reducing reagents were prepared in a

solution of 200 mM HEPES (pH 9) having 5 mM DTPA, then 650 µL of this solution was added to 250 µL of the collected supernatant. For the reduction process of thiols compounds, TCEP was used as indicated by the following reaction 1. To minimise the oxidation process, DTPA was added to the buffer solution.

$$(CH_3CH_2COOH)_3P: + RS\text{-}SR + H_2O \longrightarrow (CH_3CH_2COOH)_3P = O + 2RS\text{-}H \quad (1)$$

TCEP **oxidized thiol**

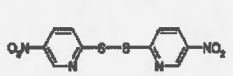
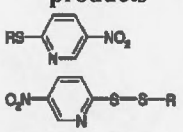
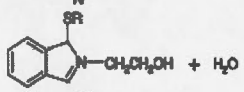
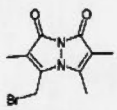
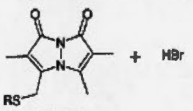
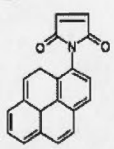
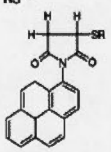
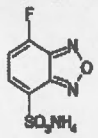
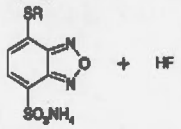
The application of a derivatization step for PCs and GSH permitted to increase the sensitivity of the analysis by using a derivatizing compound that have a minimum of interference. Many derivatizing reagents for PCs and corresponding thiol derivatives are available today and are listed in Table 2.3 (Kawakami *et al.*, 2006). In our protocol, the derivatization of PCs was done by using mBrB, a very common sulphur-specific fluorescent tag, which have a high sensitivity to naturally occurring PCs (Ahner *et al.*, 1997). The derivatization step of PCs and GSH with mBrB (in final concentration of 1 mM in acetonitrile) was carried out under dim light conditions at room temperature. This process permitted the fluorescence emission detection of PCs at 470 nm by using an excitation light at 380 nm.

Then after 15 min, the reaction products were stabilized with MSA at a final concentration of 0.1 M (Kawakami *et al.*, 2006). Finally, the product of the derivatization step was stored at 4 °C in the dark until HPLC analysis is undertaken, since it is well known that the mBrB can be stable for 6 weeks at 4 °C in darkness (Rijstenbil and Wijnholds, 1996; Tang *et al.*, 2003). In contrary to PCs which are stable for a month as bimane derivatives, GSH as a bimane derivative can have a 30% degradation after storage in the fridge (4 °C) for 15 days (see Fig. 2.8) (Kawakami *et al.*, 2006).

Table 2.2 Summary of the methodology for the analysis of phytochelatins and glutathione (oxidized + reduced forms) in natural waters (adapted from Winters *et al.*, 1995; Rijstenbil and Wijnholds, 1996; Ahner *et al.*, 1997; Tang *et al.*, 2003; Kawakami *et al.*, 2006).

Sample Collection : Acid-cleaned bottles.
Centrifugation : 13000 *g* during 20 min at 4 °C.
Extraction of thiols from filters : Addition of 1.2 mL of 0.1 M HCl and 5 mM DTPA in 1.5 mL microtube; Ultrasonication (0 °C; 6 min); Centrifugation (20 min; 13000 *g*; 4 °C).
Reduction, R-S-S-R + TCEP => 2 R-SH : Buffered with 650 µL of 200 mM HEPES and 5 mM DTPA (pH = 8.2); Addition of 25 µL of 20 mM TCEP.
Derivatization, R-SH + mBrB = R-S-mBrB + HBr : Addition of 10 µL of 100 mM mBrB in acetonitrile; Acidification with 100 µL of 1 M MSA.
HPLC Analysis : Seperation by Reverse phase using C-18 column; Fluorescence detection at 470 nm (excitation light at 380 nm).

Table 2.3 Reagents and detection methods used for the derivatization of thiols compounds (Kawakami *et al.*, 2006).

Reagents	Structures	Thiol derivatives and products	Detection	Comments
DTNP			UV	Not sensitive for PCs produced by phytoplankton
OPA			FL	Requires protection of SH moieties to avoid cross-reaction with primary amino groups
MBrB			FL	
NPM			FL	Can lead to formation of diastereomers
SBD-F			FL	

UV, Ultraviolet; FL, Fluorescence.

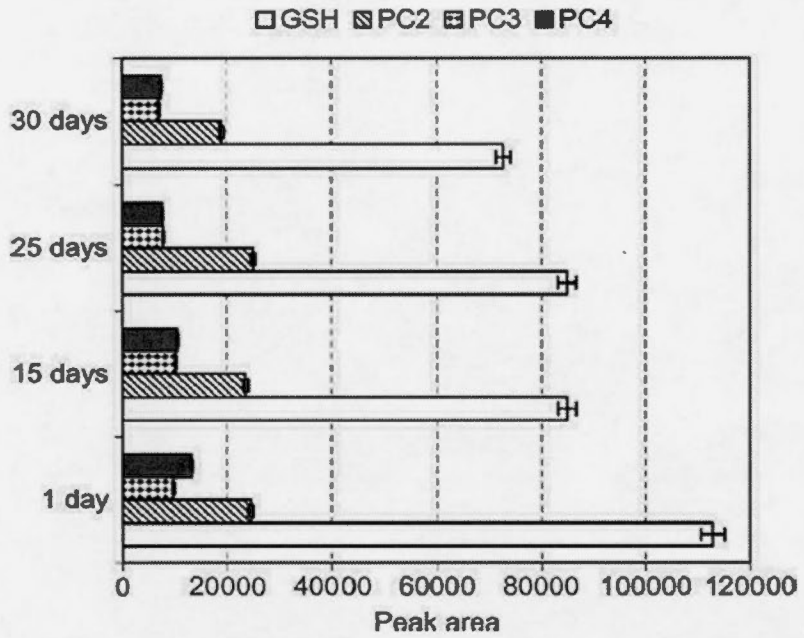


Figure 2.8 Stability of GSH and PCs as bimane derivative forms over a 30-days period (Kawakami *et al.*, 2006).

The standards of phytochelatins (PCs), cysteine (Cys), glutathione (GSH) and ɣ-glutamylcysteine (ɣ-EC) were prepared at concentrations from 0.1 to 0.8 mg/mL and also from 0.1 to 10 mM, respectively in working solutions containing 0.12 M HCl and 5 mM DTPA. To minimise the oxidation, standard solutions were made in MSA solution. The standard curves of GSH, cysteine, ɣ-glutamylcysteine, PC_2, PC_3, PC_4 and PC_5 were determined by fluorescence detection after HPLC separation of the extracts, permitting the identification and the quantification of these compounds (Figures 2.9, 2.10, 2.11, 2.12, 2.13 and 2.14).

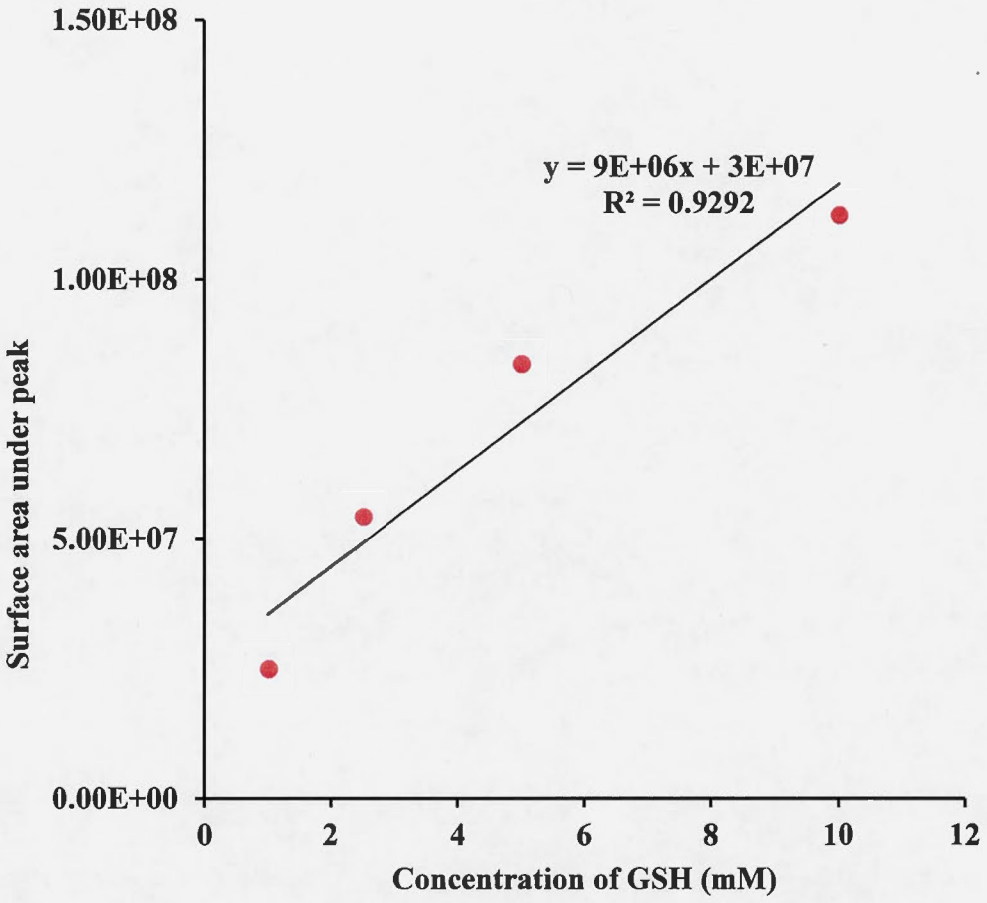


Figure 2.9 The standard curve of glutathione (GSH) determined by HPLC and fluorescence detection.

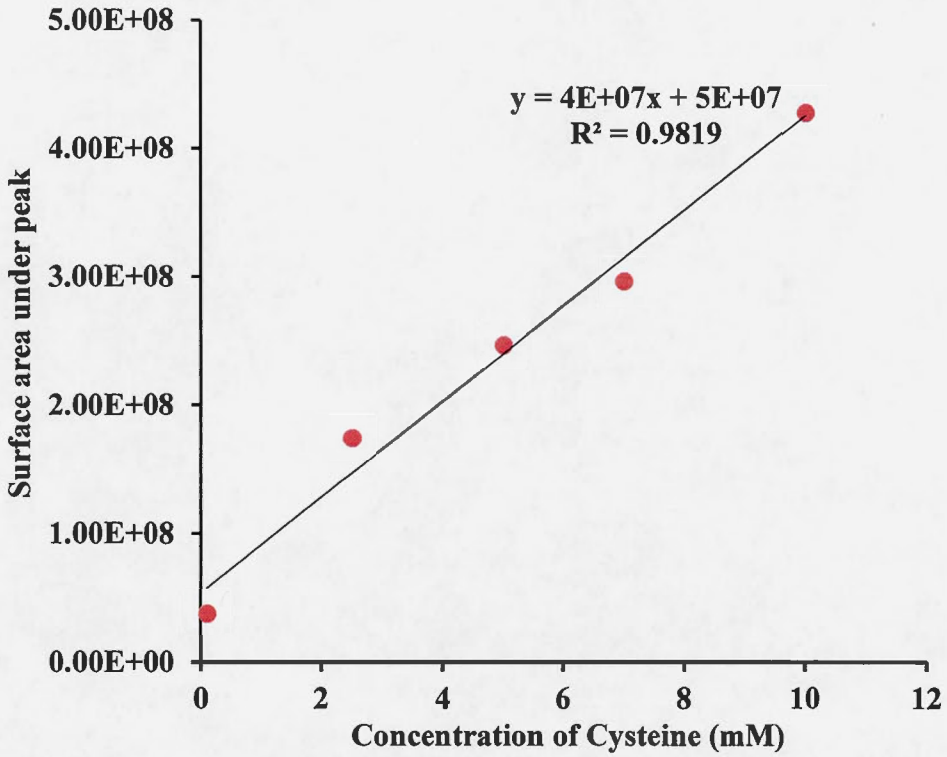


Figure 2.10 The standard curve of Cysteine determined by HPLC and fluorescence detection.

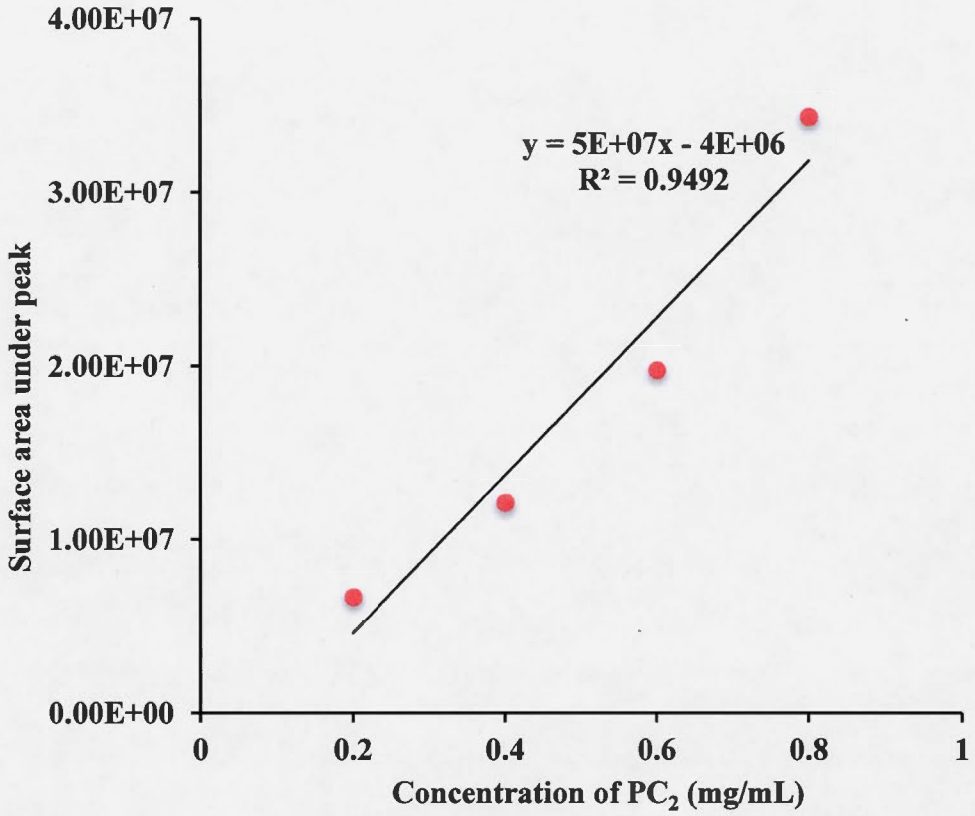


Figure 2.11 The standard curve of PC_2 determined by HPLC and fluorescence detection.

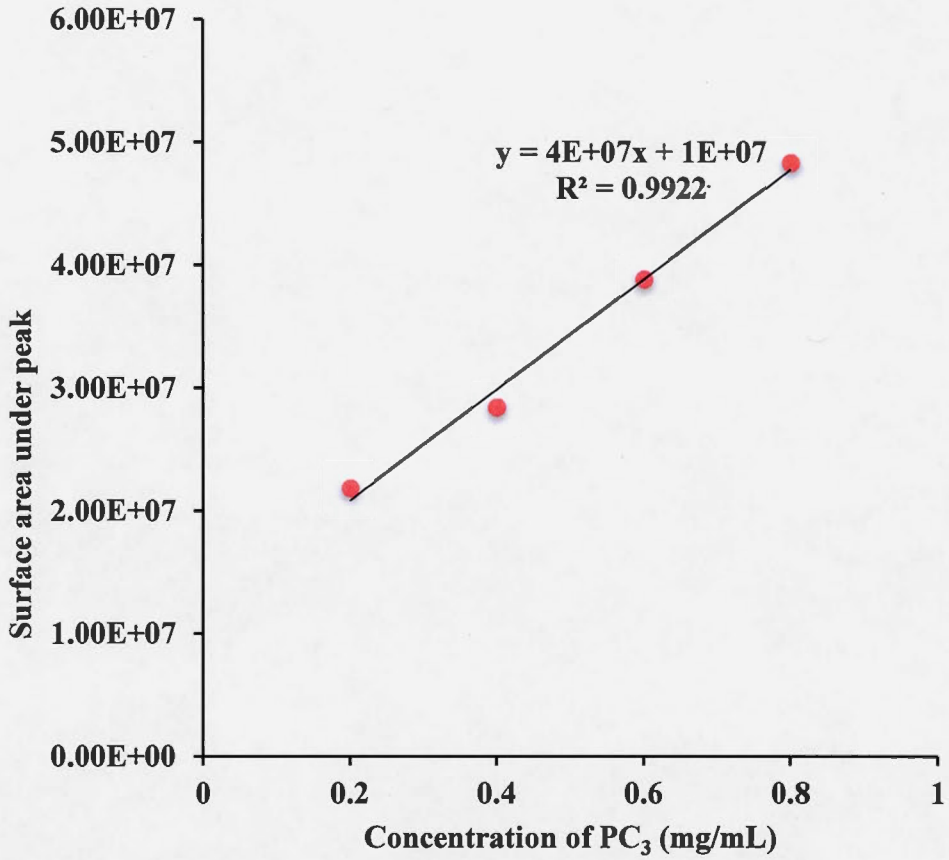


Figure 2.12 The standard curve of PC_3 determined by HPLC and fluorescence detection.

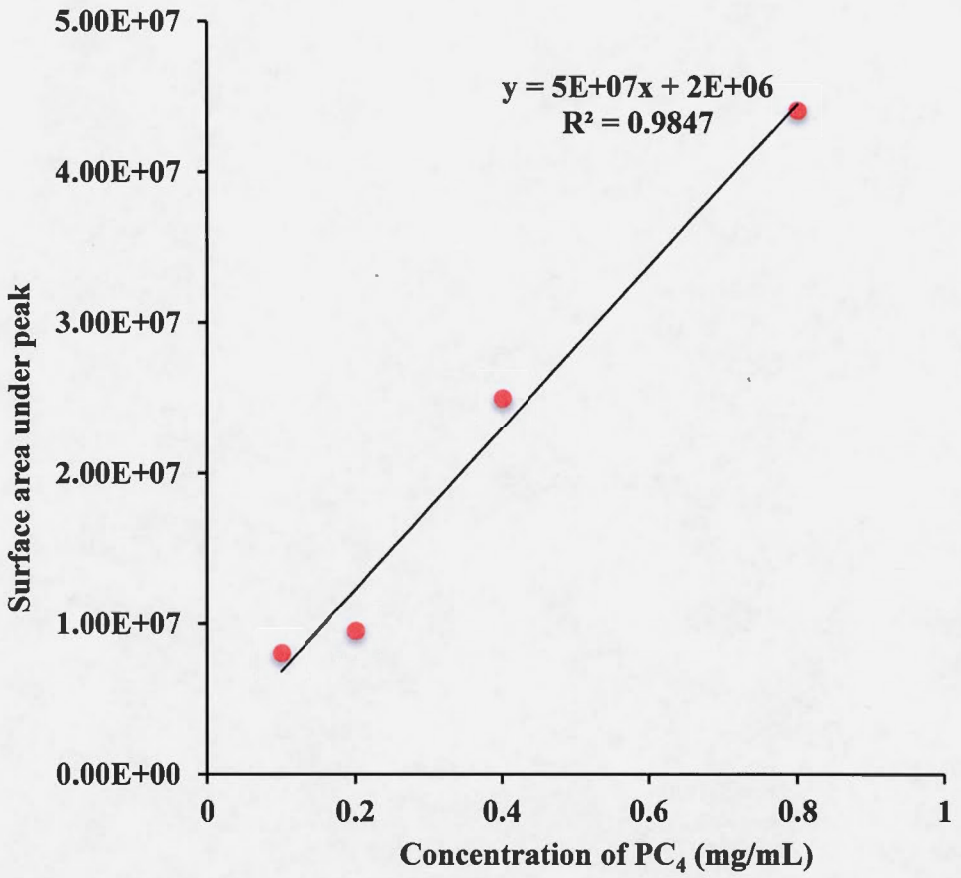


Figure 2.13 The standard curve of PC_4 determined by HPLC and fluorescence detection.

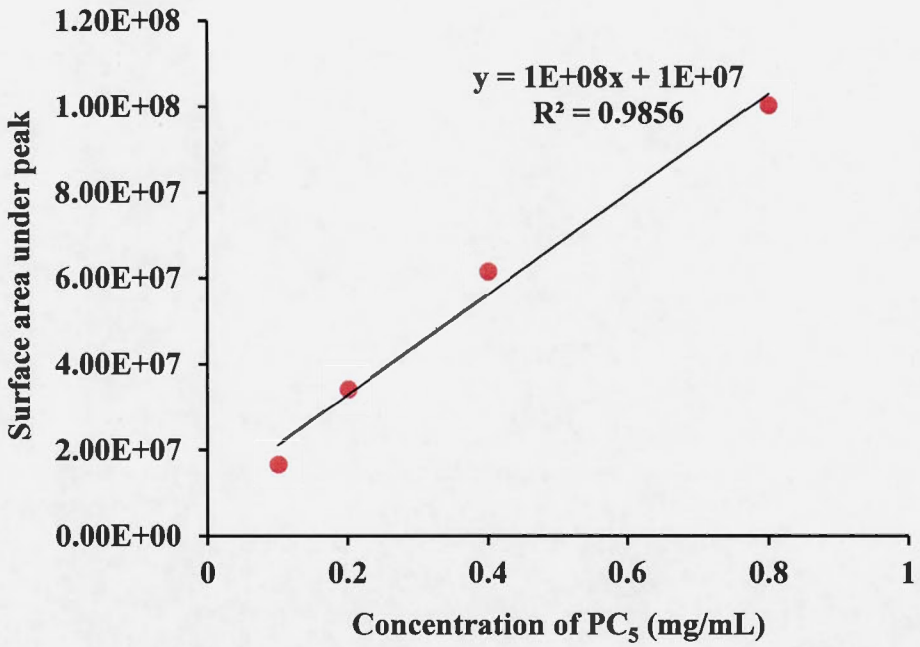


Figure 2.14 The standard curve of PC_5 determined by HPLC and fluorescence detection.

2.9 Photosynthesis measurement

2.9.1 Total chlorophyll content

The treatment condition is the same as described previously in section 2.5, but only three concentrations of $HgCl_2$ were employed for exposure: 10, 50 and 100 μM. After 24 h of exposure, 1 mL of algal sample was centrifuged at 7600 *g* for 5 min. Then, 1 mL of 100% methanol was added to the pellet which was placed in a hot water bath at 65 °C for 10 min. The last step was to centrifuge again for 5 min before taken the absorbance measurements (λ = 652.4, 665.2) with a spectrophotometer.

The amount of total chlorophyll was measured according to the following formula of Lichtenthaler (1987):
[24.93 × Abs 652.4 + 1.44 × Abs 665.2] × [vol. of methanol/vol. of algal sample]

With the total chlorophyll obtained, we were able to determine the volume necessary to filter 10 μg of total chlorophyll for each fluorometric measurement.

2.9.2 Plant Efficiency Analyzer Fluorometer (Handy PEA)

The Handy PEA (Hansatech Ltd., Norfolk, UK) is a portable fluorescence measuring instrument for plant leaves and algae with a high time resolution fluorescence detection of fast induction kinetics. Also, it uses saturating light with high intensity focused LEDs array for the determination of the maximum fluorescence level. The chlorophyll fluorescence signal is then digitized for analysis. This instrument is illustrated in figure 2.15 (http://hansatech-instruments.com).

Figure 2.15 Handy PEA (http://hansatech-instruments.com).

2.9.3 Fluorescence measurement technique and parameters

Prior to fluorescence measurements, algal samples were dark-adapted for 20 min by using manual stirring to keep oxygenation in liquid. Volume of algal samples corresponding to 10 μg of total Chl were preleved and filter using a low pressure filtration to be place algal cells on glass fiber filter (Millipore No. AP2001300). Then, rapid Chl *a* fluorescence induction from 10 μs to 1 s was measured with the Handy-PEA by using a saturating light irradiance of 3500 μmol of photons m^{-2} s^{-1}.

Different fluorescence intensity levels were determined according to Strasser *et al.* (2004) : Fluorescence intensity at 20 μs was considered to be the O transient, noted as $F_{20\mu s}$; Variable fluorescence intensities at J and I transients were determined at 2 ms (F_{2ms}) and 30 ms (F_{30ms}), respectively; The maximum fluorescence yield reached maximal value of fluorescence intensity under saturating illumination. Based on these fluorescence intensity levels, several photosynthetic-based fluorescence parameters were determined (Force *et al.*, 2003; Strasser *et al.*, 2004) : The PSII maximal quantum yield, indicating the efficiency of light energy transfer to PSII primary photochemistry, was determined by the ratio between variable fluorescence emission (F_V) and maximal fluorescence level (F_M), as $F_V/F_M = (F_M - F_{20\mu s}) / F_M$; The ratio between absorption of photons by chlorophyll antenna complex and

photochemically active PSII reaction centers was determined as ABS/RC = (M_O/V_J) / (F_V/F_M); The parameter M_O representing the net rate of PSII reduced by electron transport was determined as $M_O = [4 \times (F_{300\mu s} - F_{20\mu s}) / (F_M - F_{20\mu s})]$; The parameter V_J representing the proportion of Q_A reduced relative to the plastoquinone pool was evaluated as $V_J = [(F_{2ms} - F_{20\mu s}) / (F_M - F_{20\mu s})]$; The performance index of PSII activity was evaluated as P.I. = $[(F_{2ms} - F_{20\mu s}) / 4 \times (F_{300\mu s} - F_{20\mu s}) \times F_V/F_M] \times (F_V/F_{20\mu s}) \times ((1-V_J)/V_J)$.

2.10 Statistical analysis

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

CHAPTER III

RESULTS

3.1 Inhibition of cellular division by Hg (II)

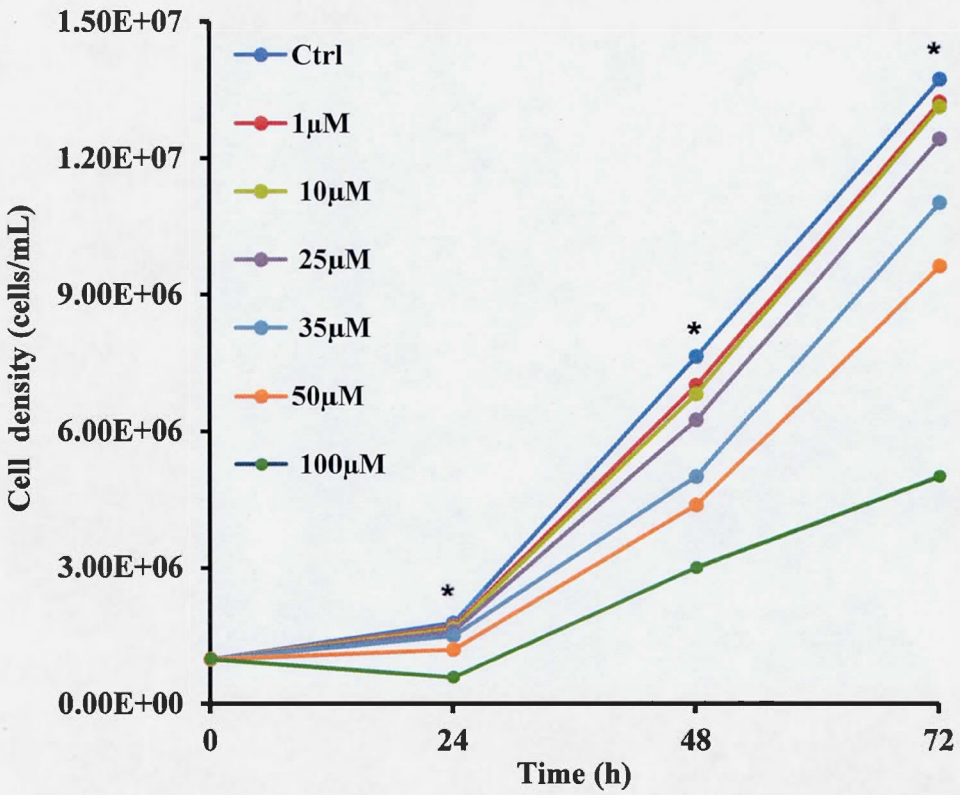


Figure 3.1 Changes in cell density of alga *C. vulgaris* exposed during 72 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$*: At 24 h, 50-100 µM; At 48-72 h, 25-100 µM.

In previous toxicological studies about the effect of mercury on several algal species, the inhibition of cellular division was an important cellular parameter used as a global indicator of the algal physiological state (Elbaz *et al.*, 2010; Afkar *et al.*, 2010; Wu and Wang, 2011; Wu and Wang, 2012). Therefore, we monitored in this study the change of algal cell density during 72 h in order to determine the effect of mercury on the state of cellular division. When algal cells were exposed during 72 h to different concentrations of $HgCl_2$, the change in cell density was measured every 24 h, and treated samples were compared to the control (Fig. 3.1). Here, the effect of 1, 10, 25, 35, 50 and 100 µM of $HgCl_2$ on the state of cellular division of alga *C. vulgaris* was examined. At 24 h of exposure, we noticed only a significant inhibitory effect on cellular division for algal cells treated to 100 µM of $HgCl_2$ in comparison to the control. Indeed, under this treatment condition, the cell density decreased by 3 times compared to control. However, when algal cells of *C. vulgaris* were exposed during 48 and 72 h, a significant inhibition of cellular division was observed even at low concentration (25 µM) of mercury (Fig. 3.1). But a strong inhibition of cellular division was noticed for treatment to 50 and 100 µM of $HgCl_2$ in comparison to the control. At 48 h, the cell density was respectively decreasing by 1.7 and 2.5 times in comparison to the control for treatment to 50 and 100 µM of $HgCl_2$. At 72 h, the cell density was respectively decreasing by 1.4 and 2.7 times in comparison to the control for treatment to 50 and 100 µM of $HgCl_2$. Therefore, the exposure to $HgCl_2$ caused a significant toxicity impact on algal cells of *C. vulgaris* at 48 and 72 h which was dependent to the concentration tested. A previous study demonstrated that the growth of *C. reinhardtii* was significantly inhibited when exposed during 96 h to 4-8 µM of $HgCl_2$ (Elbaz *et al.*, 2010). Indeed, they showed that the growth of algal cells was inhibited by 56 % in comparison to the control when treated to 4 µM of $HgCl_2$. In another study, three algal species were exposed during 72 h to different

concentrations of $HgCl_2$ (Wu and Wang, 2011). This work showed that the growth rate of *T. pseudonana*, *C. autotrophica* and *I. galbana* decreased significantly when algal cells were exposed to 36, 30 and 15 $\mu g\ L^{-1}$. However, there was a difference in sensitivity where *C. autotrophica* was the most tolerant and *T. pseudonana* the least tolerant to $HgCl_2$. Based on our results and the one found in the scientific litterature, it appears that there is a difference in sensitivity to Hg (II) depending to the algal species and the experimental conditions.

3.2 Mercury accumulation in biomass of alga *C. vulgaris*

In order to investigate the bioaccumulation efficiency of mercury by algal cells of *C. vulgaris*, we determined the content of mercury in algal biomass when *C. vulgaris* was exposed during 72 h to different concentrations of $HgCl_2$ (Fig. 3.2). Our results showed that the change of mercury content was directly related to the concentration tested of $HgCl_2$ and the time of exposure. The mercury accumulation in algal biomass of *C. vulgaris* was similar at 48 and 72 h of exposure to different concentrations of $HgCl_2$. Furthermore, when algal cells of *C. vulgaris* were exposed to low concentrations of $HgCl_2$ (1-35 μM), there were no significant differences between the accumulated content of mercury during 24 h in comparison to longer time of exposure. However, the level of mercury content in algal biomass was significantly different for cells treated during 24 h to 50-100 μM of $HgCl_2$ in comparison to 48 and 72 h. Under these treatment concentration conditions, the highest efficiency of bioaccumulation was reached already at 24 h. Based on these results, we concluded that the bioaccumulation of mercury in algal biomass reached the maximum level when *C. vulgaris* was exposed during 24 h to 100 μM of $HgCl_2$. In a previous study on marine phytoplankton, similar results on the bioaccumulation effect of mercury were noticed concerning the change in intracellular mercury

concentration (mol $cell^{-1}$) for *T. weissflogii* exposed during 96 h to two concentrations of $HgCl_2$, 8.4 and 222 µg/L. Authors found that the intracellular mercury concentrations decreased after 24 h and with the increasing time of exposure, especially at the highest concentration. According to their results, they found out that the highest efficiency of bioaccumulation of mercury was reached at 24 h and for the highest concentration of $HgCl_2$ tested, 222 µg/L (Wu and Wang, 2012).

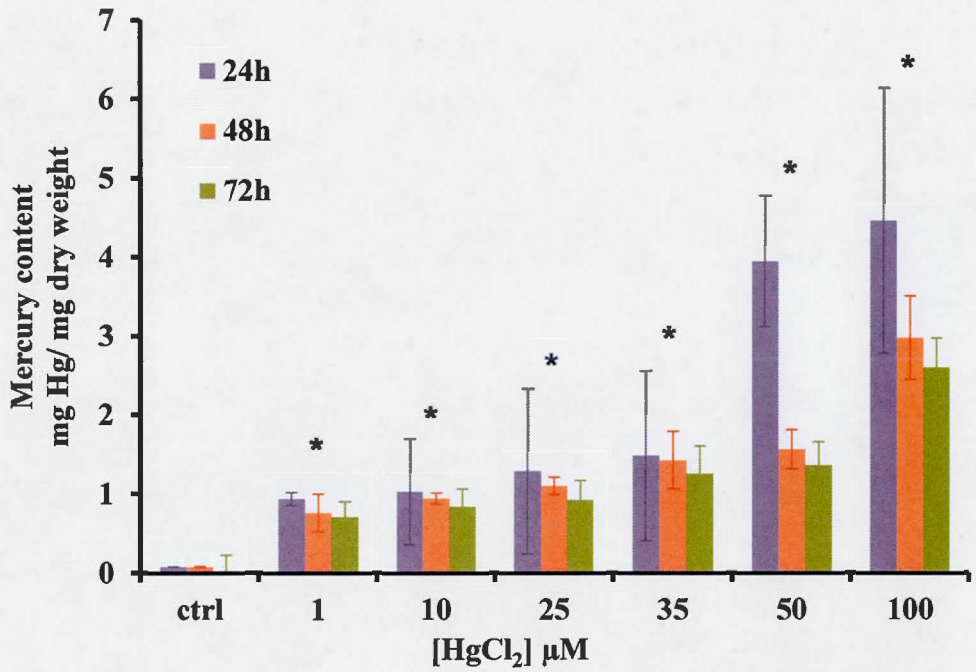


Figure 3.2 Accumulation of Hg (II) in algal biomass of *C. vulgaris* exposed during 72 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$ *.

3.3 Effect of Hg(II) on photosynthetic activity of *C. vulgaris*

3.3.1 Change in total chlorophyll content

Based on our results concerning cellular division and bioaccumulation efficiency, the change in the total chlorophyll content was determined when algal cells of *C. vulgaris* were exposed during 48 h to different concentrations of $HgCl_2$. The methodological approach used was described in the "Material and Methods" chapter. The change in total Chl content, presented in figure 3.3, showed a significant decrease in Chl content in comparison to the control for *C. vulgaris* exposed during 48 h to 10-100 μM of $HgCl_2$. Only 10 μM of $HgCl_2$ during 24 h was not able to cause a significant decrease in Chl content in comparison to the control. The control sample presented a higher Chl content at 48 compared to 24 h due to the increase in cell density. Therefore, the effect of mercury caused a decrease in total Chl content dependent to the tested concentration, indicating that the bioaccumulation of mercury inhibited the biosynthesis of chlorophylls which may affect the function of the photosystems light-harvesting energy transfer of the photosynthetic apparatus. In a previous study, the sensitivity of the green alga species *C. reinhardtii* was investigated, and the effect of different concentrations of $HgCl_2$ (1-8 μM) was examined during 96 h on the change of the cellular chlorophyll content (Elbaz *et al.*, 2010). According to their results, the amount of total chlorophyll decreased in direct relation with the increasing concentration and exposure of mercury. At low concentration of $HgCl_2$ (4 μM), a decrease on the amount of Chl compared to control was shown by 58.6 % of the control (Elbaz *et al.*, 2010).

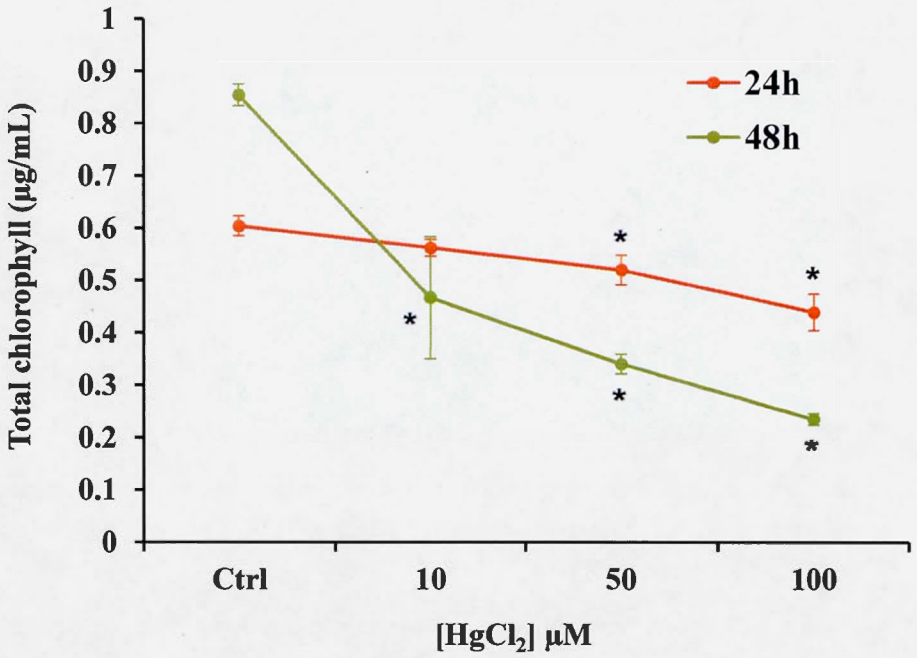
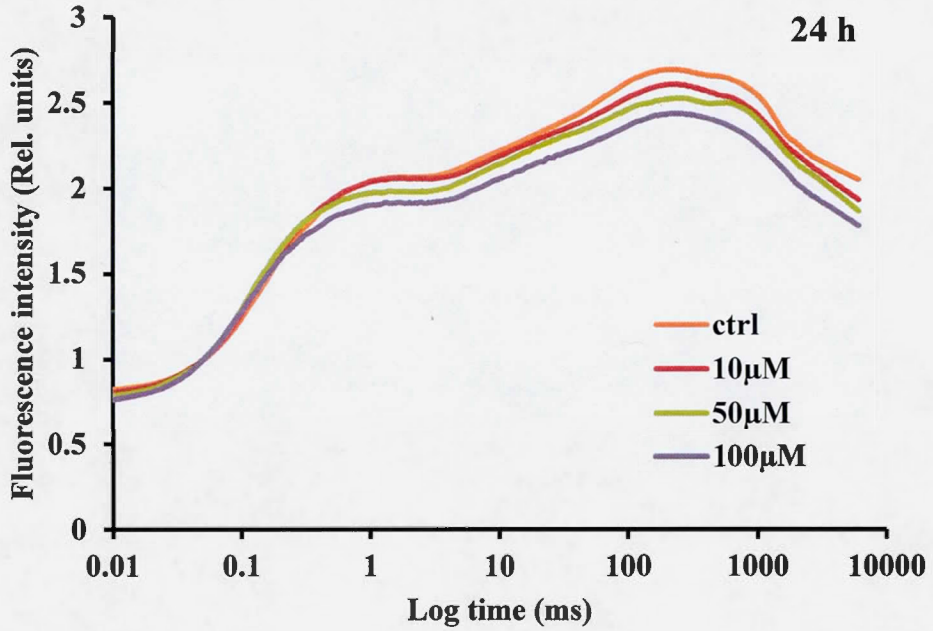


Figure 3.3 Change in the total chlorophyll content of alga *C. vulgaris* exposed during 48 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$*.

3.4 Rapid chlorophyll fluorescence emission

In this study, the change of the chlorophyll fluorescence emission was monitored when *C. vulgaris* was exposed during 24-48 h to different concentrations of $HgCl_2$. The 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 biomass growth. 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 (Popovic *et al.*, 2003, and see the introduction section). Therefore, fluorescence measurements was used here as an indication of PSII photochemistry and electron transport activity, providing information of metals interaction with photosynthesis at molecular level. In Figure 3.4, results showed the toxic effect of different concentrations of $HgCl_2$ at both 24 and 48 h of exposure on PSII photochemistry indicated by the change of variable fluorescence intensity.



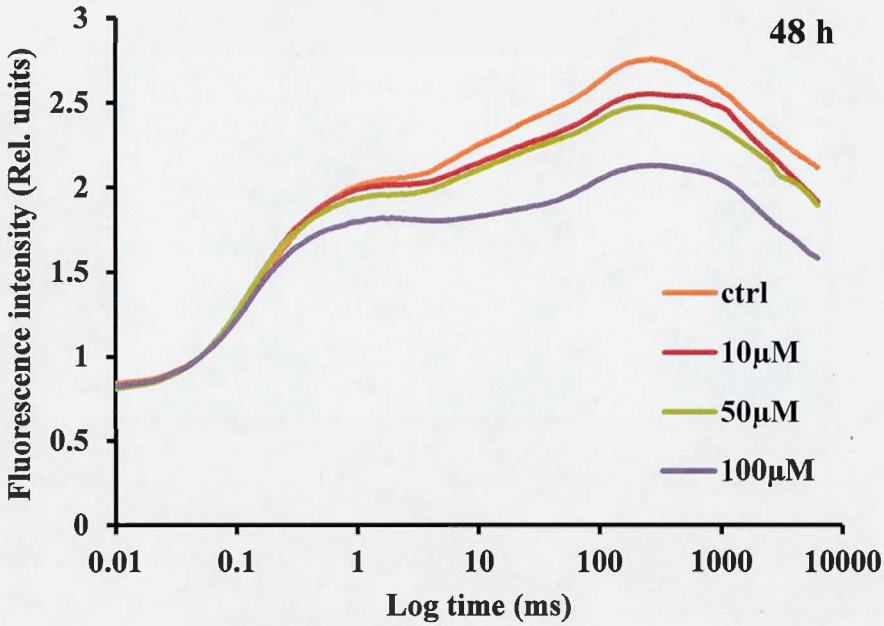


Figure 3.4 Change in the rapid chlorophyll fluorescence emission in *C. vulgaris* exposed during 48 h to different concentrations of $HgCl_2$.

According to these results, a decrease in the variable fluorescence intensity of PSII was significantly noticed in dependence to the tested concentration of $HgCl_2$ and the time of exposure. The effect of mercury was stronger at 48 h compared to 24 h of exposure. Indeed at 24 h, there was not a significant difference between the fluorescence intensity of algal cells treated to the highest concentration (100 µM) of $HgCl_2$ in comparison to the control, indicating a low inhibitory effect of mercury on PSII reaction center. However at 48 h, the inhibitory effect of mercury on PSII photochemical reactions was stronger, especially for the highest concentration of mercury (100 µM) in comparison to the control. Therefore, our results clearly indicated that, when algal cells of *C. vulgaris* was exposed during 48 h to $HgCl_2$, the

photochemical reactions of PSII, including the absorbed light energy transfer and electron transport activity, were altered by the bioaccumulation effect of mercury. In previous studies, the effect of mercury on algal photosynthesis was demonstrated by the alteration of PSII photochemical reactions and it was shown by a strong decrease of the chlorophyll fluorescence intensity (Juneau and Popovic, 1999; Juneau *et al.*, 2001; Popovic *et al.*, 2003). Moreover for another heavy metal, the bioaccumulation effect of Cd^{2+} (0 - 4.62 µM) during 24 h on algal cells of *C. reinhardtii* showed a similar decrease in the variable fluorescence intensity at all transients of the rapid induction of Chl *a* fluorescence, which was directly related to the tested Cd^{2+} concentrations: This decrease in variable fluorescence intensity caused by Cd^{2+} toxicity was indicative of a diminished proportion of active PSII reaction centres able to perform primary photochemistry and electron transport activity (Perreault *et al.*, 2011).

3.5 Change in photosynthetic based fluorescence parameters

The analysis of the rapid induction of chlorophyll fluorescence permitted to estimate several photosynthetic parameters associated with the activity of PSII photochemical reactions. For more details, see "Material and Methods" section. The change in the relative variable fluorescence at J transient (V_J) was monitored when alga *C. vulgaris* was exposed during 24-48 h to different concentrations of $HgCl_2$. The parameter V_J represents the dependent variable fluorescence reduction of Q_A and is related to the relative value of fluorescence intensity at J transient. Therefore, it is indicative of the accumulation of the reduced PSII primary electron acceptor, Q_A.

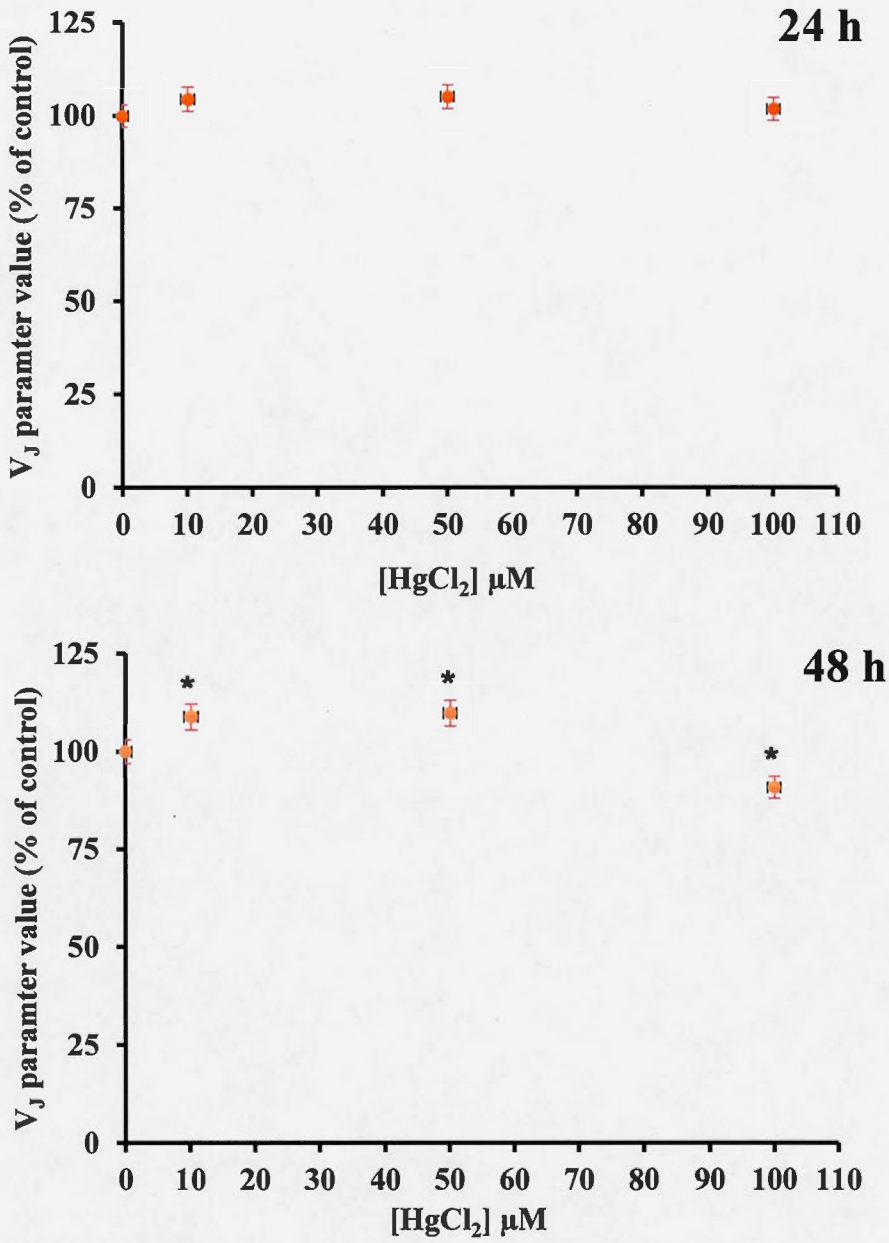


Figure 3.5 Change in the relative variable fluorescence at J transient (V_J) of alga *C. vulgaris* exposed during 48 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$ *.

The figure 3.5 shows the effect of different concentrations of $HgCl_2$ (10, 50 and 100 µM) on PSII electron transport up to Q_A reduction during 24 and 48 h of exposure time. During 24 h, the value of V_J increased in comparison to the control by 4 % for the low treatment concentration of 10 µM of $HgCl_2$, 5 % for 50 µM of $HgCl_2$ and 1 % for the highest concentration of $HgCl_2$ (100 µM). This result indicated a low non significant effect of mercury on the activity of electron flux up to the electron transporter Q_A. However, at 48 h of exposure time, we noticed that the inhibitory effect of mercury was significantly stronger in comparison to 24 h. Moreover, the value of V_J in the highest concentration decreased in comparison to the lower concentration, because of the inhibitory effect of mercury on PSII reaction center. When algal cells of *C. vulgaris* were exposed during 48 h to 10-50 µM of $HgCl_2$, the value of V_J was increased of about 10 % compared to control, indicating an inhibitory effect of mercury on electron transport carriers of PSII. However, the value of V_J significantly decreased by 9 % for the highest concentration of $HgCl_2$ (100 µM) in comparison to the control, showing the inactivation of some PSII reaction centers which are not participating in primary photochemistry and electron transport activity. In a previous study, the toxicity effect of herbicide isoproturon (0-500 µg/L) was investigated on green alga *Scenedesmus obliquus* during 24 h of exposure time (Dewez *et al.*, 2008). Results of this study showed that increasing the tested concentration of isoproturon led to the increase of V_J value due to the binding process of this herbicide with D1 protein of PSII reaction center, causing a strong inhibition of PSII electron transport.

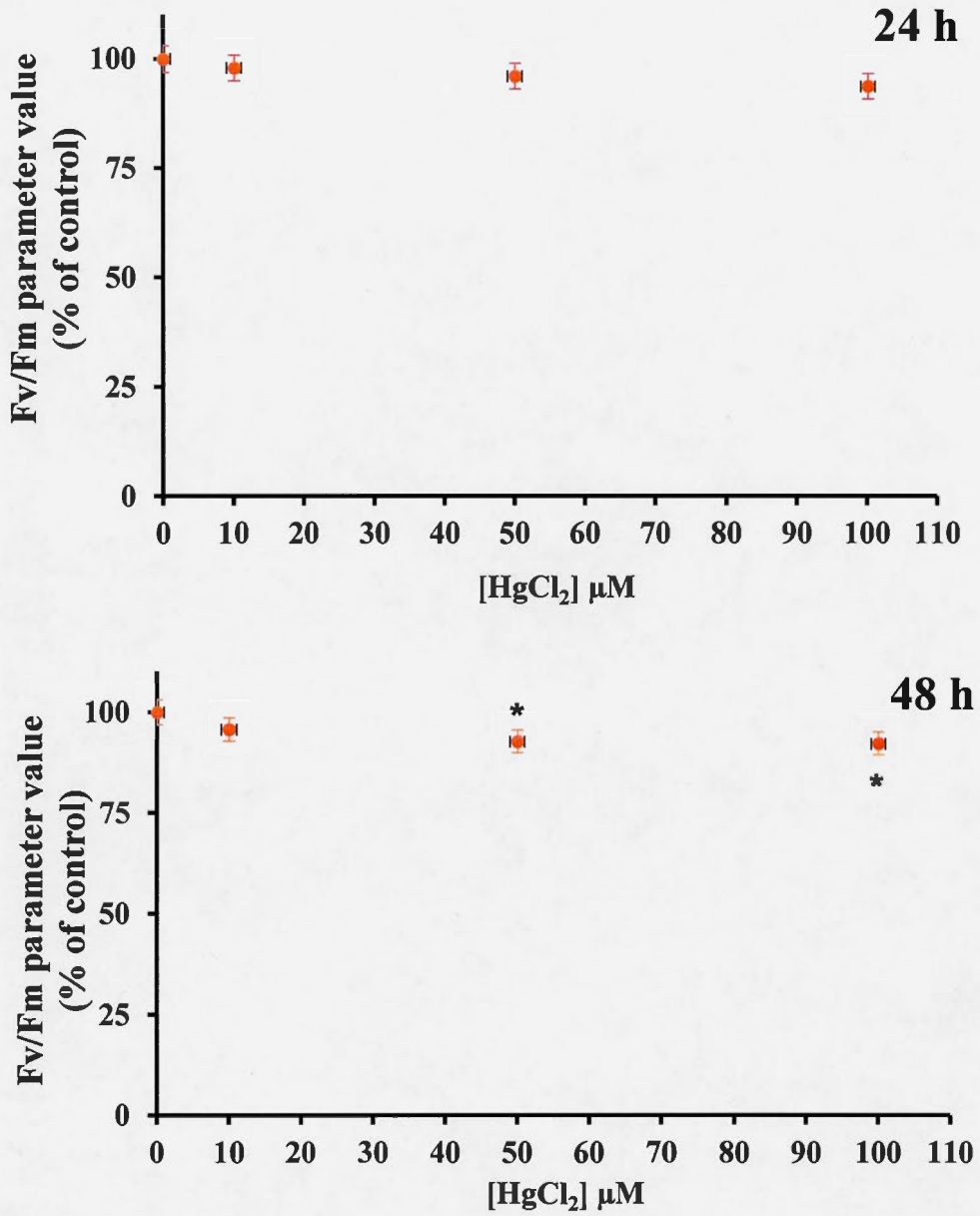


Figure 3.6 Change in the maximal PSII quantum yield (Fv/Fm) of alga *C. vulgaris* exposed during 48 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$ *.

The change in the maximal PSII quantum yield (Fv/Fm) was also investigated when alga *C. vulgaris* was exposed to different concentrations of $HgCl_2$. In figure 3.6, the change in the parameter Fv/Fm is shown for alga *C. vulgaris* treated at 24 and 48 h to $HgCl_2$. After 24 h of exposure, the value of Fv/Fm decreased slightly in relation to the concentration of $HgCl_2$, which was by 7 % compared to control when alga *C. vulgaris* was treated to the highest concentration of $HgCl_2$ (100 μM). This indicated a negligeable toxicity effect of mercury on the capacity of PSII reaction center to convert absorbed light energy into charge separation and electron transport activity at 24 h of exposure. However, at 48 h of exposure time, the effect of mercury was not stronger on the PSII reaction center. The value of Fv/Fm decreased about 8 % in comparison to control for cells treated to the highest concentration of $HgCl_2$ (100 μM). Results of previous studies on the toxicity effect of different concentration of copper (0-20 μM) during 24 h exposure time on green alga *Chlorella vulgaris* showed a direct relation between the decrease in the value of Fv/Fm with the increasing tested concentration of copper, which was explained by its mechanism of toxicity on the function of the water splitting system and the photochemical reactions of PSII (Perreault *et al.*, 2010; Oukarroum *et al.*, 2012).

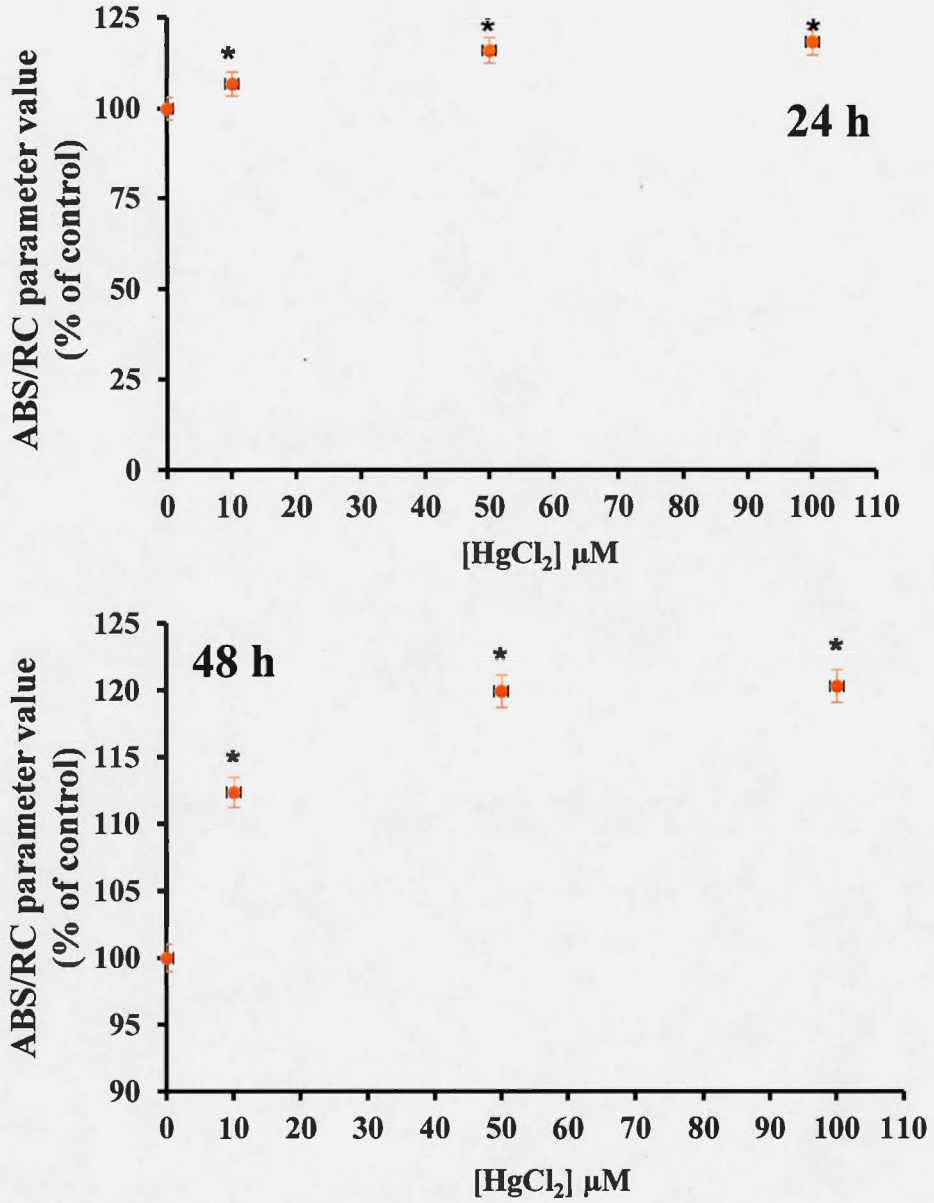
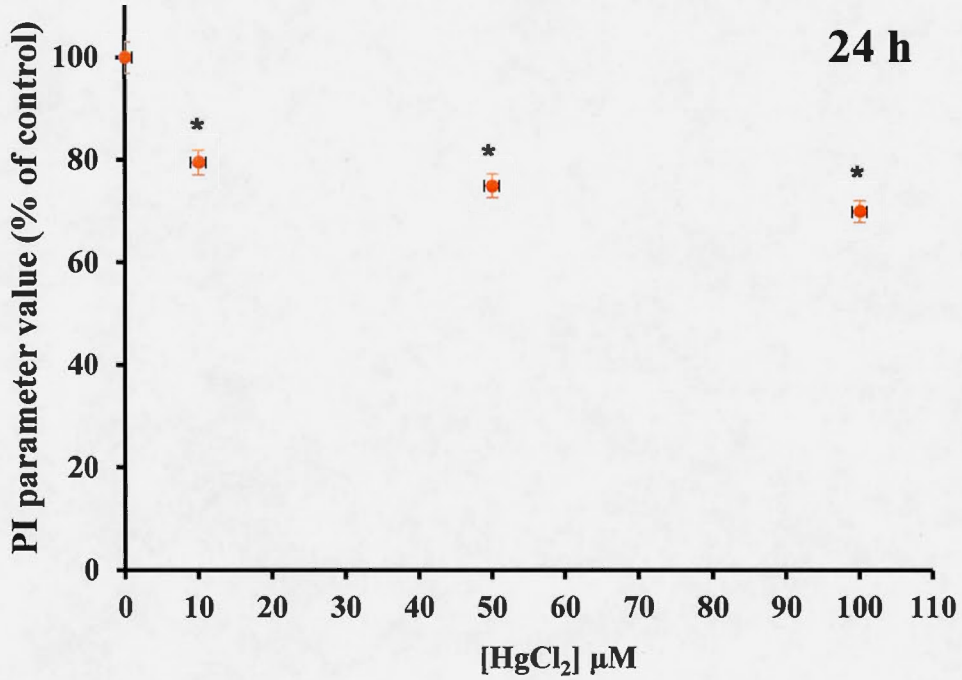


Figure 3.7 Change in the effective transfer of absorbed-light energy from antenna complexes (ABS) to PSII reaction centres (RC) of alga *C. vulgaris* exposed during 48 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$*.

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 (Appenroth *et al.*, 2001; Strasser *et al.*, 2004). In figure 3.7, we presented results of the change in the ABS/RC parameter when alga *C. vulgaris* was exposed during 48 h to different concentrations of $HgCl_2$. After 24 h of exposure, the value of ABS/RC increased by 7 % compared to control for the lowest concentration of $HgCl_2$ (10 µM) and 16 % for 50 µM of $HgCl_2$. The highest value of ABS/RC was reached for the highest concentration of $HgCl_2$ (100 µM) as 18 % compared to control, showing a change in energy transfer through non-photochemical pathway due to inactive PSII reaction centers induced by mercury effect. At 48 h of exposure, the value of ABS/RC increased even much higher in comparison to the same treatment condition at 24 h.

According to our results, this parameter was the most sensitive parameter for assessing the amount of inactive PSII reaction centers caused by mercury effect. It is well known that high amount of inactive PSII reaction centers lead to an increase of waste energy through non-photochemical pathways. Indeed, mercury had a direct effect on energy transfer through PSII reaction center. Indeed by inactivating some PSII reaction centers, the proportion of chlorophylls in antenna available per remaining active PSII reaction centers increased (Perreault *et al.*, 2010). Moreover, the change of the value of ABS/RC at 48 h increased by 12 % and 19 % in comparison to control respectively for 10 and 50 µM of $HgCl_2$ (Figure 3.7). At the highest concentration of $HgCl_2$ (100 µM), the value of ABS/RC increased by 20.5 % compared to control. This change is caused by significant changes in the energy dissipation *via* non-photochemical pathways, which indicated a higher proportion of

inactive PSII reaction centers at 48 h compared to 24 h. In a previous study, the toxicity effect of isoproturon was investigated on green algae *Scenedesmus obliquus* during 24 h of exposure time, showing an increase in ABS/RC values which was dependent to the concentration (0-500 μM) of isoproturon. This increase in ABS/RC values induced by isoproturon effect was suggested to be caused by the decrease of active PSII reaction centers (Lavergne and Lecci, 1993; Perreault *et al.*, 2010).



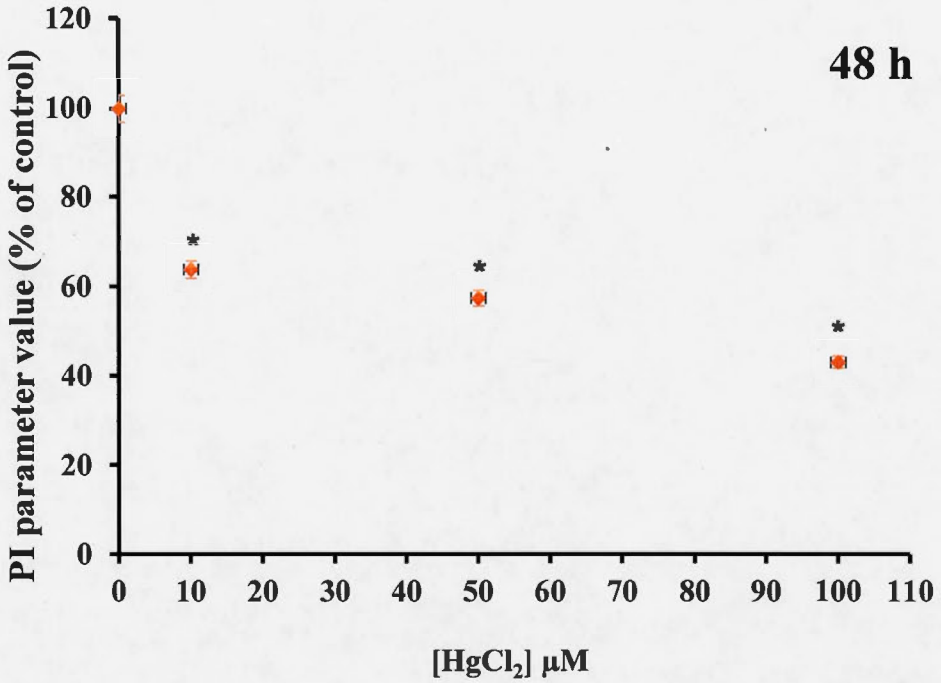


Figure 3.8 Change in the performance index of PSII activity (PI) of alga *C. vulgaris* exposed during 48 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$*.

The change in the performance index of PSII activity of alga *C. vulgaris* exposed during 48 h to different concentrations of $HgCl_2$ was determined as an overall indicator of PSII photochemical reactions (Figure 3.8). The performance index of PSII activity, PI integrates three processes which are express by these three parameters (Appenroth *et al.*, 2001):

1- The total number of active PSII reaction centres per absorption from antenna pigments (RC/ABS);

2- The yield of primary photochemistry;

3- The efficiency by which a trapped excitation can move an electron into the electron transport chain.

At 24 h of exposure, the value of PI decreased by 20 and 25 % in comparison to control, for respectively the treatment of 10 and 50 μM of $HgCl_2$. At the highest concentration of mercury (100 μM), the value of PI significantly decreased by 31 % compared to control, showing a strong toxic effect of mercury on PSII photochemical reactions. At 48 h of exposure, the effect of mercury on PSII functions was stronger in comparison to 24 h (Figure 3.8). The value of PI decreased by 37 and 43 % in comparison to control for respectively treatment of 10 and 50 μM of $HgCl_2$. The most significant decrease in the value of PI was observed for the highest concentration of $HgCl_2$ (100 μM) which was by 57 % compared to control. Similarly, it was previously shown that the toxicity effect of different concentrations of copper (0–20 μM) during 24 h of exposure time on green alga *Chlorella vulgaris* was correlated to the decrease in PI value (Oukarroum *et al.*, 2012).

3.6 Induction of cysteine, glutathione and phytochelatin synthesis

The induction of the cysteine (Cys) synthesis was investigated when algal cells of *C. vulgaris* were exposed during 24 h to different concentrations of $HgCl_2$ (Figure 3.9). We compared the changes in content of Cys between low (10 μM) and 10 times higher concentrations (100 μM) of $HgCl_2$. The cysteine was detected in control sample of algal cells since it already participates in the cellular metabolism of several syntheses of peptides and proteins. When algal cells of *C. vulgaris* were exposed during 24 h to 10 μM of $HgCl_2$, the content of Cys was not significantly different to the control, indicating that the bioaccumulated mercury was not enough to induce a change in the amount of Cys. However, under the exposure of 100 μM of $HgCl_2$, the induction of Cys was strong, more than 2 times in comparison to the control. This result indicated that 100 μM of $HgCl_2$ induced the synthesis of cysteine for cellular defence mechanisms such as the glutathione metabolic pathway. In a previous study

using the alga *T. weissflogii* exposed during 96 h to different concentrations of $HgCl_2$ (0, 8.4 and 222 µg/L), it was shown a significant increase in the cellular content of Cys for the highest concentration tested (222 µg/L) at 24 h of exposure. However, under this time of exposure, there was no significant difference between the amount of Cys at low concentration tested (8.4 µg/L) in comparison to the control (Wu and Wang, 2012). In another study on the toxic effect of mercury during 72 h on three marine phytoplanckton species, the increase of cellular Cys was dependent to the species: *I. galbana* > *C. autotrophica* > *T. weissflogii* (Wu and Wang, 2014). Therefore, it appeared that the induction of Cys synthesis was related to the concentration of $HgCl_2$ tested and the algal species.

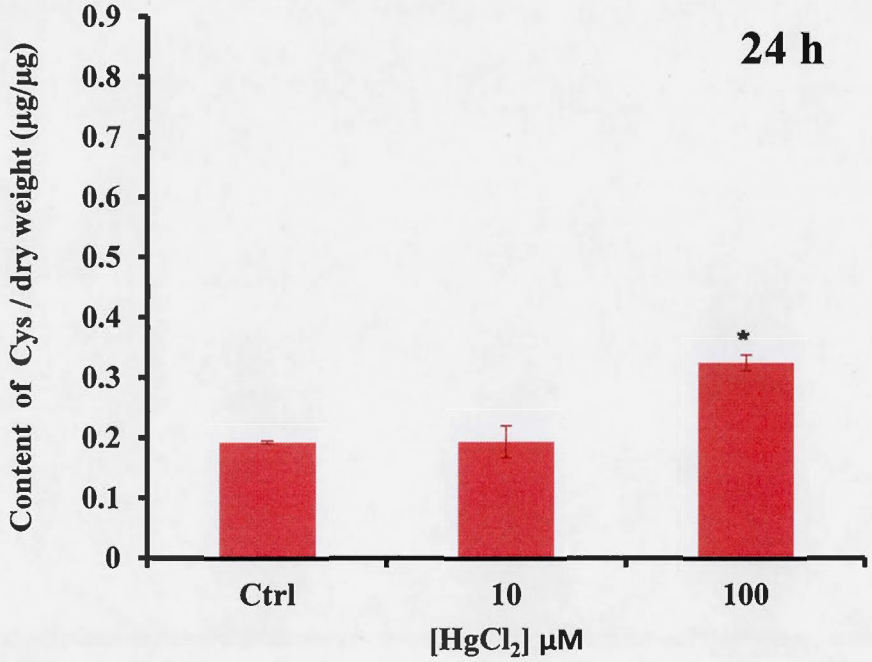


Figure 3.9 Change in the cysteine (Cys) content in algal biomass of *C. vulgaris* exposed during 24 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$*.

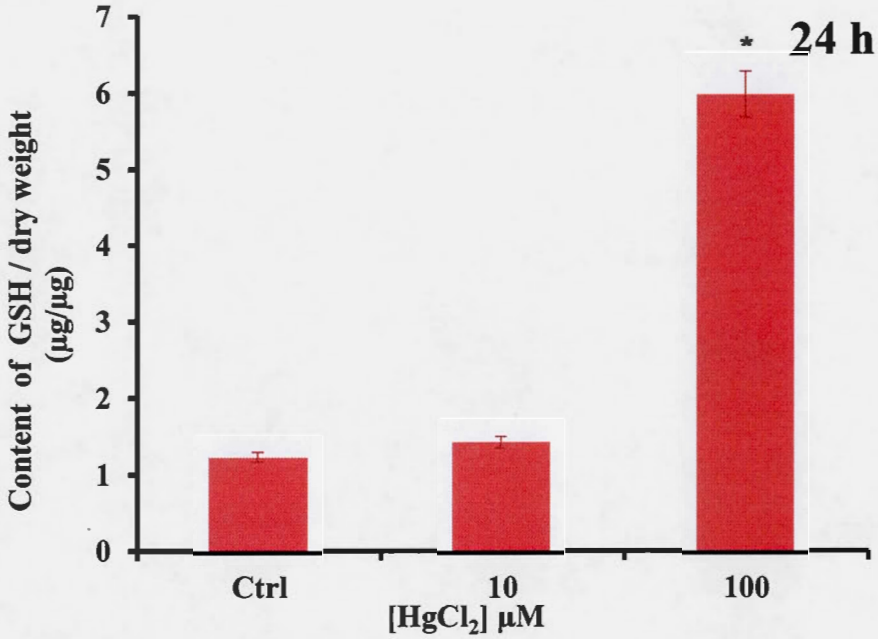


Figure 3.10 Change in the glutathione (GSH) content in algal biomass of *C. vulgaris* exposed during 24 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$*.

The induction of glutathione (GSH) synthesis was also investigated when algal cells of *C. vulgaris* were exposed during 24-48 h to different concentrations of $HgCl_2$ (Figure 3.10). The cellular content in GSH between low (10 µM) and high (100 µM) concentrations of $HgCl_2$ was compared. The GSH was detected in control cells since this compound participates in sequestration and trafficking of metals in order to maintain cellular homeostasis (Pal and Rai, 2010). When algal cells of *C. vulgaris* were exposed during 24 h to 10 µM of $HgCl_2$, the content of GSH was not significantly changed in comparison to the control, indicating that the content of GSH

was enough to deal with the bioaccumulated mercury. However, under the exposure of 100 μM of $HgCl_2$, the induction of GSH synthesis was much stronger, more than 4 times in comparison to the control. This result indicated that 100 μM of $HgCl_2$ highly induced the synthesis of GSH synthesis which is known to participate in the cellular detoxification mechanism of mercury (Le faucheur *et al.*, 2006). However, when algal cells of *C. vulgaris* were exposed during 48 h to 100 μM of $HgCl_2$, the content of GSH was not significantly changed in comparison to the control.

In a previous study, the cellular effect of mercury was demonstrated at different concentrations (0-222 μg/L) on the microalga *T. weissflogii* during 96 h of exposure (Wu and Wang, 2012). Their results showed a significant increase in the amount of glutathione especially for the highest concentration of mercury (222 μg/L) at 48 h of treatment. Furthermore, the cellular change in GSH on three marine phytoplanckton species (*I. galbana*, *C. autotrophica* and *T. weissflogii*) caused by the effect of $HgCl_2$ during 72 h was directly related to the algal species and the tested concentration (Wu and Wang, 2014). Moreover, an increase in the induction of GSH was also seen in another study concerning the effect of different concentrations of As(III) ($0\text{-}5\times10^{-5}$–2×10^{-4} M) and As(V) (8×10^{-6}, 2×10^{-5} M) on green algal cells of *S. vacuolatus* during 72 h of exposure. According to this study, there wasn't a significant difference between the amounts of GSH under low concentration exposure of As(III) in comparison to the control, but at higher concentration the synthesis of GSH increased significantly. For As (V), the content of GSH was increased under even low treatment concentration in comparison to the control and the induction significantly increased at higher concentrations (Le Faucheur *et al.*, 2006).

When algal cells of *C. vulgaris* were exposed during 24-48 h to different concentrations of $HgCl_2$, the synthesis of several species of phytochelatins (PCs) was investigated and quantified (Figure 3.11). The production of PCs was compared

under low (10 μM) and high (100 μM) concentrations of $HgCl_2$ treatment. According to our results, the synthesis of PCs was not induced in the control sample. When algal cells of *C. vulgaris* were exposed during 24 h to 10 and 100 μM of $HgCl_2$, the synthesis of PC_2 and PC_4 was detected. Their contents increased significantly in comparison to the control and the increase was dependent to the tested concentration of $HgCl_2$. Furthermore, for both tested concentration of $HgCl_2$, the bioaccumulated mercury induced a stronger increase in the synthesis of PC_4 in comparison to PC_2. However, when algal cells of *C. vulgaris* were exposed during 48 h to 10 and 100 μM of $HgCl_2$, the synthesis of PC_2 and PC_4 was not detected. Indeed, recent works examined the induction of PC-metal complexes in phytoplankton for the detoxification of metals (Dupont and Ahner, 2005; Lee *et al.*, 1996). In a previous study, the change in the amount of phytochelatins induced by mercury was determined on microalga *T. weissflogii* treated during 96 h. A high increase in the amount of PC_2 and PC_4 was already shown for the highest treatment concentration (222 μg/L) at 24 h of exposure time (Wu and Wang, 2012). Authors demonstrated a direct relation between the change in content of PC_2 and PC_4 with the concentration tested of $HgCl_2$, permitting the cellular sequestration of this metal. Moreover, in another study on three marine phytoplanckton species (*I. galbana*, *C. autotrophica*, *T. weissflogii*), the exposure of $HgCl_2$ during 72 h caused an increase in PCs (PC_2, PC_3 and PC_4) which was dependent to the species (Wu and Wang, 2014). In comparison to the two others species, alga *T. weissflogii* presented a high ability to synthesized PCs, and the PC_4 was only detected in this algal species. Therefore, it appeared that the induction of the different PCs was related to the concentration of $HgCl_2$ tested and was specific to the algal species.

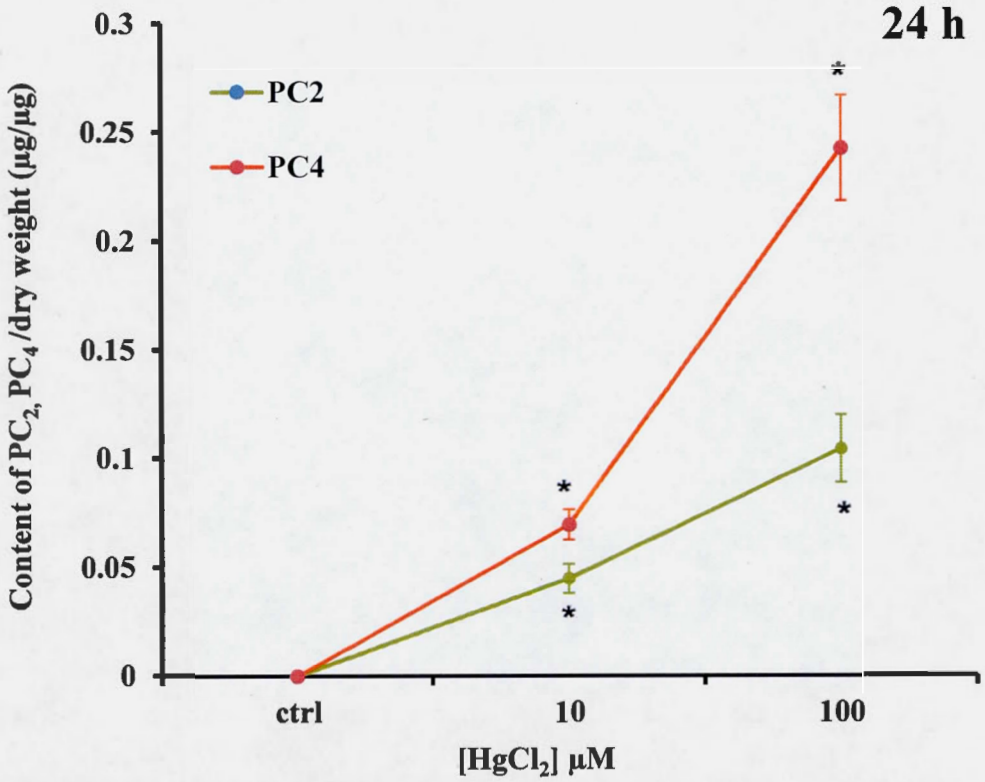


Figure 3.11 Change in PC_2 and PC_4 contents of alga *C. vulgaris* exposed during 24 h to different concentrations of $HgCl_2$. Significant differences between control and treated samples were determined for $p < 0.05$*.

CHAPTER IV

DISCUSSION / CONCLUSION

The goal of this study was to investigate the bioaccumulation effects of mercury on green alga *C. vulgaris* by analyzing the cellular division, the bioaccumulation efficiency of mercury in algal biomass, the photochemical efficiency of photosynthesis (PSII photochemistry) and also the synthesis of glutathione and phytochelatins as protective molecular mechanisms against metal toxicity. The overview conclusion concerning the change of these cell physiological parameters is summarized in the Table 4.1.

Table 4.1 Change of investigated physiological parameters when algal cells of *C. vulgaris* was exposed to 100 µM of $HgCl_2$ at 24 and 48 h.

Physiological parameters	**100 µM of $HgCl_2$ 24 h**	**100 µM of $HgCl_2$ 48 h**
Bioaccumulation efficiency	**↗ 5 × compared to 1 µM $HgCl_2$**	**↗ 4 × compared to 1 µM $HgCl_2$**
Cellular division	**↘ 30 % compared to control**	**↘ 40 % compared to control**
PSII photochemistry	**↘ 30 % compared to control**	**↘ 60 % compared to control**
GSH	**↗ 3 × compared to control**	**↘ 0.9 × compared to control**
PC_2, PC_4	**For PC_4 : ↗ 2.5 × compared to PC_2**	**No PCs**

The variation of these parameters are shown under the treatment condition when algal cells of *C. vulgaris* had the highest efficiency of Hg bioaccumulation, at 24 and 48 h of exposure. Indeed, these changes permitted to explain the efficiency of Hg bioaccumulation reached and to compare the exposure at 24 h and 48 h. The effect of mercury on the change of cell density of *C. vulgaris* was investigated when algal cells were exposed during 24-72 h to different concentrations of $HgCl_2$. The cellular division was significantly inhibited at higher concentrations of $HgCl_2$ (50 and 100 μM). During 24 h of treatment to the highest concentration of $HgCl_2$ (100 μM), cellular division was inhibited by 30 % in comparison to the control, but at 48 h of exposure, cellular division was inhibited by 40 % compared to control (Table 4.1), showing a relationship between the cell density and the concentration of mercury. In order to determine the maximum capacity of algal cells of *C. vulgaris* able to accumulate mercury, the bioaccumulation efficiency was investigated by estimating the content of mercury in algal biomass when *C. vulgaris* was exposed during 72 h to different concentrations of $HgCl_2$. According to our obtained results, the highest efficiency of bioaccumulation was reached for the highest concentration of $HgCl_2$ (100 μM) at 24 h of exposure time, and it was 5 times more efficient in comparison to the lowest concentration treatment condition, 1 μM of $HgCl_2$ (Table 4.1). However, under the same treatment concentration (100 μM of $HgCl_2$), the content of mercury in algal biomass bioaccumulated at 48 h was lower by 4 times in comparison to the content of mercury in algal biomass under the treatment condition of 1 μM of $HgCl_2$. These results demonstrated that treatment of $HgCl_2$ 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 injured and dead cells. This cellular toxicity impact may explain the lower uptake and sequestration capacity of mercury by algal cells after 24 h of exposure due to no synthesis of PCs.

Furthermore, the characterization of this cellular toxicity impact induced by the bioaccumulation effect of mercury was investigated when *C. vulgaris* was exposed during 24-48 h to different concentrations of $HgCl_2$. Several cell physiological parameters were monitored in order to better explain the bioaccumulation efficiency of mercury in algal cells. Our results showed a significant inhibitory effect of $HgCl_2$ on PSII primary photochemistry and electron transport activity at 24 and 48 h of exposure time. For *C. vulgaris* exposed during 24 h to 100 µM of $HgCl_2$, the performance index of PSII activity decreased by 30 % in comparison to control, showing the inhibitory effect of mercury on photosynthesis which may explain the decrease in cellular division. However at 48 h of exposure, under the same treatment condition, the performance index of PSII activity decreased by 60 % in comparison to control, showing a stronger effect of mercury on photosynthetic electron transport in comparison to 24 h of exposure. This stronger inhibitory effect on photosynthesis by mercury was related to a higher inhibition of cellular division at 48 h of exposure. Therefore, our results indicated that the performance index of PSII activity was used as a reliable biomarker of the bioaccumulation effects of mercury at cellular level, and which was highly related to the treatment concentration of $HgCl_2$ and the time of exposure. Also, the change of this photosynthetic-based fluorescence parameter was related to the efficiency of the bioaccumulation of mercury in algal biomass, which reached the highest level at 24 h of exposure and decreasing at 48 h and after.

Moreover, in order to better understand the cellular toxicity impact of mercury in algal cells of *C. vulgaris*, the synthesis of thiol peptides, such as glutathione (GSH) and phytochelatins (PCs), was investigated, since it is well known that these cysteine containing peptides are involved in cellular homeostasis and detoxification mechanisms of metals accumulation in the cytoplasm (Cobbett and Goldsbrough, 2003; Le Faucheur *et al.*, 2006). Obtained results showed that the content of thiol peptides in algal cells, related to either GSH or PCs, was affected by the exposure of

C. vulgaris to different concentrations of $HgCl_2$. According to our results, the amount of GSH in algal cells of *C. vulgaris* exposed during 24 h to 100 µM of $HgCl_2$ was 3 times more compared to control, but at 48 h of exposure, the amount of GSH was lower by 0.9 times in comparison to the control (Table 4.1). Furthermore, the synthesis of PCs which are produced from GSH molecules was also measured to determine the importance of this cellular detoxification mechanism in algal cells during treatment. Our results indicated that PC_2 and PC_4 were detected at 24 h of exposure when algal cells were treated to 100 µM of $HgCl_2$. Under this treatment condition, the content of PC_4 was 2.5 times more than PC_2, indicating the formation of high molecular weight phytochelatin-mercury complexes which permitted the efficient sequestration of mercury in the cellular vacuole (Table 4.1). However, when algal cells were exposed during 48 h to 100 µM of $HgCl_2$, the induction of both PC_2 and PC_4 was not detected, which may be due to the strong toxic effect of mercury causing damages on the cellular system and affecting enzymatic activities. Finally, these results can explain the algal cell level of tolerance against the bioaccumulation effect of mercury at 24 h and the stronger toxic effect of mercury on injuring the cellular system at 48 h.

In conclusion, according to the obtained results in this study, it is most likely that this species of green algae, *C. vulgaris* was enough resistant to the effect of $HgCl_2$ up 100 µM and until 24 h of exposure in order to maintain its maximum bioaccumulation efficiency. Indeed, under this condition, algal biomass had the highest capacity of mercury bioaccumulation although we detected an inhibition of cellular division and a decrease in PSII activity due to the bioaccumulation toxic effect of mercury. Therefore, algal cellular system was partially protected during 24 h against the toxicity of mercury by the high induction of GSH and PCs permitting the efficient sequestration of mercury inside the cell. However, this molecular protective

mechanism was lost at 48 h of exposure, as indicated by the much stronger toxicity of bioaccumulated mercury on photosynthesis and cellular division.

Therefore, this study done at laboratory scale showed the cellular limitation of green algae *C. vulgaris* in the development of phycoremediation technology for the treatment of wastewater contaminated by mercury. However, future works need to focus on the determination of the bioaccumulation efficiency for others metals (Cd, Zn, Pb) and mixtures too, by using *C. vulgaris* and others species. Moreover, this study must be performed at the industrial level for the determination of the efficiency of the wastewater treatment at larger scales.

REFERENCES

Abdel-Raouf, N., Al-Homaidan, A. A., Ibraheem, I. B. M. (2012). Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences, 19* (3), 257-275.

Aderhold, D., Williams, C. J., Edyvean, R. G. J. (1996). The removal of heavy-metal ions by seaweeds and their derivatives. *Bioresource Technology, 58* (1), 1-6.

Adriano, D. C., Bolan, N. S., Vangronsveld J., Wenzel. W. W. (2005). Heavy metals. In D. Hillel (Ed.): *Encyclopedia of Soils in the Environment,* 175-182. Amsterdam: Elsevier.

Afkar. E., Ababna, H., Fathi, A. A. (2010). Toxicological response of the green alga *Chlorella vulgaris* to some heavy metals. *American Journal of Environmental Sciences, 6* (3), 230-237.

Ahner, B. A, Morel, F. M. M., Moffett, J. W. (1997). Trace metal control of phytochelatin production in coastal waters. *Limnology and Oceanography, 42*, 601-608.

Appenroth, K.-J., Stöckel, H., Srivastava, A., Strasser, R. J. (2001). Multiple effect of chromate on the photosynthetic apparatus of *Spirodela polyrhiza* as probed by OJIP Chlorophyll *a* fluorescence measurements. *Environmental pollution, 115*, 49-64.

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annual Review of Plant Biology, 59*, 89-113.

Baumann, H. A., Morrison, L., Stengel, D. B. (2009). Metal accumulation and toxicity measured by PAM--chlorophyll fluorescence in seven species of marine macroalgae. *Ecotoxicology and Environmental Safety, 72* (4), 1063-1075.

Beaty, R. D., Kerber, J. D. (1993). Concepts, instrumentation and techniques in atomic absorption spectrophotometry. 2^{sd} Ed. The Perkin-Elmer Corporation, Norwalk, CT, U.S.A. 95 p.

Beldowski, J., Pempkowiak, J. (2003). Horizontal and vertical variabilities of mercury concentration and speciation in sediments of the Gdansk Basin, Southern Baltic Sea. *Chemosphere, 52* (3), 645-654.

Bernard, A. (2011). Renal and neurological effects heavy metals in the environment. In J. O Nriagu (Ed.): *Encyclopedia of Environmental Health,* 801-805. Burlington: Elsevier.

Blair, M. F., Kokabian, B., Gude, V. G. (2014). Light and growth medium effect on Chlorella vulgaris biomass production. *Journal of Environmental Chemical Engineering, 2*, 665-674.

Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology, 70* (1-3), 313-321.

Buchanan, B. B., Gruissem, W., Jones, R. L. (2000). *Biochemistry & Molecular Biology of Plants*. American Society of Plant Physiologists, Rockville, MD.

Calderon, R. L., (2000). The epidemiology of chemical contaminants of drinking water. *Food Chemistry and Toxicology, 38*, S13-20.

Chan, H. M., Egeland, G. M., (2004). Fish consumption, mercury exposure, and heart diseases. *Nutrition Review, 62* (2), 68-72.

Cobbett, C. S., Goldsbrough, (2003). Phytochelatins and metallothioneins; role in heavy metal detoxification and homeostasis. *Annual Review in Plant Biology, 53*, 159-182.

Crawford, N. M., Kahn, M. L., Leustek, T., Long, S. R. (2000). Nitrogen and sulfur. In B. B. Buchanan, W. Gruissem, R. L. Jones (Eds): *Biochemistry and molecular biology of plants*, 786-849. Maryland, Rockville: American Society of Plant Physiologists.

Dewez, D., Didur, O., Vincent-Herroux, J., Popovic, R. (2008). Validation of photosynthetic-fluorescence parameters as biomarkers for isoproturon toxic effect on alga *Scenedesmus obliquus*. *Environmental Pollution, 151*, 93-100.

Domagalski, J. L., Alpers, C. N., Slotton, D. G., Suchanek, T. H., Ayers, S. M. (2004). Mercury and methylmercury concentrations and loads in the Cache Creek watershed, California. *Science of Total Environment, 327* (1-3), 215-237.

Dupont, C. L., Ahner, B. A. (2005). Effects of copper, cadmium and zinc on the production and exudation of thiols by *Emiliania huxleyi*. *Limnology and Oceanography, 50*, 508-515.

Eccles, H. (1999). Treatment of metal-contaminated wastes: why select a biological process? *Tibtech december, 17* (12), 462-465.

Elbaz, A., Wei, Y. Y., Meng, Q., Zheng, Q., Yang, Z. M. (2010). Mercury induced oxidative stress and impact on antioxidant enzymes in *Chlamydomonas reinhardtii*. *Ecotoxicology, 19*, 1285-1293.

Environment Canada, (2011). Mercury and the Environment. http://www.ec.gc.ca/mercure-mercury/default.asp?lang=En&n=DB6D2996-1.

Fabiani, C. (1992). Recovery of metal ions from waters and sludges. *ENEA [rapporti tecnici] RT/AMB/92/32*.

Fathi, A. A., Azooz, M. M., Al-Fredan, M. A. (2013). Phycoremediation and the potential of sustainable algal biofuel production using wastewater. *American Journal of Applied Sciences, 10* (2), 189-194.

Force, L., Critchley, C., Van Rensen, J. J. S. (2003). New fluorescence parameters for monitoring photosynthesis in plants. *Photosynthesis Research, 78*, 17-33.

Fujita, Y. (2005). Chlorophylls. *Encyclopedia of life sciences*. John Wiley & Sons, Ltd. www.els.net.

Gadd, G. M. (2009). Heavy metal pollutants: environmental and biotechnological aspects. In Moselio Schaechter (Ed.): *Encyclopedia of microbiology,* 321-334. Oxford: Elsevier.

González-Barreiro, O., Rioboo, C., Herrero, C., Cid, A. (2006). Removal of triazine herbicides from freshwater systems using photosynthetic microorganisms. *Environmental Pollution, 144*, 266-271.

Görs, M., Schumann, R., Hepperle, D., Karsten, U. (2010). Quality analysis of commercial *Chlorella* products used as dietary supplement in human nutrition. *Journal of Applied Phycology, 22*, 265-276.

Govindjee, kern, J. F., Messinger, J., Whitmarsh, J. (2010). Photosystem II. *Encyclopedia of life sciences*. John Wiley & Sons, Ltd. www.els.net.

Grill, E., Winnacker, E.-L., Zenk, M. H. (1987). Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proceedings of the National Academy of Sciences of the United States of America, 84*, 439-443.

Grill, E., Loffler, S., Winnacker, E.-L., Zenk, M. H. (1989). Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific ɤ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proceedings of the National Academy of Sciences of the United States of America, 86* (18), 6838-6842.

Hansen, J. C., Dasher, G. (1997). Organic mercury: an environmental threat to the health of dietary exposed societies? *Review Environmental Health, 12*.

Harada, M., Nakachi, S., Cheu, T., Hamada, H., Ono, Y., Tsuda, T., Yanagida, K., Kizaki, T., Ohno, H., 1999. Monitoring of mercury pollution in Tanzania: relation between head hair mercury and health. *Science of Total Environment*, 227 (2–3), 249-256.

Harada, M., Nakachi, S., Tasaka, K., Sakashita, S., Muta, K., Yanagida, K., Doi, R., Kizaki, T., Ohno, H. (2001). Wide use of skin-lightening.

Hu, S., Lau, K. W. K., Wu. M. (2001). Cadmium sequestration in *Chlamydomonas reinhardtii. Plant Science, 161* (5), 987-996.

Jackson, T. A. (1998). Mercury in aquatic ecosystems. In W. J. Langston, M. J. Bebianno (Eds): *Metal metabolism in aquatic environments*, 77-158. Springer: US.

Jarup, L. (2002). Cadmium overload and toxicity. *Nephrology Dialyse Transplantation, 17* (S2), 35-39.

Juneau, P., Popovic, R. (1999). Evidence for the rapid phytotoxicity and stress evaluation using the PAM fluorometric method : importance and future application. *Ecotoxicology, 8*, 449-455.

Juneau, P., Dewez, D., Matsui, S., Kim, S.-G., Popovic, R. (2001). Evaluation of different algal species sensitivity to mercury and metolachlor by PAM-fluorometry. *Chemosphere, 45*, 589-598.

Kawakami, S. K., Gledhill, M., Achterberg, E. P. (2006). Determination of phytochelatins and glutathione in phytoplankton from natural waters using HPLC with fluorescence detection. *Trends in Analytical Chemistry, 25*, 133-142.

Kazantzis, G. (2002). Mercury exposure and early effects: an overview. *Med. Lav., 93* (3), 139-147.

Kobayashi, R., Yoshimura, E. (2006). Differences in the binding modes of phytochelatin to cadmium(II) and zinc(II) ions. *Biological Trace Element Research, 114* (1-3), 313-318.

Lavergne, J., Lecci, E. (1993). Properties of inactive photosystem II centers. *Photosynthesis research, 35*, 323-343.

Lavoie, M., Faucheur, S. L., Fortin, C., Campbell, P. G. C. (2009). Cadmium detoxification strategies in two phytoplankton species: metal binding by newly synthesized thiolated peptides and metal sequestration in granules. *Aquatic Toxicology, 92* (2), 65-75.

Lazár, D. (2006). The polyphasic chlorophyll *a* fluorescence rise measured under high intensity of exciting light. *Functional Plant Biology, 33* (1), 9.

Lee. J. G., Ahner, B. A., Morel, F. M. M. (1996). Export of cadmium and phytochelatin by the marine diatom *Thalassiosira weissflogii*. *Environmental Science Technology, 30*, 1814-1821.

Leermakers, M., Baeyens, W., Quevauviller, P., Horvat, M. (2005). Mercury in environmental samples: Speciation, artifacts and validation. *Trends in Analytical Chemistry, 24*, 383-393.

Le Faucheur, S., Schildknecht, F., Behra, R., Sigg, L. (2006). Thiols in *Scenedesmus vacuolatus* exposure to metals and metalloids. *Aquatic Toxicology, 80*, 335-361.

Leopold, K., Foulkes, M., Worsfold, P. (2010). Methods for the determination and speciation of mercury in natural waters – A review. *Analytical Chimica Acta, 663*, 127-138.

Levine, S. (2004). Who will stop the mercury rain? *US News World Report, 136* (11), 70-71.

Liang, Y., Sarkany, N., Cui, Y. (2009). Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters, 31*, 1043-1049.

Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids: pigments of photosynthetic membranes. *Methods Enzymology, 148*, 349-382.

Lubitz, W., Reijerse, E. J., Messinger, J. (2008). Solar water-splitting into H_2 and O_2: design principles of photosystem II and hydrogenases. *Energy & Environmental Science, 1* (1), 15-31.

Macklin, M. G., Ridgway, J., Passmore, D. G., Rumsby, B.T. (1994). The use of overbank sediment for geochemical mapping and contamination assessment: results from selected English and Welsh floodplains. *Applied Geochemistry, 9*, 689-700.

Malkin, R., Niyogi, M. (2000). Photosynthesis. In B. B. Buchanan, W. Gruissem, R. L. Jones (Eds): *Biochemistry and molecular biology of plants,* 568-628. Maryland, Rockville: American Society of Plant Physiologists.

Mallick, N., Mandal, S., Singh A. K., Dash, M. B. A. (2011). Green microalga *Chlorella vulgaris* as a potential feedstock for biodiesel. *Journal Chemistry Technology Biotechnology, 87*, 137-145.

Miller, J. R., Lechler, P. J., Hudson-Edwards, K. A., Macklin, M. G. (2002). Lead isotopic fingerprinting of heavy metal contamination, Rio Pilcomayo basin, Bolivia. In R. Allan, A. Horowitz, J. R. Miller (Eds): *Mining and Metals in the Environment. Geochemistry: Explor., Environ., Analysis, 2*, 225-233.

Morel, F. M. M., Kraepiel, A. M. L., Amyot, M. (1998). The chemical cycle and bioaccumulation of mercury. *Annual Review of Ecology and Systematics, 29*, 543-566.

Muñoz, R., Guieysse, B. (2006). Algal-bacterial processes for the treatment of hazardous contaminants: a review. *Water Research, 40* (15), 2799-2815.

Nishikawa, K., Onodera, A., Tominaga, N. (2006). Phytochelatins do not correlate with the level of Cd accumulation in *Chlamydomonas spp. Chemosphere, 63* (9), 1553-1559.

Oukarroum, A., Perreault, F., Popovic, R. (2012). Interactive effect of temperature and copper on photosystem II photochemistry in *Chlorella vulgaris. Journal of photochemistry and photobiology B : Biology, 110*, 9-14.

Ou-yang, H.-L., Kong, X.-Z., Lavoie, M., He, W., Qin, N., He, Q.-S., Yng, B., Wang, R., Xu, F.-L. (2013). Photosynthetic and cellular toxicity of cadmium in *Chlorella vulgaris. Environmental Toxicology and Chemistry, 32* (12), 2762-2770.

Ort, D. R., Whitmarsh, J. (2001). Photosynthesis. *Encyclopedia of life sciences.* Nature Publishing Group, 1-7. www.els.net

Ozdemir, C., Karatas, M., Dursun, S., Argun, M. E., Dogan, S. (2005). Effect of $MnSO_4$ on the chromium removal from leather industry wastewater. *Environmental Technology, 26*, 397-400.

Pal, R., Rai, J. P. N. (2010). Phytochelatins: peptides involved in heavy metal detoxification. *Applied Biochemistry and Biotechnology, 160* (3), 945-963.

Pehlivan, E., Altun, T. (2006). The study of various parameters affecting the ion exchange of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Pb^{2+} from aqueous solution on Dowex 50W synthetic resin. *Journal of Hazard, 30* (1-3), 149-56.

Perreault, F., Dewez, D., Fortin, C., Juneau, P., Diallo, A., Popovic, R. (2010). Effect of aluminum on cellular division and photosynthetic electron transport in *Euglena gracilis* and *Chlamydomonas acidophila. Environmental Toxicology and Chemistry, 29* (4), 887-892.

Perreault, F., Dionne, J., Didur, O., Juneau, P., Popovic, R. (2011). Effect of cadmium on photosystem II activity in *Chlamydomonas reinhardtii*: alteration of O-J-I-P fluorescence transients indicating the change of apparent activation energies within photosystem II. *Photosynthesis Research, 107*, 151-157.

Ponnuswamy, I., Madhavan, S., Shabudeen, S. (2013). Isolation and characterization of green microalgae for carbon sequestration, waste water treatment and bio-fuel production. *International Journal of Bio-Science and Bio-Technology, 5* (2).

Popovic, R., Dewez, D., Juneau, P. (2003). Application of Chlorophyll fluorescence in ecotoxicology, heavy metals, herbicides and air pollutions. Chapter 5, 152-176.

Priya, S. (2012). Analysis of value–added biochemical compounds and antimicrobial activity of green algae *Chlorella vulgaris. Journal of Chemical and Pharmaceutical Research, 4*, 2577-2579.

Pulz, O. (2001). Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology, 57* (3), 287-293.

Rice, D., Barone Jr., S. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental Health Perspective, 108* (S3), 511-513.

Richmond, A. (2004). *Handbook of microalgal culture: Biotechnology and Applied Phycology*. Oxford: Wiley-Blackwell, 566 p.

Rijstenbil, J. W., Wijnholds, J. A. (1996). HPLC analysis of nonprotein thiols in planktonic diatoms: pool size, redox state and response to copper and cadmium exposure. *Marine Biology, 127*, 45-54.

Rioboo, C., González, O., Herrero, C., Cid. A. (2002). Physiological response of freshwater microalga (*Chlorella vulgaris*) to triazine and phenylurea herbicides. *Aquatic Toxicology, 59* (3-4), 225-235.

Rivas, J. D. L., Balsera, M., Barber, J. (2004). Evolution of oxygenic photosynthesis: genome-wide analysis of the OEC extrinsic proteins. *TRENDS in Plant Science, 9* (1), 18-25.

Ruiz, J., Arbib, Z., Álvarez-Díaz, P. D., Garrido-Pérez, C., Barragán, J., Perales, J. A. 2014. Photobiotreatment model (PhBT): a kinetic model for microalgae biomass growth and nutrient removal in wastewater. *Environmental Technology*.

Sanità di Toppi, L., Gabbrielli, R. (1999). Response to cadmium in higher plants. *Environmental and Experimental Botany, 41* (2), 105-130.

Selin, N. E. (2009). Global biogeochemical cycling of mercury: A review. *Annual Review of Environment and Resources, 34*, 43-63.

Sharma, R., Singh, G. P., Sharma, V. K. (2011). Comparison of different media formulations on growth, morphology and chlorophyll content of green alga, *Chlorella vulgaris. International Journal of Pharma and BioSciences, 2*, 509-516.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering, 101* (2), 87-96.

Stein, J. (Ed.), (1973). Handbook of Phycological methods. *Culture methods and growth measurements.* Cambridge University Press. 448 pp.

Stoiber, T. L., Shafer, M. M., Armstrong, D. E. (2010). Differential effects of copper and cadmium exposure on toxicity endpoints and gene expression in *Chlamydomonas reinhardtii. Environmental Toxicology and Chemistry, 29* (1), 191-200.

Strasser, B. J., Strasser, R. J. (1995). Measuring fast fluorescence transients to address environmental questions: the JIP-test. In P. Mathis (Ed.): *Photosynthesis: from light to biosphere*, 977-980. Dordrecht: Kluwer Academic Publishers.

Strasser, R. J., Tsimilli-Michael, M., Srivastava, A. (2004). Analysis of the chlorophyll a fluorescence transient. In G. C. Papageorgiou, Govindjee (Ed.): *Chlorophyll Fluorescence: a signature of photosynthesis*, 321-362. Dordrecht: Springer.

Swennen, R., Van Keer, I., De Vox, W. (1994). Heavy metal contamination in overbank sediments of the Geul river (East Belgium): its relation to former Pb–Zn mining activities. *Environmental Geology, 24*, 12-21.

Taiz, L., Zeiger, E. (2010). Plant Physiology. 5th Ed. Sunderland, MA: Sinauer Associates. 782 p.

Tang, D. G., Shafer, M. A., Vang, K., Kamer, D. A., Amstrong, D. E. (2003). Determination of dissolved thiols using solid-phase extraction and liquid chromatographic determination of fluorescently derivatized thiolic compounds. *Journal of Chromatography, 998*, 31-40.

Ugwu, C. U., Aoyagi, H., Uchiyama, H. (2008). Photobioreactors for mass cultivation of algae. *Bioresource Technology, 99* (10), 4021-4028.

Vymazal, J. (1995). Algae and element cycling in wetlands. Lewis publishers Boca raton, pp. 689.

Wang, X. D., Liu, X. J., Yang, S., Li, A. L., Yang, Y. L. (2007). Removal and toxicological response of Triazophos by *Chlamydomonas reinhardtii*. *Bulletin of Environmental Contamination and Toxicology, 78* (1), 67-71.

Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., Ruan, R. (2010). Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. *Applied Biochemistry Biotechnology, 162*, 1174-1186.

Watt, G. C. M., Britton, A., Gilmour, H. G., Moore, M. R., Murray, G. D., Robertson, S. J. (2000). Health implications of new guidelines for lead in drinking water: a case study in an area with historically high water lead levels. *Food Chemistry and Toxicology, 8*, S73-79.

Westphal, G., Hallier, E., (2003). Mercury in infants given vaccines containing thiomersal. *Lancet, 361* (93-58), 699.

Whitmarsh, J., Govindjee (2002). Photosystem II. *Encyclopedia of life sciences*. Oxford: Nature Publishing group, 13 p.

Winters, R. A, Zukowski, J., Ercal, N., Matthews, R. H., Spitz, D. R. (1995). *Analytical Biochemistry, 227*, 14.

Wu, Y., Wang, W.-X. (2011). Accumulation, subcellular distribution and toxicity of inorganic mercury and methylmercury in marine phytoplankton. *Environmental Pollution, 159*, 3097-3105.

Wu, Y., Wang, W.-X. (2012). Thiol compounds induction kinetics in marin phytoplankton during and after mercury exposure. *Journal of Hazardous Materials* 217-218.

Wu, Y., Wang, W.-X. (2014) Intracellular speciation and transformation of inorganic mercury in marine phytoplankton. *Aquatic Toxicology, 148*, 122-129.

Xu, L., Weathers, P. J., Xiong, X.-R., Liu, C.-Z. (2009). Microalgal bioreactors: Challenges and opportunities». *Engineering in Life Sciences, 9* (3), 178-189.

Zahir, F., Rizwi, S. J., Haq, S. K., Khan, R. H. (2005). Low dose mercury toxicity and human health. *Environmental Toxicology and Pharmacology, 20*, 351-360.

Zhang, M. Q., Zhu, Y. C., Deng, R. W. (2002). Evaluation of mercury emissions to the atmosphere from coal combustion, China. *Ambio, 31* (6), 482-484.

Zhang, S., Qiu, C. B., Zhou, Y., Jin, Z. P., Yang, H. (2011). Bioaccumulation and degradation of pesticide fluroxypyr are associated with toxic tolerance in green alga *Chlamydomonas reinhardtii*. *Ecotoxicology, 20*, 337-347.