Induction of phytochelatins by lead in the alga

*Chlamydomonas reinhardtii*

A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

Presented by

Christian Scheidegger

Master of Science in Biochemistry, University of Bern

born on March 30, 1982

Citizen of Trub (BE)

Accepted on the recommendation of

Prof. Dr. Laura Sigg, examiner

Prof. Dr. Bernhard Wehrli, co-examiner

Prof. Dr. Claude Fortin, co-examiner

Dr. Renata Behra, co-examiner

2010
# Table of content

Summary ...................................................................................................................... V  
Zusammenfassung ..................................................................................................... IX  

## 1 Introduction .............................................................................................................. 1  
1.1 Metals in the aquatic environment .............................................................. 3  
   1.1.1 Metal speciation ................................................................................................. 3  
   1.1.2 Metal speciation and bioavailability to algae ..................................................... 4  
   1.1.3 Intracellular metal homeostasis and detoxification ............................................ 7  
1.2 Phytochelatins ............................................................................................. 8  
   1.2.1 Structure and biosynthesis ................................................................................. 8  
   1.2.2 Induction of phytochelatin synthesis in algae .................................................... 9  
   1.2.3 Role of phytochelatins in metal detoxification and formation of  
       metal-phytochelatin complexes ....................................................................... 11  
1.3 Scope of the thesis .................................................................................... 12  
1.4 References ................................................................................................. 15  

## 2 Phytochelatin formation kinetics and toxic effects in the freshwater alga  
*Chlamydomonas reinhardtii* upon short- and long-term exposure to lead(II) .. 23  
2.1 Abstract ..................................................................................................... 25  
2.2 Introduction ............................................................................................... 25  
2.3 Material and Methods ............................................................................... 27  
   2.3.1 Chemicals ......................................................................................................... 27  
   2.3.2 Test organism and growth conditions .............................................................. 27  
   2.3.3 Experimental setup to determine short-term phytochelatin synthesis kinetics.  28  
   2.3.4 Experimental setup for chronic lead exposures ................................................ 28  
   2.3.5 Growth rate determination and photosynthetic yield measurements .......... 29  
   2.3.6 Cell homogenization and non protein thiol analysis ........................................ 29  
   2.3.7 Total and intracellular metal measurements ..................................................... 30  
2.4 Results ....................................................................................................... 31  
   2.4.1 Total and intracellular lead in *Chlamydomonas reinhardtii* under lead  
       exposure ........................................................................................................... 31  
   2.4.2 Kinetics of phytochelatin synthesis in the green alga *Chlamydomonas reinhardtii*  
       as a function of free lead ions ................................................................. 32  
   2.4.3 Effects of lead on growth, photosynthetic yield and phytochelatin  
       production upon long-term exposure ............................................................... 35  
2.5 Discussion ................................................................................................. 37  
2.6 References ................................................................................................. 40  

## 3 Characterization of lead induced metal-phytochelatin complexes in  
*Chlamydomonas reinhardtii* ................................................................................... 43  
3.1 Abstract ......................................................................................................... 45  
3.2 Introduction .................................................................................................... 45
Summary
Lead is a non-essential trace metal, occurring in elevated concentrations in many aquatic systems. Uptake of non-essential trace metals in algae leads to interactions with macromolecules, potentially inducing toxic effects. Algae possess protective mechanisms as a response to metal uptake and toxicity. One of these mechanisms is the control of intracellular metal speciation by synthesis of metal-binding ligands, such as phytochelatins. Phytochelatins (PCs) are metal-binding oligopeptides with the general structure \((\gamma\text{-Glu-Cys})_n\text{-Gly} (n = 2 – 11)\), which are enzymatically synthesized from glutathione. Metal detoxification by PC is assumed to result through immobilization of metals, preventing non-specific binding of metals to important biomolecules, followed by the transport of these complexes into vacuoles. The aim of this project was to examine induction of PC synthesis by Pb in the unicellular freshwater alga *Chlamydomonas reinhardtii* as a function of the Pb speciation and to explore the role of phytochelatins in Pb detoxification. Furthermore, Pb induced metal-phytochelatin complexes and standard Pb-PC complexes were analyzed to examine whether Pb is actually bound to phytochelatins.

Phytochelatin formation kinetics as function of the free Pb concentration was examined at free Pb ion concentrations ranging from \(10^{-11} – 10^{-7}\) M. Pb accumulation after 6 h showed an increase of intracellular Pb with increasing \([\text{Pb}^{2+}]\). PC₂ – PC₄ were present at low concentrations in *C. reinhardtii*, grown under optimal growth conditions. Upon short-term exposure, PC₂ and PC₃ synthesis was induced within minutes at \([\text{Pb}^{2+}] \geq 10^{-8}\) M, and PC₄ after a lag phase at \(10^{-7}\) M. The PC₂, PC₃ and PC₄ concentrations increased with increasing \([\text{Pb}^{2+}]\) and continuously over 6 h. In contrast, upon long-term exposure, induction of PC synthesis was also detected at \(10^{-9}\) M and the production of PC with a higher degree of polymerization was observed (PC₅).

Comparisons of PC concentrations and intracellular Pb content showed that PC is not present at sufficiently high concentration to immobilize accumulated Pb. Inhibition of photosynthesis and growth up to 100 % was observed upon long-term exposure; whereas no Pb toxicity was observed in short-term experiments.

To examine whether Pb is bound by PCs in *C. reinhardtii*, metal-phytochelatin complexes induced by Pb were isolated by size-exclusion chromatography and the collected fractions were analyzed for their PC and metal content by HPLC and ICP-MS. PC₂ and PC₃ were detected in a molecular weight range from 500 – 5300 Da, indicating the formation of complexes with various stoichiometries. Cu, Zn and Pb were observed in PC containing fractions, suggesting the formation of complexes with...
these metals. Molecular weight considerations allowed the prediction of putative metal-phytochelatin complex stoichiometries. The prevalent stoichiometries Me-(PC$_2$)$_2$ and Me-(PC$_3$)$_2$ were assumed. This study suggests that PCs play a minor role in Pb detoxification, yet from the obtained results, phytochelatins are assumed to be involved in Cu and Zn homeostasis. As unambiguous identification of complex stoichiometry and composition requires higher mass resolution than size-exclusion chromatography, a nano-electrospray ionization mass spectrometry method was developed to further characterize Me-PC complexes. Analysis of standard Pb-PC$_2$ indicated the formation of four different complexes at m/z 746.10, 952.06, 1285.24 and 1491.20, corresponding to singly charged Pb-PC$_2$, Pb$_2$-PC$_2$, Pb-(PC$_2$)$_2$ and Pb$_2$-(PC$_2$)$_2$. Their m/z indicated coordination of Pb through thiol groups of PC cysteine as well as through the carboxyl groups of PC glutamic acid. For both standard PC$_3$ and PC$_4$, the singly charged complexes Pb-PC$_3$, Pb$_2$-PC$_3$, Pb-PC$_4$, and Pb$_2$-PC$_4$ and the twicly charged Pb-PC$_4$ were detected. The isotopic patterns of all complexes were identical to the theoretical patterns. Competition experiments between Zn and Pb indicated the appearance of a singly charged Zn-PC$_2$ peak as well as the reduction of the Pb-PC$_2$ peak.

The results of this work indicate that phytochelatins are involved in Zn and Cu homeostasis rather than in Pb detoxification in *C. reinhardtii* at environmentally relevant Pb concentrations. Furthermore, the study shows that toxic effects of Pb to *C. reinhardtii* mainly appear upon long-term exposure, which suggests the importance of long-term exposures to predict Pb toxicity, as *C. reinhardtii* is an often used model organism.
Zusammenfassung

Die Kinetik der Phytochelatin Synthese wurde in Funktion der freien Pb-Konzentration \([\text{Pb}^{2+}]\) zwischen \(10^{-11} – 10^{-7} \text{ M}\) untersucht. Die Pb Akkumulation nach 6 h Exposition zeigte einen Anstieg in der intrazellulären Konzentration mit ansteigender \([\text{Pb}^{2+}]\). PC2 – PC4 waren in C. reinhardtii, gewachsen unter optimalen Wachstumsbedingungen, in tiefen Konzentrationen vorhanden. Unter kurzzeitiger Pb-Exposition wurde die PC2 und PC3 Synthese innerhalb von Minuten induziert bei \([\text{Pb}^{2+}] \geq 10^{-8} \text{ M}\) und PC4 nach einer Lagphase bei \(10^{-7} \text{ M}\). Die PC2 – PC4 Konzentrationen stiegen mit steigender \([\text{Pb}^{2+}]\) und kontinuierlich über 6 h. In Langzeitexperimenten hingegen wurde die Induktion der PC Synthese auch bei \(10^{-9} \text{ M}\) festgestellt und PC Oligomere mit höherem Polymerisierungsgrad wurden beobachtet (PC3). Der Vergleich zwischen PC Konzentrationen und intrazellulärem Pb zeigte, dass PC nicht in genügender Menge vorhanden ist um alles akkumulierte Pb zu immobilisieren. Die Inhibition von Photosynthese und Wachstum bis 100 % wurde in Langzeitexperimenten beobachtet, während in Kurzzeitexperimenten keine toxischen Effekte von Pb auftraten.

Um herauszufinden ob Pb in C. reinhardtii von PCs gebunden wird, wurden Pb-induzierte Me-PC Komplexe mit size-exclusion Chromatographie isoliert und die gesammelten Fraktionen wurden mit HPLC und ICP-MS auf ihren PC- und
Zusammenfassung

Chapter 1

Introduction
1.1 Metals in the aquatic environment

Metals are typically released into the aquatic environment from either natural processes (e.g. erosion of rocks, volcanic activity or dissolution from soil) or due to anthropogenic activities, such as industrial discharge. Trace metals are divided into two major classes: essential (e.g. Cu, Zn, Mn, Fe) and non-essential metals (e.g. Cd, Pb, Hg) (Rand, 1995). Essential metals are important to sustain the biological functions for aquatic organisms. However, these metals become toxic at elevated concentrations, whereas non-essential metals may be toxic already at low concentrations (Tessier and Turner, 1995).

Pb is a non-essential heavy metal and considered to be a major pollutant in aquatic systems. It is introduced into the aquatic system by natural weathering processes and from direct or indirect anthropogenic sources. In the last century, the concentrations of Pb in natural waters increased drastically until mid 1960s or early 1970s concomitant with the increased use of leaded gasoline. The limitation of the Pb concentrations in gasoline led to a continuous decrease of environmental concentrations in the last decades (Nriagu, 1990). The main sources besides the use leaded gasoline are fertilizers, dissolution from soils, mining and smelting of metallic ores (Sharma and Dubey, 2005). In pristine mountain streams with only atmospheric deposition of Pb, background concentrations as low as $10^{-12}$ M were reported (Erel et al., 1991). Surface waters in more populated areas show Pb concentrations up to $10^{-7}$ M (Warnken et al., 2009). The bioavailability, and hence uptake and effects of elevated Pb concentrations, is mainly dependent on the speciation of Pb in the water (Morel and Hering, 1993; Tessier and Turner, 1995).

1.1.1 Metal speciation

In natural waters, metals occur in a variety of chemical forms or species, primarily complexed by ligands, in particulate form, or as free metal ions (Buffie, 1988; Sigg and Xue, 1994). Both inorganic and organic ligands bind metals, resulting in dissolved metal-ligand complexes. Inorganic ligands include water, hydroxide, carbonate and chloride (Sigg and Stumm, 1989; Sigg and Behra, 2005). Natural organic ligands are present in a wide range of low and high molecular weight compounds. The small organic ligands include carboxylic acids (citrate, acetate, oxalate and malonate), amino acids, phenols and catechols, which result from
decomposition of organic matter or are excreted by organisms. The most important larger ligands are humic and fulvic acids, which have binding sites for metals such as phenolic, carboxylic, nitrogen and sulfur containing groups. Other ligands include synthetic organic ligands like EDTA and NTA that are introduced into the aquatic environment by anthropogenic activities. The bulk particulate matter consists of oxides and hydroxides of iron, manganese, aluminum, silicates, clay minerals, carbonates and organic matter (bacteria, algae, organic debris) (Sigg and Behra, 2005). Trace metals may be bound by adsorption to functional groups on the surface of these particulate phases.

As a consequence in natural waters metal species with different complexing properties, with respect to kinetic lability and thermodynamic stability, are present. Kinetic lability allows the distinction between labile and “inert” complexes based on the dissociation rate of metals from the complexes. Compared to “inert” complexes, which have high stability constants and low dissociation rates, labile complexes have lower stability constants and a higher dissociation rates. These complexes are operationally defined and therefore their categorization depends on the method used to determine stability constants and dissociation rates. Metal speciation can either be measured with various speciation techniques or it can be estimated with chemical equilibrium models.

1.1.2 Metal speciation and bioavailability to algae

Metal uptake into algae generally includes three steps (Tessier and Turner, 1995; Campbell et al., 2002). In the first step, the free metal ion or metal-ligand complex diffuses through the diffusion layer and the cell wall to the plasma membrane. At the plasma membrane free metal ions bind very fast to transport sites of the membrane, following chemical equilibrium. The fast adsorption is followed by the slower, and uptake limiting, transport through the plasma membrane (Fig. 1.1). The transport can be mediated by transmembrane proteins, such as ion channels, which allow diffusion along an electrochemical gradient, or ion transporters, which are energy dependent. Once inside the cell, metals are either used in metabolism or accumulated in the algal cell (Tessier and Turner, 1995).
To conceptually describe metal uptake into cells, the Free Ion Activity Model (FIAM) was established. FIAM states that biological uptake and effects on aquatic organisms depend on the activity of free metal ions in chemical equilibrium rather than on the total dissolved metal concentration (Morel and Hering, 1993; Campbell, 1995). Binding of metal ions to biological metal carriers is assumed to be in competition with the ligands in solution. Therefore, at chemical equilibrium between these various ligands, the binding to metal carriers is dependent on the free metal ion activity. The main goal of this model is to predict the bioavailability of a metal as a function of its metal speciation. The FIAM was successfully tested for various metals in algae in laboratory experiments using well defined culture media (Anderson et al., 1978; Sunda and Huntsman, 1992; Knauer et al., 1997). Furthermore, the applicability of the FIAM was also shown in algae and periphyton exposed to Cd and Zn under freshwater conditions (Meylan et al., 2003; Töpperwien et al., 2007; Bradac et al., 2009).

However, since its development, limitations and exceptions to the FIAM were reported. Identified exceptions are the formation of metal-ligand complexes that are internalized into the cell by other mechanisms than metal cation transporters or by passive diffusion. Uptake by passive diffusion of lipophilic metal-organic complexes across the plasma membrane was, for example, demonstrated in *Thalassiosira weissflogii* involving copper, lead and cadmium (Phinney and Bruland, 1994). For
silver, increased accumulation was detected in presence of thiosulfate. It was concluded that silver-thiosulfate complexes are transported across the plasma membrane via anion transporters responsible for thiosulfate / sulfate transport (Fortin and Campbell, 2001). Similarly, enhanced uptake of cadmium and zinc as metal-citrate complex were reported (Errécalde et al., 1998; Errécalde and Campbell, 2000). Pb uptake into algae was observed to be mainly governed by the free ion concentration, following the FIAM in the presence of citrate and malonate (Slaveykova and Wilkinson, 2002). In presence of natural organic matter, such as humic acid and fulvic acid, however, Pb uptake increased compared to the amount predicted by FIAM. It was suggested that changes in the algal surface charge through binding of fulvic acid (Slaveykova et al., 2003) or the formation of a ternary complex, between the Pb-humic acid complex and the internalization sites on the algal surface, might be responsible for the enhanced Pb uptake (Lamelas et al., 2005; Lamelas and Slaveykova, 2007; Lamelas et al., 2009).

Because the composition of natural waters is much more complex than synthetic media, the FIAM was further developed into the Biotic Ligand Model (BLM). The BLM takes other cations and natural ligands into account. Cations (e.g. H\(^+\), Mg\(^{2+}\), Na\(^+\), Ca\(^{2+}\)) can compete with the metal of interest for the binding to the biotic ligand (uptake site) and environmental ligands can alter the speciation of both cations and metals (Di Toro et al., 2001; Bell et al., 2002; Campbell et al., 2002) (Fig. 1.2).

![Figure 1.2: Conceptual scheme of the Biotic Ligand Model (BLM) (Di Toro et al., 2001).](image)
Both FIAM and BLM are models based on the assumption of chemical equilibrium and do not consider kinetic effects. They assume equilibrium between free metal ion concentration in solution and metal adsorbed to the biotic ligand and further, that diffusion of metal species to the cell membrane is faster than internalization. In contrast, non-equilibrium based models consider diffusion and internalization fluxes (van Leeuwen, 1999; Slaveykova and Wilkinson, 2005; van Leeuwen et al., 2005). If diffusion to the cell membrane is rate limiting, metal uptake is controlled by the concentration of chemically labile species, which have low stability and high dissociation rate (Hudson, 1998; van Leeuwen, 1999; Slaveykova and Wilkinson, 2005). This was confirmed for silver uptake in \textit{Chlamydomonas reinhardtii} (Fortin and Campbell, 2000) and for copper and cadmium uptake in periphyton under freshwater conditions (Meylan et al., 2003; Bradac et al., 2009).

\textbf{1.1.3 Intracellular metal homeostasis and detoxification}

The need for essential trace metals in organisms is linked to their function; they are essential as cofactor of enzymes, for electron transport, for catalysis of redox reactions and for the maintenance of conformations and tertiary structures of proteins (Lehninger, 1982). Aquatic organisms have specific requirements in terms of trace metal concentrations to sustain vital processes and optimal development. Nevertheless, these trace metals become toxic for organisms at concentrations above the requirement or deficiencies arise at too low concentrations. In contrast, non-essential metals are not used in the metabolism and already toxic at low concentrations.

Therefore, intracellular metal concentrations are tightly regulated in order to maintain physiological functions and to prevent toxic effects. Once metals are inside the cell, they are rapidly bound by ligands, such as chaperones and chelators (Mason and Jenkins, 1995; Clemens, 2001). Essential metals are bound by chaperones that are involved in metal trafficking and deliver them to specific organelles and metal-requiring proteins. Uptake into organelles is catalyzed by metal transporters that directly interact with specific chaperones. To store metals or to prevent toxic effects of essential and non-essential metals, metal ions can be directly sequestered into the vacuole or bound to chelators (metallothioneins, phytochelatins, glutathione, organic acids, amino acids), which can be also transported into vacuoles or excreted from the cell (Lee et al., 1996; Clemens, 2001).
Chapter 1

The Subcellular Partitioning Model (SPM) has been developed to predict metal toxicity to aquatic organisms, such as algae (Wang and Rainbow, 2006). It assumes that toxicity depends on the distribution of metal among different operationally defined subcellular fractions. It is hypothesized in SPM that elevated metal concentrations saturate detoxifying ligands, leading to a redistribution of metals to sensitive ligands, resulting in toxicity (Wang and Wang, 2008). A few recent studies have tried to relate subcellular metal distribution to toxicity in marine phytoplankton (Miao and Wang, 2007; Wang and Wang, 2008) and freshwater species (Lavoie et al., 2009).

The toxicity of Pb is assumed to arise from non-specific binding to important biomolecules, due to the high affinity of Pb to oxygen, nitrogen and sulfur functional groups (Sharma and Dubey, 2005). Therefore, it can substitute essential metal ions or directly bind to functional groups. Binding to functional groups can have an effect on enzyme activity, by reducing or suppressing its biological activity. In addition, Pb can affect photosynthesis by inhibition of chlorophyll synthesis due to impaired uptake of Mg and Fe and by inhibition of electron transport in photosystem II (Sharma and Dubey, 2005). Pb can also cause toxicity by production of reactive oxygen species (Szivak et al., 2009). Due to the high affinity of Pb to various functional groups, Pb is bound to various ligands, including metal detoxifying ligands and metal sensitive ligands. To explore the mechanisms involved in Pb detoxification, Pb distribution among the different operationally defined subcellular fractions have to be examined.

1.2 Phytochelatins

1.2.1 Structure and biosynthesis

Phytochelatins (PCs) are heat stable, metal-binding polypeptides with the general structure \((\gamma\text{-Glu-Cys})_n\text{-Gly}\) where \(n = 2 – 11\) (Robinson, 1989; Rauser, 1990; Steffens, 1990; Cobbett, 2000) (Fig. 1.3). On the basis of the number of \(-\text{Glu-Cys}\) units, PCs have been classified as PC2, PC3, PC4 etc (Grill et al., 1985; Rauser, 1995). PCs are synthesized enzymatically by the addition of glutathione (GSH) to \((\gamma\text{-Glu-Cys})_n\) by the phytochelatin synthase to produce a \(n + 1\) oligomer. The activation of phytochelatin synthase occurs by the binding of metal ions and metal-glutathione
complexes (Cobbett, 2000; Vatamaniuk et al., 2000). Experiments have shown that phytochelatin synthase is activated by a wide range of metals and metalloids such as Cd, Ag, Pb, Zn, Cu, and Hg (Rauser, 1995).

1.2.2 Induction of phytochelatin synthesis in algae

Phytochelatin synthesis was observed in almost all algal species tested under exposure to various metals and metalloids. The strength of induction, however, varies with the involved metal, its concentration and the algal species. Furthermore, phytochelatin synthesis is a metal specific response not induced upon exposure to other stressors. Cd was reported to be the most potent inducer in plants. Therefore, most studies examined the induction of phytochelatins under Cd exposure. Studies using Cd as an inducer of phytochelatin synthesis reported a highly species specific response in various freshwater and marine algal species (Gekeler et al., 1988; Ahner et al., 1995; Le Faucheur, 2005). PC synthesis varied by more than a factor of 10 in different species, however, Cd was found to induce large amounts of phytochelatins in all species. Comparison to other metals showed that Cd induces the largest amount of phytochelatins in marine algae (Ahner and Morel, 1995), and the highest degree of phytochelatin polymerization was observed under Cd exposure (Allen et al., 1980). The only algal species without detectable induction of PC synthesis under Cd exposure were *Achnanthes brevipes* and *Prorocentrum micans* (Pistocchi et al., 2000). Pb was shown to be a good inducer of phytochelatin synthesis in both freshwater and marine algal species (Ahner and Morel, 1995; Pawlik-Skowronska, 2000; Morelli and Scarano, 2001; Pawlik-Skowronska, 2001; Pawlik-Skowronska, 2002; Le Faucheur et
al., 2006). Under Pb exposure mainly short chain phytochelatins such as PC$_2$ and PC$_3$ were observed and the highest degree of polymerization detected was PC$_6$ in *Phaeodactylum tricornutum* (Morelli and Scarano, 2001). Furthermore, time dependence of PC induction was examined and revealed that shorter chain oligomers are induced first, followed by longer chain oligomers. In addition, fast degradation of PC oligomers when transferred to metal free medium was observed (Morelli and Scarano, 2001; Pawlik-Skowronska, 2001).

Examination of PC synthesis under Cu exposure was mainly examined in marine algae and reported inductions of PC$_2$ – PC$_4$ (Ahner and Morel, 1995; Rijstenbil and Wijnholds, 1996; Ahner et al., 1997; Ahner et al., 2002; Wei et al., 2003). Only in one freshwater alga phytochelatins were quantified under Cu exposure, showing an increase of total PC$_n$ from $1 – 2$ µmol / g chl a at pCu15 to a maximum $20$ µmol / g chl a at pCu9 (Knauer et al., 1997).

PC synthesis induced by Zn was extensively studied in various *Stigeoclonium* species (Pawlik-Skowronska, 2001; Pawlik-Skowronska, 2003). For various strains, similar amounts of PCs were quantified, but the tolerant species produced a novel peptide, differing from phytochelatins by an additional cysteine. In *T. weissfolgii*, PC content was found to decrease under Zn limiting conditions, whereas high Zn concentrations did not lead to increased PC production (Ahner and Morel, 1995). In addition, other species only showed induction of PC synthesis at very high Zn concentrations (pZn 7.2 in *Skeletonema costatum*) (Ahner and Morel, 1997) or no induction in *Chlorella kessleri* (Hassler et al., 2005).

Studies on induction of PC synthesis by other metals are scarce. Low PC$_2$ concentrations were observed upon Ag exposure in *Pseudokirchneriella Subcapitata* (Hiriart-Baer et al., 2006), but no induction of PCs was detected in *Scenedesmus vacuolatus*, *C. reinhardtii* and *T. weissflogii* (Howe and Merchant, 1992; Ahner and Morel, 1995; Le Faucheur et al., 2006). PC$_n$ formation was slightly induced in *Chlorella fusca* by Co and Ni (Gekeler et al., 1988). Hg was found to induce PC synthesis in *T. weissflogii* (Ahner and Morel, 1995; Morelli et al., 2009) but not in *C. reinhardtii* (Howe and Merchant, 1992). Furthermore, induction of PC synthesis was reported upon exposure to the metalloids As(V), As(III) and Sb(III) in the freshwater species *S. vacuolatus* (Pawlik-Skowronska et al., 2004; Morelli et al., 2005; Le Faucheur et al., 2006) and marine algae (Pawlik-Skowronska et al., 2004; Morelli et al., 2005).
1.2.3 Role of phytochelatins in metal detoxification and formation of metal-phytochelatin complexes

First evidence on the role of PC in metal detoxification were obtained by using yeast (*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) and plant mutants (*Arabidopsis spp.*) that were deficient in PC synthase genes (Clemens et al., 1999; Ha et al., 1999). Indeed, these species were more sensitive to Cd, As(V) and Cu than the wild-type. It is assumed, that the role of PC in metal detoxification is the formation of a metal-phytochelatin (Me-PC) complex, used to avoid metal binding to other target sites, followed by its transport into vacuoles (Fig. 1.4). Formation of Me-PC complexes was detected *in vitro* for Cd, Pb, Ag, Cu, As(V), As(III) and Hg (Mehra et al., 1995; Maitani et al., 1996; Rauser, 1999; Schmoger et al., 2000). In more recent studies *in vitro* Me-PC complexes formed with Pb, Cd, Zn and Hg were analyzed by differential pulse polarography and voltammetry reporting the formation of Me-PC complexes with various stoichiometries for the examined metals (Chekmeneva et al., 2008; Cruz et al., 2001; Cruz et al., 2005; Alberich et al., 2007a; Alberich et al., 2007b; Chekmeneva et al., 2007). It was found that PCs interact with metals through the thiol (-SH) group of PC cysteine. It was reported that an increase in the degree of polymerization of PCs led to increased binding stability of Me-PC complexes (Mehra et al., 1995).

Most studies in plants (Kneer and Zenk, 1997; Yen et al., 1999; Chen et al., 2007) and algae (Hu and Wu, 1998; Hu et al., 2001; Morelli et al., 2002; Scarano and Morelli, 2002) were carried out examining Cd-PC complexes. These studies revealed the presence of two kinds of complexes, low molecular weight complexes (LMW) and high molecular weight complexes (HMW). LMW complexes are defined as complexes formed by Cd (or other metals) bound to a single phytochelatin oligomer. The composition of HMW complexes was found to be from the formation of a CdS core, generated by Cd and acid labile sulfur (S-), surrounded by PCs (mainly PC₂ and PC₃). The role of PC coating is to stabilize the CdS core. The presence of HMW complexes increases metal tolerance, as shown in the yeast *Candida glabrata*, where a strain with enhanced CdS production revealed increased Cd resistance compared to the wildtype (Barbas et al., 1992; Mehra et al., 1994).

Complexes identified *in vivo* were mainly Cd-PC complexes. Studies on Me-PC complexes induced by Pb *in vivo* are very scarce in plants (Andra et al., 2009) and algae (Scarano and Morelli, 2002). In the marine alga *P. tricornutum* formation of Pb-
PC complexes with PC₃ – PC₆, but not with PC₂, was observed (Scarano and Morelli, 2002). This literature review indicates that there are many open questions concerning the role of PCs in Pb detoxification.

![Phytochelatin biosynthesis pathway and possible fate of Me-PC complexes within the cell.](image)

**Figure 1.4:** Phytochelatin biosynthesis pathway, and possible fate of Me-PC complexes within the cell. Enzymes involved in PC synthesis are GCS = Glutamyl-cysteinyl synthase, GS = Glutathione synthase and PCS = Phytochelatin synthase. Transport into the vacuoles and formation of HMW complexes was observed to be involved in Cd detoxification and was not observed with other metals. Excretion of Me-PC complexes and the homeostatic function of PC were not proven so far.

### 1.3 Scope of the thesis

Due to the ecotoxicological importance of Pb in aquatic systems, the scope of this work was to study the induction of PC synthesis as a function of the free Pb ion concentration in the exposure medium. Additionally, toxic effects of Pb were analyzed to explore the role of PCs in Pb detoxification. Pb induced metal-phytochelatin complexes were examined for their stoichiometry and composition to evaluate whether Pb is bound by phytochelatins.
1. Phytochelatin formation kinetics and toxic effects in the freshwater alga *Chlamydomonas reinhardtii* upon short- and long-term exposure to lead(II) (Chapter 2)

Pb is a toxic and abundant non-essential metal in the environment. Many studies on phytochelatin induction by various metals were carried out, but only a few were conducted on the induction of phytochelatins by Pb in freshwater algae. In this study, phytochelatin synthesis, toxic effects and Pb accumulation as a function of the free Pb ion concentration in the medium were examined. To that aim, *C. reinhardtii* was exposed to various free Pb ion concentrations, and formation kinetic of phytochelatins and Pb accumulation was investigated in short-term experiments. Furthermore, the role of PCs in Pb detoxification was explored by investigating how PC synthesis relates to effects of Pb on growth and photosynthetic yield, upon long-term exposure to Pb.

2. Characterization of lead induced metal-phytochelatin complexes in *Chlamydomonas reinhardtii* (Chapter 3)

In the first study of this work, induction of phytochelatin synthesis was observed at elevated free Pb concentrations and toxic effects were observed upon long-term exposure. Therefore, the aim of this study was to examine whether Pb is actually bound to phytochelatins, in order to explore the role of PCs in Pb detoxification. For that purpose, Me-PC complexes were extracted under native conditions from *C. reinhardtii*. The homogenate was separated by gel filtration and collected fractions were analyzed for non protein thiols by HPLC and metals by ICP-MS to determine, if Pb co-eluted with PCs. Furthermore, results suggested putative stoichiometries and compositions of Me-PC complexes and conclusions about the role of PCs in Pb detoxification were drawn.

3. Characterization of standard lead-phytochelatin complexes by nano-electrospray ionization mass spectrometry (nano-ESI-MS) (Chapter 4)

In this study a method was developed to identify Me-PC complexes under non denaturing conditions by nano-ESI-MS. This method is being used to identify the precise molecular weight of Me-PC complexes and to further examine formed complexes for their stoichiometry and composition. To identify Me-PC complexes, Pb-PC complexes were formed using phytochelatin standards (PC₂ – PC₄) and Pb
nitrate. The complexes formed were analyzed under various conditions using direct infusion nano-ESI-MS. The study indicated the formation of four different Pb-PC$_2$ and two Pb-PC$_3$ and Pb-PC$_4$ complexes which were stable enough to be detected by this method. Furthermore, the results indicate that Pb is coordinated through thiol groups of PC cysteine and in some cases additionally through carboxyl groups of PC glutamic acid.
1.4 References


Chapter 1

Chekmeneva, E., Prohens, R., Diaz-Cruz, J.M., Arino, C., Esteban, M., 2008, Thermodynamics of Cd$^{2+}$ and Zn$^{2+}$ binding by the phytochelatin ($\gamma$-Glu-Cys)$_4$-Gly and its precursor glutathione. Anal. Biochem. 375, 82-89


Chapter 2

Phytochelatin formation kinetics and toxic effects in the freshwater alga *Chlamydomonas reinhardtii* upon short- and long-term exposure to lead(II)

This chapter will be published in *Aquatic toxicology*
2.1 Abstract

Phytochelatins (PC) are metal-binding ligands synthesized by algae in response to elevated concentrations of various metals, such as Pb. Kinetics of PC synthesis and Pb accumulation in *Chlamydomonas reinhardtii* were investigated as a function of \([\text{Pb}^{2+}] = 10^{-11} - 10^{-7} \text{ M (pPb11 – pPb7.1)}\) in the exposure medium for up to 6 h. The role of PC in Pb detoxification was explored by relating PC synthesis to the effects of Pb on growth and photosynthetic yield upon exposure to pPb9 and pPb8.3 for up to 72 h. Pb accumulation increased with increasing \([\text{Pb}^{2+}]\), reaching a maximum concentration of 596 ± 77 amol / cell (intracellular concentration 2.98 mM) at pPb7.1. Low concentrations of PC2 – PC4 were present in *C. reinhardtii* grown in control media without Pb addition. Upon short-term exposure, PC2 and PC3 synthesis was induced within minutes at \([\text{Pb}^{2+}] \geq \text{pPb8}\) and PC4 synthesis after a lag phase at pPb7.1. Cellular PC2 – PC4 concentrations increased with time over 6 h and with increasing \([\text{Pb}^{2+}]\). PC concentrations after 6 h exposure to pPb7.1 were 28.5 ± 0.2 amol / cell (142 µM) PC2, 2.8 ± 0.05 amol / cell (14 µM) PC3 and 0.30 ± 0.01 amol / cell (1.5 µM) PC4. Upon long-term exposure, induction of PC synthesis was detected at pPb9 and synthesis of PCs with a higher degree of polymerization was observed (PC5). PC concentrations were lower than intracellular Pb and were thus not present at sufficiently high concentrations to immobilize accumulated Pb. Inhibition of photosynthesis and growth up to 100 % was observed upon long-term exposure; whereas in short-term experiments no inhibitory effects were detected.

2.2 Introduction

Lead, a non essential and toxic heavy metal, enters the environment through various sources like smelting of metallic ores, industrial fabrication and mainly the use of leaded gasoline (Nriagu, 1988; Sharma and Dubey, 2005). Pb concentrations in the range 1 – 8 x 10^{-12} M were reported in remote mountain streams, which are only exposed to atmospheric Pb deposition (Erel et al., 1991). Rivers in more populated areas contain Pb concentrations up to 10^{-7} M (Warnken et al., 2009). It has been shown that Pb accumulates in algae (Crowder, 1991; Gupta et al., 1995; Debelius et al., 2009). Pb uptake into animal cells might occur through Ca or Mg channels (Kerper and Hinkle, 1997), which are also assumed to be involved in Pb uptake into algal cells. Furthermore, concentration dependent effects of Cu on Pb internalization...
were reported (Chen et al. 2010), indicating a role of Cu-transporters in Pb uptake.
Upon elevated concentrations of Pb, toxic effects on ultrastructure, metabolism and
the production of reactive oxygen species (ROS) were observed in plants (Sharma and
Dubey, 2005) and algae (Sharma and Dubey, 2005; Szivak et al., 2009). The
mechanisms leading to Pb toxicity in algae are unknown and assumed to be caused by
non-specific binding of Pb to physiologically important molecules and the production
of ROS species.
Algae possess extracellular and intracellular mechanisms to prevent metal toxicity.
Extracellularly, metal uptake into algal cells is limited by lowering the metal
bioavailability through excretion of non-specific ligands (Soldo et al., 2005) or by
altering concentration and affinity of metal carrier proteins. Furthermore, chemical
transformations such as oxidation, reduction and methylation of metals may play a
role in metal detoxification, because these transformation reactions may influence the
solubility and toxicity of a metal. In addition, intracellular metal detoxification can
occur through precipitation or immobilization of metals by ligands (Mason and
Jenkins, 1995).
Specific ligands synthesized to immobilize metals within the cell are phytochelatins
(PCs). PCs are metal-binding oligopeptides with a general structure (γ-Glu-Cys)\(_n\)-Gly
\((n = 2 – 11)\), which are synthesized upon elevated metal concentrations in plants
(Rauser, 1995; Zenk, 1996) and algae (Gekeler et al., 1988; Ahner et al., 1995a; Le
Faucheur et al., 2005). Cd was shown to be the strongest inducer of phytochelatin
synthesis, but induction of PC synthesis by other metals and metalloids (Pb, Cu, Zn,
Ag, As(V)) was also observed (Gekeler et al., 1988; Ahner and Morel, 1995b; Le
Faucheur et al., 2006). PC is synthesized post-translationally from glutathione (GSH)
by the enzyme phytochelatin synthase. The expression of phytochelatin synthase is
constitutive. Enzyme activation occurs by the binding of inducing metal ions and
metal-glutathione complexes (Cobbett, 2000; Vatamaniuk et al., 2000). Immobilization of metals by PCs occurs through complex formation of PCs with
metals through thiol coordination of the PC cysteine.
Induction of PC synthesis is a metal specific response not induced by other chemicals,
but the role of PCs in metal detoxification is not fully understood. Few studies on
induction of PCs by Pb in algae were carried out (Ahner and Morel, 1995b; Scarano
and Morelli, 2002; Le Faucheur et al., 2006) and reported phytochelatin synthesis at
various Pb concentrations. In the alga \textit{Stichococcus bacillaris}, PCs increased
continuously with exposure time at nominal concentrations up to 20 µM (Pawlik-Skowronska, 2000). The present study aimed to investigate the kinetics of PC synthesis in the alga *Chlamydomonas reinhardtii* as a function of [Pb²⁺] by measuring cellular PC concentrations and intracellular Pb concentrations during exposure over several hours, using exposure media which are controlled and buffered with respect to Pb speciation. A further aim was to explore the role of PCs in Pb detoxification by investigating how PC synthesis relates to effects of Pb on growth and photosynthetic yield, which were measured during exposure for several days.

### 2.3 Materials and methods

#### 2.3.1 Chemicals

Metal salts for metal solutions, ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA) and most other chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Supelco (Bellefonte, PA, USA). Nitric acid (HNO₃; 65 %), hydrochloric acid (HCl; 30 %) and hydrogen peroxide (H₂O₂; 30 %) were suprapure chemicals from Merck (Darmstadt, Germany). Phytochelatin standards (PC₂, PC₃ and PC₄) were obtained from Invitrogen (San Diego, CA, USA).

Glassware and polycarbonate containers were presoaked in 0.01 M HNO₃ and rinsed with nanograde water (18 MΩ-cm, Q-H₂O grade, Barnstead Nanopure, Alleschwil, Switzerland) before use. All solutions and media were prepared in nanograde water in autoclaved containers.

#### 2.3.2 Test organism and growth conditions

Experiments were carried out with cultures of the unicellular green alga *C. reinhardtii* (strain CC125). Stock cultures were obtained from the Chlamydomonas Genetics Center (Durham, NC, USA).

Algae were grown in glass Erlenmeyer flasks in a HT Infors shaker (Infors, Bottmingen, Switzerland) at 25 °C under continuous illumination of 120 µEm⁻²s⁻¹ provided by cool white fluorescent lamps and were shaken at 90 rpm. For the experiments and exposure to Pb, algae were acclimatized to the growth medium by transferring an inoculum of exponentially growing algae to the growth medium in at least two successive batch cultures.
Chapter 2

*C. reinhardtii* were cultured in Talaquil, a complete growth medium containing major nutrients and trace metals buffered by EDTA (Knauer et al., 1997), in which pH is buffered using MOPS (3-morpholinopropanesulfonic acid) as a non-metal complexing buffer. The total concentrations in the growth medium are the following: $2 \times 10^{-5}$ M Na$_2$EDTA; $5 \times 10^{-4}$ M CaCl$_2 \cdot 2$H$_2$O; $1.5 \times 10^{-4}$ M MgSO$_4 \cdot 7$H$_2$O; $1.2 \times 10^{-3}$ M NaHCO$_3$; $5 \times 10^{-5}$ M K$_2$HPO$_4 \cdot 3$H$_2$O; $1 \times 10^{-3}$ M NH$_4$Cl; $5 \times 10^{-8}$ M CoCl$_2 \cdot 6$H$_2$O; $5 \times 10^{-5}$ M H$_3$BO$_3$; $8 \times 10^{-8}$ M Na$_2$MoO$_4 \cdot 2$H$_2$O; $1.63 \times 10^{-7}$ M CuSO$_4 \cdot 5$H$_2$O; $1.22 \times 10^{-6}$ M MnCl$_2 \cdot 4$H$_2$O; $1.58 \times 10^{-7}$ M ZnSO$_4 \cdot 7$H$_2$O and $9 \times 10^{-7}$ M FeCl$_3 \cdot 6$H$_2$O in $1 \times 10^{-2}$ M MOPS buffer (pH 7.5). All media were prepared at least 24 h before use to allow equilibration. Free ion concentrations of Cu$^{2+}$, Mn$^{2+}$, Zn$^{2+}$ and Fe$^{3+}$ in Talaquil, modeled using the speciation software VMINTEQ (Gustafsson, 2005), are $3 \times 10^{-14}$, $1.7 \times 10^{-8}$, $9 \times 10^{-12}$ and $1 \times 10^{-20}$ M.

2.3.3 Experimental setup to determine short-term phytochelatin synthesis kinetics

For the kinetics experiments, exponentially growing algae were exposed for 0 – 6 h to various pPb (pPb = -log [Pb$^{2+}$]; pPb11, pPb10, pPb9, pPb8, pPb7.5 and pPb7.1). The cell density was $\sim 8.4 \times 10^5$ cells / ml for all experiments. The experimental medium used for Pb exposures was Talaquil without trace metal mix, containing Pb buffered with 20 µM EDTA (pPb11 – pPb8) or NTA (> pPb8) to keep [Pb$^{2+}$] constant. Trace metals were omitted to avoid metal competitions and cumulative effects resulting from the presence of various metal ions. Total metal concentrations in the experimental medium were measured by ICP-MS and [Pb$^{2+}$] was modeled using VMINTEQ (Gustafsson, 2005).

A sample was taken at the start of exposure (time 0) and later at 30, 60, 120, 180, 240, 300 and 360 min after start of exposure. At every time point non protein thiols (GSH, PCs), photosynthetic activity, cell number and cell volume were measured. Cellular concentrations of PCs were calculated using the average algal cell volume of 200 fL measured by the electronic particle counter (Orifice, 50µm; Multisizer II; Beckham Coulter, Fullerton, CA, USA). All experiments were carried out in triplicate cultures.

2.3.4 Experimental setup for chronic lead exposures

To examine long-term effects of lead on growth, photosynthesis and non protein thiols algae were exposed in a modified Talaquil (containing trace metal mix) at pPb9
and pH 8.3. Free metal ion concentrations for all trace metals were maintained the same as in the growth medium by adjusting their total concentration. Chronic exposure was done over three subsequent cultures and was started by exposing exponentially growing algae up to late exponential growth phase (24 – 27 h). Then, an inoculum of this culture was transferred to fresh exposure medium (subculture 1) and from subculture 1 to subculture 2, again until late exponential growth phase. The initial cell density in each of the three replicate cultures was 8.4 x 10^5 cells / ml in order to allow exponential growth of *C. reinhardtii*. Growth, photosynthesis and PCs were measured in each subculture.

### 2.3.5 Growth rate determination and photosynthetic yield measurements

Cell number was measured with an electronic particle counter (Orifice, 50µm; Multisizer II; Beckman Coulter, Fullerton, CA, USA) at all time points in short-term and chronic exposure experiments. For chronic exposures the growth rate of the cultures was determined by measuring cell density in each culture every 2 h during exponential phase and calculating the growth rate \( \mu \) (\( N_t = N_0 \times e^{\mu t} \); \( N_t \) = cell number at time \( t \); \( N_0 \) = initial cell number; \( \mu \) = growth rate; \( t \) = time).

To measure the photosynthetic yield of *C. reinhardtii*, algal cultures were diluted with the corresponding experimental medium to 10^5 cells / ml and the photosynthetic yield was read on the Maxi-Imaging-PAM (IPAM, Walz GmbH, Germany) by applying three saturation pulses each 20 s after acclimatization to actinic light. For each time point and concentration triplicate measurements of the photosynthetic yield were done.

### 2.3.6 Cell homogenization and non protein thiol analysis

For non protein thiol analysis 50 ml algal cells (8.4 x 10^5 cells / ml) were harvested by centrifugation for 10 min at 5000 x g. The cells were resuspended in 200 µl HCl / DTPA (diethylenetriamine-pentaacetic acid) (0.12 M / 5 mM) to allow protein precipitation and the suspension was homogenized for 2 x 45 s at 6.0 m / s by mechanical breaking of the cells with silica beads using a Fast prep instrument (FP120, Savant Instruments, Inc., Holbrook, NY, USA). Cell breakage was determined microscopically after staining with methylene blue. The homogenate was centrifuged for 10 min at 9900 x g and 4 °C to pellet precipitated proteins and cell
fragments. The resulting supernatant (first extract) was removed and kept on ice prior to derivatization and thiol analysis. A second extract was obtained through repetition of the previously described extraction procedure. Both extracts were derivatized and analyzed for non-protein thiols. The recovery of the second extract was 5 – 10 % of the first extract. Additional extraction steps resulted in recoveries of < 1 % of the first extract.

Thiols were measured by reversed phase high performance liquid chromatography (HPLC) after derivatization of thiol groups with monobromobimane as fluorescent tag following the procedure described in (Le Faucheur et al., 2005). For fluorescence detection an Agilent 1200 series liquid chromatography system with a fluorescence detector (Agilent Technologies, Santa Clara CA, United States) was used. The separation of the derivatized sample was carried out on a reversed phase octadecylsilica column (Nucleosil C18, 5 µm, 250 x 4 mm) obtained from Macherey und Nagel and a flow rate of the mobile phase of 1 ml / min. Trifluoroacetic acid (0.1 %) and a gradient from 10 – 36 % acetonitrile over 65 min were used as the mobile phase. Between each measurement the column was cleaned with 100 % acetonitrile for 5 min, followed by 10 min at 10 % acetonitrile for equilibration. For sample injection a 100 µl loop was used resulting in a detection limit of ~ 0.1 pmol thiol groups (~ 0.02 amol / cell). Thiols were detected by fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 470 nm. The retention time of the PC oligomers was verified by PC2 – PC4 standards. For quantification of GSH and PCs a calibration curve relating GSH concentrations of standard solutions and resulting peak area was used.

2.3.7 Total and intracellular metal measurements

The total (cell wall associated and intracellular Pb) and intracellular Pb concentration in *C. reinhardtii* was quantified after 6 h exposure by acidic digestion. For determination of total cellular Pb, 20 ml aliquots of algal cultures were filtered on acid-washed (cellulose nitrate; pore size 0.45 µm; Sartorius, Goettingen, Germany) and dried (15 h; 50 °C) filters. After filtration the filters were dried at 50 °C for one day. The filters were placed in Teflon digestion flasks, 1 ml H₂O₂ (30 %) and 4 ml HNO₃ (65 %) were added and digestion was performed in a high performance microwave digestion unit (mls1200 mega; Microwave Laboratory Systems, Oberwil, Switzerland) for 30 min. The metal concentrations were measured after appropriate
dilution by inductively coupled plasma mass spectrometry (ICP-MS; Perkin-Elmer; Perkin Elmer Sciex, Ueberlingen, Germany), using rhodium as an internal standard. The ICP-MS measurements were checked using SLRS-4 reference water (River Water Reference Material for Trace Metals; National Research Council Canada, Ottawa). The remaining algal culture was washed by addition of 4 mM EDTA (final concentration) for 10 min to remove metals adsorbed to the cell walls. The remaining metal content is considered to be intracellular (nonexchangeable with EDTA) (Meylan et al., 2004). Cell wall adsorbed metal can be calculated as the difference between the total and the intracellular Pb. For each Pb concentration three replicate cultures were used. In addition, three filter replicates of each algal culture were done for both total and intracellular Pb. The cellular Pb concentration was calculated using the average algal cell volume of 200 fL.

2.4 Results
2.4.1 Total and intracellular lead in *Chlamydomonas reinhardtii* under lead exposure
The total Pb content in *C. reinhardtii* after short-term exposure (6 h) increased with increasing [Pb$^{2+}$] from $7.79 \pm 0.63$ amol / cell (amol / cell = $10^{-18}$ mol / cell) in control cultures (pPb15.8) to $476 \pm 32$ amol / cell at pPb9 and further to $684 \pm 50$ amol / cell at pPb7.1 (Fig. 2.1). Intracellular Pb also increased in the same manner from $3.89 \pm 1.49$ amol / cell in control algae to $476 \pm 10$ amol / cell at pPb9, reaching a maximum of $596 \pm 77$ amol / cell at pPb7.1 (Fig. 2.1). Cell wall adsorbed Pb increased from 0 amol / cell at pPb9 to 88 amol / cell (13 %) at pPb7.1.
Chapter 2

2.4.2 Kinetics of phytochelatin synthesis in the green alga *Chlamydomonas reinhardtii* as a function of free lead ions

In *C. reinhardtii* grown under optimal conditions PC$_2$ – PC$_4$ were detected at concentrations of 5.4 ± 1.0 amol / cell for PC$_2$, 0.5 ± 0.1 amol / cell for PC$_3$ and 0.1 ± 0.02 amol / cell for PC$_4$ (Fig. 2.2). Similar PC contents were measured in algae exposed to [Pb$^{2+}$] lower than pPb8. Synthesis of cellular PC was induced at pPb8, pPb7.5 and pPb7.1 (Fig. 2.2a / Fig. 2.2b). Induction of PC$_2$ synthesis occurred within minutes after the start of lead exposure. At pPb7.1 the PC$_2$ concentration increased linearly from the control level to 28.5 ± 0.2 amol / cell (Fig. 2.2a). Induction of PC$_3$ synthesis was detectable after exposure for 60 – 120 min, reaching a concentration of 2.8 ± 0.05 amol / cell at pPb7.1 (Fig. 2.2b). Induction of PC$_4$ synthesis was observed after exposure for 4 h to pPb7.1. The cellular concentration measured after 6 h was 0.30 ± 0.01 amol / cell (Fig. 2.2c). PC$_2$ content after 6 h exposure increased with increasing [Pb$^{2+}$] (Fig. 2.2a), whereas for PC$_3$ the measured cellular concentration after 6h was less for pPb7.5 (1.56 amol / cell) than for pPb8 (2.78 amol / cell) (Fig. 2.2b).
Figure 2.2: Kinetic of phytochelatin (PC2 – PC4) synthesis and GSH from 0 – 6 h exposure to various [Pb^{2+}] (pPb7.1 = ■, pPb7.5 = ▲, pPb8 = ▼, pPb9 = ●, pPb10 = ○, pPb11 = ×) and control cultures (Control = △). The cellular concentrations of PC2 – PC4 and GSH during the time of exposure are shown in Figure 2.2a – 2.2d (PC2, PC3, PC4, GSH). Error bars indicate the standard deviation of three replicate cultures.

The calculated rates of PC2 synthesis increased with increasing [Pb^{2+}] from 0.05 amol cell^{-1} min^{-1} at pPb8 to 0.09 amol cell^{-1} min^{-1} at pPb7.1. The rate of PC3 synthesis was 10 times lower and similar at pPb8 and at pPb7.1. The rate for PC4 synthesis at pPb7.1 was 0.001 amol cell^{-1} min^{-1} (Table 2.1). GSH concentrations measured in control algae were 837 ± 153 amol / cell and were similar at all [Pb^{2+}] concentrations and exposure times (Fig. 2.2d).
Table 2.1: Rates of PC-synthesis (PC₂, PC₃, PC₄) for various [Pb²⁺] from 0 – 6 h of exposure. At concentrations lower than pPb8 no induction of PC-synthesis was detected. PC₄ was only induced at pPb7.1. The rates of synthesis were calculated for the time with detectable PC induction.

<table>
<thead>
<tr>
<th>[Pb²⁺]</th>
<th>PC₂ (amol cell⁻¹ min⁻¹)</th>
<th>PC₃ (amol cell⁻¹ min⁻¹)</th>
<th>PC₄ (amol cell⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPb7.1</td>
<td>8.7 x 10⁻²</td>
<td>7.3 x 10⁻³</td>
<td>1.2 x 10⁻³</td>
</tr>
<tr>
<td>pPb7.5</td>
<td>4.8 x 10⁻²</td>
<td>3.7 x 10⁻³</td>
<td>x</td>
</tr>
<tr>
<td>pPb8</td>
<td>4.7 x 10⁻²</td>
<td>6.2 x 10⁻³</td>
<td>x</td>
</tr>
<tr>
<td>pPb9 – pPb11</td>
<td>no induction of PC synthesis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The calculated ratio of the sum of total non-protein thiols (\(\sum_{\text{SHtot}}\)) to intracellular lead ([Pb(II)]_{intra}) was ~ 2 and similar at all [Pb²⁺] concentrations. The ratio of total thiol groups of PCₙ [\(\sum_{\text{SH (PCₙ)}}\)] to [Pb(II)]_{intra} increased from 0.011 at pPb9 to 0.11 at pPb7.1 (Fig. 2.3a). A large increase from 5.3 amol / cell to 66.7 amol / cell in [\(\sum_{\text{SH (PCₙ)}}\)] was measured in comparison to [Pb(II)]_{intra} at pPb9 and pPb7.1 (Fig. 2.3b).

Figure 2.3: a) Ratio of PC - thiols ([\(\sum_{\text{SH (PCₙ)}}\)] = 2 x PC₂ + 3 x PC₃ + 4 x PC₄) to intracellular lead for various [Pb²⁺] after 6 h exposure. b) Total cellular PC - thiols ([\(\sum_{\text{SH (PCₙ)}}\)]) as a function of intracellular Pb.
2.4.3 Effects of lead on growth, photosynthetic yield and phytochelatin production upon long-term exposure

Pb did not show any effects on photosynthetic yield in the green alga *C. reinhardtii* upon exposure to pPb11 – pPb7.1 for up to 6 h (data not shown). However, inhibitory effects on photosynthetic yield and growth were observed in long-term experiments at pPb9 and pPb8.3. A first effect on the photosynthetic yield was detected in the first subculture, after 45 h of Pb exposure, showing an inhibition of 50 – 60 % at both Pb concentrations. After 70 h of exposure (second subculture) photosynthetic yield was inhibited by ~ 90 % at pPb9 and 100 % at pPb8.3 (Fig. 2.4a).

Compared to control cultures, the growth rate of Pb exposed *C. reinhardtii* increased to 150 % compared to control cultures in the first culture (0 – 27 h), followed by an inhibition of 20 % in the first subculture (27 – 52 h) and up to 50 % in the second subculture (52 – 76 h) (Fig. 2.4b).

![Figure 2.4: Effects of chronic lead exposure in modified growth medium (pPb9 = ●, pPb8.3 = ▼) on a) photosynthetic yield (■ = control) and b) growth rate as % of the growth rate of control culture. Error bars are the standard deviations of three replicate cultures.](image)

PC concentrations were higher upon long-term Pb exposure than in control cultures. PC2 and PC3 concentrations were found to be similar to control cultures at both time points (24 h and 48 h). PC4 concentrations showed a 10-fold increase to 0.7 – 0.8 amol / cell after 24 h of exposure to pPb9 and pPb8.3, decreasing to 0.3 amol / cell after 48 h (Table 2.2). PC5, which was not detected upon short-term exposure, was detected at concentrations up to 3.3 amol / cell at pPb9 and 2.7 amol / cell at pPb8.3.
after 24 h and decreased to 1.3 amol / cell at pPb9 and 1.4 amol / cell at pPb8.3 after 48 h (Table 2.2). After 24 h and 48 h exposure to pPb8.3 and pPb9, $\sum_{SH(PC_n)}$ was similar at both concentrations. Compared to control cultures $\sum_{SH(PC_n)}$ was approximately twofold under lead exposure (Table 2.2). Comparison of $\sum_{SH(PC_n)}$ of short- and long-term exposures shows a decrease by almost 50 % and a large difference in PC oligomer distribution (Fig. 2.2, Table 2.2).

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>Phytochelatins (amol / cell)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>PC&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>PC&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>PC&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$\sum_{SH(PC_n)}$</td>
<td>13.0 ± 2.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>pPb9</th>
<th>pPb8.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3.6 ± 1.3</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>PC&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.2 ± 0.07</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>PC&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.8 ± 0.03</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>PC&lt;sub&gt;5&lt;/sub&gt;</td>
<td>3.3 ± 0.15</td>
<td>2.7 ± 0.14</td>
</tr>
<tr>
<td>$\sum_{SH(PC_n)}$</td>
<td>27.5 ± 2.7</td>
<td>22.8 ± 0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>pPb9</th>
<th>pPb8.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6.4 ± 0.1</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>PC&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.6 ± 0.04</td>
<td>0.6 ± 0.10</td>
</tr>
<tr>
<td>PC&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.3 ± 0.03</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>PC&lt;sub&gt;5&lt;/sub&gt;</td>
<td>1.3 ± 0.06</td>
<td>1.4 ± 0.19</td>
</tr>
<tr>
<td>$\sum_{SH(PC_n)}$</td>
<td>22.3 ± 0.4</td>
<td>24.2 ± 1.2</td>
</tr>
</tbody>
</table>

Table 2.2: PC<sub>2</sub> - PC<sub>5</sub> and $\sum_{SH(PC_n)}$ ($\sum_{SH(PC_n)} = 2 \times PC_2 + 3 \times PC_3 + 4 \times PC_4 + 5 \times PC_5$) detected in control cultures and after 24h and 48h exposure to pPb9 and pPb8.3 in modified Talaquil.
2.5 Discussion

A more than 100-fold increase of [Pb(II)]_intra was observed between control algae and pPb9. However, only a slight increase of [Pb(II)]_intra from pPb9 to pPb7.1 was observed. This slight increase upon a 100-fold increase of [Pb^{2+}] in the exposure medium might indicate saturation of Pb accumulation or saturation of involved uptake transporters at these concentrations (Fig. 2.1). Furthermore, the use of a simplified medium for accumulation experiments, in order to reach a well defined Pb speciation, might lead to an overestimation of lead uptake compared to more natural water composition. The absence of certain essential nutrients in this medium could also cause deficiency influencing Pb uptake. Similar results for saturation of Pb accumulation at higher [Pb^{2+}] (> pPb6) were obtained with the freshwater alga *Chlorella kessleri* after 35 min exposure (Lamelas and Slaveykova, 2007). The obtained results suggest, that in addition to [Pb^{2+}] in the exposure medium, excretion and the subcellular distribution of Pb in *C. reinhardtii* might influence Pb uptake. The measured [Pb(II)]_intra were very high, reaching calculated cellular concentrations of 2.98 mM at pPb7.1. These high [Pb(II)]_intra may indicate precipitation of Pb solid phases inside the cell. Intracellular Pb precipitation might be involved in Pb detoxification (Mason and Jenkins, 1995) and was observed in the prawn *Penaeus monodon* (Vogt and Quinitio, 1994).

The detection of PC2 and the larger oligomers PC3 and PC4 in control algae indicate that PCs appear to be constitutive in *C. reinhardtii*. Similarly, PC_n have been found to occur constitutively in marine algae (Ahner et al., 1995a; Ahner et al., 2002; Morelli et al., 2009). Compared to *C. reinhardtii* (PC2 = 27 µM; PC3 = 2.7 µM; PC4 = ~ 0.5 µM), similar molar concentrations were detected in Thalassiosira Pseudonana (PC2 = 44 µM; PC3 = 5.3 µM; PC4 = 2.2 µM) (Ahner et al., 2002). For the other six algal species examined in this study lower concentrations were detected. The presence of constitutive PCs might indicate the role of PCs in metal homeostasis.

Under Pb exposure the induction of PC2 synthesis was very fast and detectable within 30 min of exposure to [Pb^{2+}] ≥ pPb8 and increased continuously during 6 h (Fig. 2.2a). Comparable kinetics of PC2 induction were reported in Stichococcus bacillaris (Pawlik-Skowronska, 2000) and Phaeodactylum tricornutum (Morelli and Scarano, 2001). The rate of PC2 synthesis increased with increasing [Pb^{2+}] in the exposure medium. From pPb8 to pPb7.1 a twofold increase of the PC2 synthesis rate was
observed. PC3 synthesis rates were lower than those of PC2 and similar at various [Pb²⁺]. These similar PC3 synthesis rates at various [Pb²⁺] may result from the limited availability of PC2 for PC3 synthesis (Vatamaniuk et al., 2000). The lowest [Pb²⁺] inducing PC synthesis in short-term experiments was pPb8. However, in long-term experiments induction of PC synthesis was also observed at pPb9. Induction of PC synthesis was observed at ≥ pPb8 in short-term experiments, even though [Pb(II)]_{intra} was similar at pPb9, suggesting that induction of PC synthesis is dependent on the subcellular distribution of Pb. Induction of PC synthesis at pPb9 upon long-term exposure might occur because of additional Pb uptake with increased exposure time or because of changes in the subcellular distribution of Pb. Similarly, induction of PC2 synthesis was observed in Scenedesmus vacuolatus at pPb10 after 72 h exposure (Le Faucheur et al., 2006).

While in short-term experiments PC2 and PC3 were detected, long-term exposure showed induction of PC oligomers up to PC5 at even lower [Pb²⁺]. Consequently, the degree of polymerization of PC oligomers increased with increasing [Pb²⁺] and time. Furthermore, synthesis of PC oligomers, with a higher degree of polymerization than PC2, are induced after a lag phase, which increases with the chain length (PC3 = 1 h; PC4 = 4 h; PC5 = > 6 h). This lag phase may appear because PC2 is the substrate for PC3 synthesis or, in more general terms, PCn is substrate for PC_{n+1} synthesis (Vatamaniuk et al., 2000). The synthesis of PC oligomers with increasing chain length is believed to occur because of the higher stability of metal complexes (Mehra et al., 1995).

For $\frac{\sum_{SH(\text{PC}_n)}}{[Pb(\text{II})]_{\text{intra}}}$ a continuous increase with increasing [Pb²⁺] was observed. This increase results from the enhanced induction of PC synthesis at higher [Pb²⁺], with only a slight increase in [Pb(II)]_{intra}. The ratio $\frac{\sum_{SH(\text{PC}_n)}}{[Pb(\text{II})]_{\text{intra}}}$ increased to a maximum of 0.11 at pPb7.1 indicating that PCs synthesized in C. reinhardtii were not present at a sufficiently high concentration to fully immobilize the intracellular accumulated Pb. The ratio $\frac{\sum_{SH_{\text{tot}}}}{[Pb(\text{II})]_{\text{intra}}}$ was approximately two and similar for all [Pb²⁺], because the GSH concentration was much higher than the PC concentration. Although it is known that GSH can form complexes with Pb, it remains unclear whether GSH is directly involved in Pb detoxification (Cruz et al., 2001).

Despite the high [Pb]_{intra} and the production of reactive oxygen species (ROS) upon Pb exposure (Szivak et al., 2009), no effects on photosynthesis were observed in
short-term experiments. However, upon long-term exposure to Pb, strong inhibition of the photosynthetic yield was observed. Furthermore, damaging effects on growth were detected in long-term experiments. The inhibition of photosynthetic yield and growth increased with exposure time. These time dependent effects may result as a consequence of the high affinity of Pb to sulfur and oxygen functional groups (Mason and Jenkins, 1995). In other words, Pb binds non-specifically to functional groups of proteins containing sulfur or oxygen, inducing various effects that may affect photosynthesis and growth upon long-term exposure. In other studies using the freshwater alga Chlorella vulgaris or Emiliania huxleyi, a marine alga, no significant inhibition of growth under short-term and long-term Pb exposure was detected (Rosko and Rachlin, 1977; Vasconcelos and Leal, 2001; Baumann et al., 2009). Debelius et al. (2009) reported inhibition of growth up to 100% in five marine algae after 72 h of exposure at nominal Pb concentrations between 1.5 – 30 µM. Fujiwara et al. (2008) reported growth inhibition of 50% for much higher [Pb^{2+}] of 146 µM in Chlorella kessleri. This variability indicates strong differences in sensitivity to Pb for different algal species. In summary, this study showed no damaging effects of Pb under short-term exposure, although [Pb(II)]_{intra} were much higher than cellular PC concentrations. This suggests a minor role of PC in Pb detoxification and that other mechanisms must be involved to protect cells from short-term damaging effects. However, these Pb detoxification mechanisms are not effective enough to avoid damaging effects upon long-term exposure.

At concentrations of pPb9, an environmentally relevant concentration, Pb is accumulated in algal cells, reaching a high concentration after short exposure times. Because of delayed toxic effects induced by Pb, which are not detected under short-term exposure, it is important to examine long-term exposures to assess the toxicity of Pb on organisms. Further research needs to be done to show whether PC synthesis is actually induced as a direct response to Pb exposure or because of indirect effects, such as metal redistribution. Furthermore, it is unclear whether Pb is actually bound by PCs or if other metals are bound due to metal redistribution and to which other functional groups Pb may be bound.
Chapter 2

2.6 References


Chapter 3

Characterization of lead induced metal-phytochelatin complexes in *Chlamydomonas reinhardtii*

This chapter will be submitted to *Environmental Toxicology and Chemistry*
3.1 Abstract

Phytochelatins (PCs) are metal-binding oligopeptides with the general structure \((\gamma\text{-Glu-Cys})_n\text{-Gly} \ (n = 2 – 11)\), which are synthesized enzymatically from glutathione upon exposure of algae to various metals. Accumulation of Pb as well as induction of phytochelatin synthesis was observed in *Chlamydomonas reinhardtii* upon Pb(II) exposure. The aim of this study was to examine whether Pb(II) is bound by PCs in *C. reinhardtii* and to examine formed complexes for their stoichiometry and composition. Metal-phytochelatin (Me-PC) complexes induced by Pb were isolated by size-exclusion chromatography in 13 collected fractions, which were analyzed for their PC and metal content by HPLC and ICP-MS. A recovery of over 90% of Pb from standard Pb-PC2 complexes within the total volume of the size-exclusion column indicated the adequacy of the method for Pb-PCn complex separation and characterization. PCs were mainly detected in a molecular weight range from 1000 – 5300 Da, indicating the formation of complexes with various stoichiometries. Approximately 72% of total PC2 eluted in the range 1000 – 1600 Da and 80% of total PC3 eluted in the molecular weight range 1600 – 2300 Da. The distribution of Cu, Zn and Pb showed that over 70% of these metals were associated with the high molecular weight fractions. Cu, Zn and Pb were also observed in PC containing fractions, suggesting the formation of various Me-PC complexes. Molecular weight considerations suggested that complexes with one or two metals bound by two PC2 or PC3 were formed. PCs present in higher molecular weight fractions are assumed to be involved in the formation of high molecular weight complexes. The results of this study indicate that the role of PCs in Pb detoxification is minor as only 13% of total Pb was associated with PCs. The observed formation of Me-PC complexes with Cu and Zn suggests a homeostatic role of PC.

3.2 Introduction

Phytochelatins (PCs) are metal-binding oligopeptides with the general structure \((\gamma\text{-Glu-Cys})_n\text{-Gly} \ (n = 2 – 11)\). They are produced enzymatically from glutathione by the enzyme phytochelatin synthase, which is activated by binding of metal ions and metal-glutathione complexes (Grill et al., 1985; Grill et al., 1987; Vatamaniuk et al., 2000). PC synthesis is induced in various plants (Rauser, 1995; Zenk, 1996) and algae (Gekeler et al., 1988; Ahner et al., 1995) as a response to metal exposure. Besides Cd,
which is the most potent inducer of phytochelatins, other metals and metalloids (e.g. Pb, Cu, Zn, Hg, Ag, As(III), As(V)) were found to induce PC synthesis in algae (Gekeler et al., 1988; Ahner and Morel, 1995; Le Faucheur et al., 2006). Pb was observed to be a strong inducer of phytochelatin synthesis in marine and freshwater algae (Ahner and Morel, 1995; Pawlik-Skowronska, 2000; Morelli and Scarano, 2001; Pawlik-Skowronska, 2002; Le Faucheur et al., 2006).

Because of their high cysteine content, PCs bind metals through thiolate coordination and are assumed to be involved in metal homeostasis and detoxification (Grill et al., 1985; Rauser, 1995; Zenk, 1996; Clemens, 2001). The first convincing evidence of PC involvement in metal detoxification was obtained using yeast mutants (Schizosaccharomyces pombe and Saccharomyces cerevisiae) and plants (Arabidopsis spp.) that are deficient in PC synthase genes (Clemens et al., 1999; Ha et al., 1999). These species were more sensitive to Cd, As(V) and Cu than the wild-type. Studies on Cd-PC complexes formed in plants (Kneer and Zenk, 1997; Yen et al., 1999), yeast (Reese and Winge, 1988; Mehra et al., 1994) and algae (Hu and Wu, 1998; Hu et al., 2001; Morelli et al., 2002; Scarano and Morelli, 2002) under Cd exposure revealed the presence of two types of complexes, defined as Low Molecular Weight (LMW) and High Molecular Weight (HMW) complexes. LMW complexes consist of metals bound to PCs, whereas HMW complexes are composed of Cd and acid labile sulfur (S-) forming a CdS core surrounded by PCs. The role of the PC coating is to stabilize the CdS core and to avoid its precipitation. Formation of HMW complexes increases metal tolerance in the yeast Candida glabrata, where a strain with enhanced CdS production revealed increased Cd resistance compared to the wildtype (Barbas et al., 1992; Mehra et al., 1994). Therefore, the role of PC in Cd detoxification is assumed to result from immobilization of metals, preventing non-specific binding of cadmium to important biomolecules, followed by the transport of Cd-PC complexes into the vacuole.

The role of PC in detoxification of metals, other than Cd, is unclear because, for most metals that were found to induce PC synthesis (e.g. Pb, Zn, Ag, Cu, As(V), As(III) and Hg), the formation of metal-phytochelatin complexes (Me-PC) was only examined in vitro but not in vivo (Rauser, 1999; Schmoger et al., 2000; Alberich et al., 2007; Chekmeneva et al., 2007). Studies on Me-PC complexes induced by Pb are very scarce (Scarano and Morelli, 2002; Andra et al., 2009). In the marine alga Phaeodactylum tricornutum formation of Pb-PC complexes including PC₃ – PC₆ but
not PC2 was suggested (Scarano and Morelli, 2002). *In vitro* formation of Pb-PC complexes and examination by differential pulse polarography indicated the formation of Pb1-PC2 or Pb2-PC2 complexes (Alberich et al., 2007), and an increased complex stability with increasing number of coordinating thiols was demonstrated.

In a previous study, PC synthesis was induced in short-term experiments at concentrations of pPb8 (pPb = -log[Pb2+]) and higher (Chapter 2). The aim of the present study is to examine whether Pb is bound by PCs in *C. reinhardtii* and to characterize putative complexes for their stoichiometry and composition. In order to examine Me-PC complexes, size-exclusion chromatography coupled off-line with reversed phase high pressure liquid chromatography (RP HPLC) and inductively coupled plasma mass spectrometry (ICP-MS) was used to separate native Me-PC complexes and to determine thiol and metal content.

### 3.3 Materials and methods

#### 3.3.1 Chemicals and materials

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 3-morpholinopropanesulfonic acid (MOPS), diethylenetriamine-pentaacetic acid (DTPA), standard peptides (aprotinin, gastrin, (Glycine)6, (Glycine)3) and metal salts used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Supelco (Bellefonte, PA, USA). Nitric acid suprapure (HNO3; 65 %) and protease inhibitor cocktail III (EDTA free), containing serine, aspartic and cysteine proteases and aminopeptidases, was ordered from Merck (Darmstadt, Germany). Phytochelatin standards (PC2, PC3 and PC4) were obtained from Invitrogen (San Diego, CA; USA). Bradford protein assay and Chelex was from BioRad laboratories (Muenchen, Germany). Glassware and polycarbonate containers were presoaked in 0.01 M HNO3 and rinsed with deionized water (18 MΩ-cm, Q-H2O grade, Barnstead Nanopure, Allschwil, Switzerland) before use. All solutions and media were prepared in deionized water in autoclaved containers.

#### 3.3.2 Test organism, culture and experimental conditions

The unicellular green alga *Chlamydomonas reinhardtii* (CC125) was obtained from the Chlamydomonas Genetics Center (Durham, NC, USA) and was grown in glass Erlenmeyers in a HT Infors shaker (Infors, Bottmingen, Switzerland) at 25 °C with
continuous illumination of 120 µEm⁻²s⁻¹, provided by cool white fluorescent lamps, and shaken at 90 rpm.

For experiment and exposure to Pb, algae were acclimatized to the growth medium by transferring an inoculum of exponentially growing algae to the growth medium in at least two successive batch cultures. *C. reinhardtii* were cultured in Talaquil, a complete growth medium containing major nutrients and trace metals buffered by EDTA (Knauer et al., 1997), in which pH is buffered using MOPS (3-morpholinopropanesulfonic acid) as a non-metal complexing buffer. The total concentrations in the growth medium are the following: 2 x 10⁻⁵ M Na₂EDTA; 5 x 10⁻⁴ M CaCl₂ · 2H₂O; 1.5 x 10⁻⁴ M MgSO₄ · 7H₂O; 1.2 x 10⁻³ M NaHCO₃; 5 x 10⁻⁵ M K₂HPO₄ · 3H₂O; 1 x 10⁻³ M NH₄Cl; 5 x 10⁻⁸ M CoCl₂ · 6H₂O; 5 x 10⁻⁵ M H₃BO₃; 8 x 10⁻⁸ M Na₂MoO₄ · 2H₂O; 1.63 x 10⁻⁷ M CuSO₄ · 5H₂O; 1.22 x 10⁻⁶ M MnCl₂ · 4H₂O; 1.58 x 10⁻⁷ M ZnSO₄ · 7H₂O and 9 x 10⁻⁷ M FeCl₃ · 6H₂O in 1 x 10⁻² M MOPS buffer (pH 7.5). All media were prepared at least 24 h before use to allow equilibration. Free ion concentrations of Cu²⁺, Mn²⁺, Zn²⁺ and Fe³⁺ in Talaquil, modeled using the speciation software VMINTEQ (Gustafsson, 2005), are 3 x 10⁻¹⁴, 1.7 x 10⁻⁸, 9 x 10⁻¹² and 1 x 10⁻²⁰ M.

As experimental medium, growth medium containing Pb buffered with 20 µM NTA to keep [Pb²⁺] constant was used. Other trace metals were omitted from the experimental medium in order to avoid metal competition and cumulative effects resulting from the presence of various metal ions. Exponentially growing algae were transferred to the experimental medium and exposed to pPb7.5 (pPb = -log [Pb²⁺]) for 14 h with an initial cell density of 8.4 x 10⁵ cells / ml. Free metal concentrations were computed using the speciation software VMINTEQ (Gustafsson, 2005).

### 3.3.3 Preparation of algal cell extracts

Algal cells were harvested at the end of exposure by centrifugation (5000 x g for 10 min at 25 °C) and resuspended in 1 ml of 50 mM MOPS (pH 7.5) containing 1 mM TCEP (antioxidant) and protease inhibitor cocktail III, all degassed with argon prior to the experiment.

The algal suspension was then homogenized for 4 x 30 s at 100 % sonication intensity on ice using an ultrahomogenizer (Labsonic® M, Sartorius Stedim Biotech, France) with titanium tip to obtain native Me-PCₙ. Between each sonication step the samples were cooled on ice for 3 min. The homogenate was centrifuged at 9900 x g for 10 min
(4 °C). After centrifugation, the supernatant was transferred to a clean Eppendorf vial, followed by a second centrifugation step under the same conditions. This procedure was repeated up to five times to obtain a clear cytosolic fraction. Before analysis of supernatant using size-exclusion chromatography (SEC), total protein concentration was measured photometrically at 595 nm using a Bradford protein assay as dye reagent after performing calibration curves with diluted BSA solutions. In addition, metal concentrations and total PCs in the algal supernatant homogenate were measured by inductively coupled plasma mass spectrometry (ICP-MS) and high pressure liquid chromatography (HPLC).

### 3.3.4 Size-exclusion chromatography

For SEC, a pre-packed Superdex peptide HR 10/30 column (Amersham), designed for separation of small peptides (100 – 10000 Da), was used. The SEC system and solvents were stored and operated at 4 °C. The flow rate of the eluent, 150 mM ammonium nitrate (NH₄NO₃) and 50 mM MOPS (pH = 7.5), was 0.15 ml / min. All eluents were filtered on 0.22 µm, degassed with nitrogen and cleaned from metal contamination by using a Chelex 100 Resin. The void volume (V₀) of SEC was determined using a solution of blue dextran and the total volume (Vₜ) using CuCl₂. The calibration curve for molecular weight determination were performed by measuring the elution volumes (Vₑ) of standard peptides (aprotinin (6.5 kDa), gastrin (2.1 kDa), PC₄ (1004 Da), PC₃ (772 Da), PC₂ (540 Da), (Glycine)₆ (360 Da), (Glycine)₃ (189 Da)), calculating their partition coefficient Kₐᵥ (Kₐᵥ = \frac{Vₑ - V₀}{Vₜ - V₀}) and plotting their Kₐᵥ values versus the logarithm of their molecular weight (log Mᵣ).

For separation of algal peptides and Me-PCₙ complexes 100 µl algal homogenate (protein concentration: 6.21 mg / ml) was applied to the SEC. The eluate was collected in fractions (Fraction 1 (F₁) = 3600 µl, F₂ = 1800 µl, F₃ = 900 µl, F₄ = 2700 µl, F₅ = 1800 µl, F₆ – F₁₁ = 900 µl, F₁₂ = 1800 µl and F₁₃ = 3600 µl) and absorbance at 254 nm was recorded. Between different samples, the column was cleaned with 0.1 % HCl and then flushed with 10 column volumes eluent containing 1 mM EDTA, to remove remaining metals adsorbed to the column. After the EDTA wash, the column was equilibrated with 10 column volumes eluent.
3.3.5 Metal recovery experiments

Elution and possible adsorption of Pb and Pb-PC complexes to the column Superdex peptide HR 10/30 column were tested in independent experiments. To investigate the recovery of inorganic Pb(II), 100 µl of 15 µM Pb(NO₃)₂ in the elution buffer (150 mM ammonium nitrate (NH₄NO₃) and 50 mM MOPS; pH = 7.5) was applied to the SEC column. During the elution of Vᵣ, fractions were taken and analyzed for their metal content by ICP-MS. After the elution of Vᵣ, 100 µl elution buffer containing 2 mM EDTA was injected to remobilize Pb adsorbed to the column. Fractions taken during the elution of EDTA were analyzed for their Pb content by ICP-MS.

To investigate the elution of Pb-PC₂ complexes in SEC, a solution of 30 µM PC₂ standard and 15 µM Pb(NO₃)₂ in elution buffer were mixed and incubated for 15 min at 20 °C to allow complex formation, followed by the injection of 100 µl to the SEC column. The Pb and PC₂ concentration were determined in each of the collected eluate fractions.

3.3.6 Metal and thiol analysis in SEC fractions by ICP-MS and HPLC

Metal and thiol concentrations of each fraction were determined off-line of SEC. After appropriate dilution of a 250 µl fraction aliquot, metal concentrations were analyzed by ICP-MS (Perkin-Elmer; Perkin Elmer Sciex, Ueberlingen, Germany) using rhodium as an internal standard. ICP-MS measurements were checked using SLRS-4 reference water (River Water Reference Material for Trace Metals; National Research Council Canada, Ottawa).

For thiol determination, the remaining fraction was lyophilized for 15 h using a Lyovac GT2 (Leybold, Switzerland). The lyophilized sample was resuspended in 50 µl HCl / DTPA (120 mM / 5 mM) before pre-column derivatization with monobromobimane according to the procedure explained elsewhere (Le Faucheur et al., 2005). Thiols were measured by reverse-phase high performance liquid chromatography (HPLC) equipped with a fluorescence detector (Agilent 1200 series, Agilent Technologies, Santa Clara CA, USA). The separation of the derivatized sample was carried out on a reversed phase octadecysilica column (Nucleosil C18, 5 µm, 250 x 4 mm) obtained from Macherey und Nagel (Düren, Germany) with a mobile phase flow rate of 1 ml / min. The mobile phase was composed of 0.1 % trifluoroacetic acid and a gradient of 10 – 36 % acetonitrile during 65 min. Between each measurement the column was cleaned with 100 % acetonitrile for 5 min,
followed by 10 min at 10 % acetonitrile for equilibration. A 100 µl sample injection loop was used, resulting in a detection limit of approximately 0.1 pmol for thiol groups. Thiols were detected by fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 470 nm. The retention times of the PC oligomers were verified using PC$_2$ – PC$_4$ standards. For quantification of PCs a calibration curve relating GSH concentrations of standard solutions to resulting peak areas was used.

3.4 Results

3.4.1 Calibration and recovery of size-exclusion chromatography

The calibration curve for the size-exclusion chromatography (SEC) column shows that PC$_n$ standards and other standard peptides elute according to their molecular weight (M$_r$) under the applied elution conditions (Fig. 3.1).

As Pb is known to be reactive and has a high affinity to many functional groups, the recovery of Pb in SEC was tested (Table 3.1). Pb applied to the SEC in inorganic form resulted in a recovery of 3 % within V$_t$ (total volume of the column). EDTA injection to remobilize adsorbed Pb resulted in a recovery of 30 % of total Pb. The
recovery of Pb injected as Pb-PC₂ complexes was > 90% within V₁ (Table 3.1). This recovery indicated that SEC is adequate for separation and characterization of Pb-PC complexes.

<table>
<thead>
<tr>
<th>Pb (µg)</th>
<th>Pb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied on SEC as Pb²⁺</td>
<td>0.29</td>
</tr>
<tr>
<td>Recovery in V₁</td>
<td>0.01</td>
</tr>
<tr>
<td>Recovery in V₁; EDTA wash</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Total Pb recovery</strong></td>
<td>0.10</td>
</tr>
</tbody>
</table>

| Pb applied as Pb-PC₂ | 0.29 | 100 |
| Recovery >10 kDa – 3 kDa | 0.0003 | 0.1 |
| Recovery 3 kDa – 700 Da | 0.26 | 89.8 |
| Pb recovery < 700 Da | 0.001 | 0.5 |
| **Total Pb recovery** | 0.26 | **90.4** |

Table 3.1: Recovery of Pb and standard Pb-PC complexes in SEC during the elution of V₁. The recovery of inorganic Pb during V₁ and EDTA wash was monitored after injection of 100µl of 15µM Pb(NO₃)₂. Recovery and stability of standard Pb-PC₂ complexes during SEC was tested by injection of 15 µM Pb and 30 µM PC₂ forming Pb-PC₂ complexes.

3.4.2 Peptide fractionation by size-exclusion chromatography and fraction analysis

The UV-absorbance at 254 nm was recorded on-line during peptide separation by size-exclusion chromatography (Fig. 3.2). The numbers in Figure 3.2 correspond to the collected eluate fractions (1 – 13) with the Mr range shown in Table 3.2. The absorption spectrum shows that macromolecules with Mr > 10 kDa elute at retention times from 45 – 65 min, which correspond to the void volume of the column (V₀) (Fig. 3.2). The absorption peaks at retention times of 120 – 140 min correspond to a molecular weight of 100 – 1000 Da. The last absorption peak (retention time = 150 min) corresponds to V₁ and molecular weights < 70 Da. The negative absorbance after
135 – 140 min is explained by the elution of MOPS, which was present in the algal sample.

![Graph showing UV-absorption as function of elution time.](image)

**Figure 3.2:** UV-absorption as function of elution time, recorded on-line at 254 nm during fractionation of *C. reinhardtii* homogenate grown in pH7.5 for 14 h. Numbers 1 – 13 indicate the collected fractions during peptide separation.

<table>
<thead>
<tr>
<th>SEC - fraction</th>
<th>M&lt;sub&gt;r&lt;/sub&gt; range (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>2</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>3</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>4</td>
<td>&gt;10000 - 5300</td>
</tr>
<tr>
<td>5</td>
<td>5300 - 2300</td>
</tr>
<tr>
<td>6</td>
<td>2300 - 1600</td>
</tr>
<tr>
<td>7</td>
<td>1600 - 1000</td>
</tr>
<tr>
<td>8</td>
<td>1000 - 700</td>
</tr>
<tr>
<td>9</td>
<td>700 - 450</td>
</tr>
<tr>
<td>10</td>
<td>450 - 300</td>
</tr>
<tr>
<td>11</td>
<td>300 - 200</td>
</tr>
<tr>
<td>12</td>
<td>200 - 100</td>
</tr>
<tr>
<td>13</td>
<td>&lt; 100</td>
</tr>
</tbody>
</table>

**Table 3.2:** Molecular mass range (M<sub>r</sub> range) of the eluate fractions collected during the separation of algal peptides
Upon analysis of the SEC fractions by HPLC, PC_2 and PC_3 were detected in various fractions. PC_2 was detected in the Mr range 450 – 5300 Da. The sum of PC_2 quantified in all fractions was 640 pmol, with 72 % of total PC_2 eluting in the Mr range 1000 – 1600 Da and 19 % in the Mr range 700 – 1000 Da. PC_2 detected in other fractions resulted in less than 10 % (Fig. 3.3a). PC_3 was detected in the Mr range between 1000 – 2300 Da. The sum of PC_3 quantified in all fractions was 27 pmol with 80 % of total PC_3 eluting in the Mr range 1600 – 2300 Da and 20 % in the range 1000 – 1600 Da (Fig. 3.3b). Similar distributions of PC_2 and PC_3 were detected in replicate experiments, but the absolute concentration varied (data not shown). In the Mr range 450 – 700 Da an unidentified thiol was detected during HPLC analysis. This unidentified peptide was only observed in this specific fraction and was present in a higher concentration than PC (data not shown).

Examination of the collected fractions for their metal content showed elution of Pb and Cu in every fraction and Zn in most fractions (Fig. 3.4). The amount of Cu, Zn and Pb detected in Mr > 10 kDa corresponded to 68 % Cu, 90 % Zn and 71 % Pb of the total amount eluted in SEC. In all other fractions the measured Cu amount corresponded to 2 – 5 % of total Cu (Fig. 3.4a). Pb quantified in other fractions was 0.5 – 3 % of total Pb, except in the Mr range 400 – 750, where 10 % of the total eluted Pb was recovered (Fig. 3.4c). Approximately 3 % Zn was present in Mr range 700 – 1000 Da and 5.5 % in Mr < 300 Da (Fig. 3.4b). Figure 3.3 and Figure 3.4 show the
concentrations of PCs and metals in µmol / l fraction. The molar ratio ($\sum$SH : $\sum$metals) calculated from the sum of phytochelatin thiols ($\sum$SH (PCₙ)) and the sum of metals ($\sum$Cu, Zn, Pb) ranged from 0.17 to 18 (in fraction 7) and was higher than 1 in fractions 6, 7 and 8 (Figure 3.5).

Figure 3.4: Concentrations of a) Cu, b) Zn and c) Pb in the SEC eluate fractions (µmol / l fraction) quantified by ICP-MS
The recovery in SEC was higher than 80 % for metals and PCs except Pb. In the case of Pb, 30 % of the amount measured in the homogenate was recovered in the SEC fractions (Table 3.2).

<table>
<thead>
<tr>
<th></th>
<th>Total metals or PC\textsubscript{n} in homogenate (µg)</th>
<th>( \sum \text{metals or PC}_n ) in eluate fractions (µg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>741</td>
<td>1047</td>
<td>141.2</td>
</tr>
<tr>
<td>Zn</td>
<td>2108</td>
<td>1780</td>
<td>84.4</td>
</tr>
<tr>
<td>Pb</td>
<td>3125</td>
<td>1066</td>
<td>34.1</td>
</tr>
<tr>
<td>PC\textsubscript{2}</td>
<td>0.38</td>
<td>0.35</td>
<td>90.5</td>
</tr>
<tr>
<td>PC\textsubscript{3}</td>
<td>0.025</td>
<td>0.021</td>
<td>81.3</td>
</tr>
</tbody>
</table>

Table 3.3: Calculated metal and PC recovery between homogenate applied to the SEC column and the total quantified in SEC fractions.
3.5 Discussion

In the present study Pb induced PCs in *C. reinhardtii* were examined with respect to the formation of Pb-PCs complexes, their molecular weight and their possible stoichiometries.

The detection of PCs standards according to their molecular weight in the calibration of SEC column confirmed that the ionic strength in the eluent was high enough to avoid non-specific interactions between peptides and the Superdex matrix (Irvine, 1997). Furthermore, the resolution of the SEC method was sufficient to separate PC₂ – PC₄ standards in single peaks (data not shown). Recovery experiments on the elution of inorganic Pb from the SEC column resulted in a low recovery, indicating adsorption of Pb to the column matrix. Strong interaction and adsorption of inorganic Pb to the column was expected due to the high reactivity of Pb. Control experiments carried out with standard Pb-PC₂ complexes, however, resulted in over 90 % Pb recovery. This indicates the elution of intact Pb-PC₂ complexes without complex dissociation under the applied SEC conditions.

Analysis of non-protein thiols from algal extracts in the eluate fractions showed PC₂ mainly in Mᵣ > 700 Da and PC₃ in Mᵣ >1000 Da, indicating complex formation. PC₄ and larger PC oligomers were not detected, even though their induction was previously observed at exposure times > 6 h and under similar exposure conditions (Chapter 2). We assume that the concentration of these oligomers was below the detection limit. Detection of the unidentified thiol peptide, present in higher concentrations than PC in the Mᵣ range 450 – 700 Da (fraction 9; data not shown), suggests complex formation of metals with other thiol peptides than PCs. From the retention time in HPLC analysis, a possible candidate is γ-Glu-Cys or another small thiol peptide. The presence of γ-Glu-Cys as a precursor of GSH and PCs would indicate induction of PC synthesis. In *Phaeodactylum tricornutum*, a marine alga, co-elution of Pb with unidentified low molecular weight compounds was reported, but no specific compound was suggested (Scarano and Morelli, 2002).

Quantification of Cu, Zn and Pb showed that the highest concentrations are present in fractions with Mᵣ > 10000 Da. Around 70 % of total Cu and Pb and 90 % of total Zn was detected in these fractions. For Cu and Zn this was expected due to their role in metabolic processes and as cofactors of enzymes (Waldron et al., 2009). The elution of 70 % Pb in fractions with Mᵣ > 10000 Da also indicates that a major amount of
cellular Pb is bound to macromolecules, most probably due to the high affinity of Pb to oxygen, nitrogen and sulfur containing functional groups. The recovery of Zn and Cu in the SEC was > 80 %, whereas only 30 % Pb was recovered. Considering the Pb recovery of 3 % for inorganic Pb and > 90 % for standard Pb-PC2 complexes, dissociation of non-specifically bound Pb from high M_r complexes and Pb adsorption to the column matrix could have occurred, whereas the Pb-PC2 complexes were probably recovered. Cu, Zn and Pb were also observed in PC containing fractions, suggesting the formation of Me-PC complexes. Cu and Zn are known as inducers of PC synthesis at elevated concentrations, and complex formation was demonstrated in vitro (Rauser, 1995; Maitani et al., 1996; Chekmeneva et al., 2007). In the fraction with the highest PC concentration, 1000 – 1600 Da, Cu and Zn concentrations were 10-fold and 3-fold higher compared to Pb (Fig. 3.4). The high relative concentrations of Cu and Zn in this fraction and the induction of PC synthesis observed under Pb exposure suggests various possible functions of PCs. Firstly, PCs might be involved in Cu and Zn homeostasis, which is supported by the results obtained in a previous study, showing that PCs are also present in C. reinhardtii grown under optimal growth conditions (Chapter 2). Secondly, induction of PC synthesis by Pb might cause a cellular redistribution of these metals, leading to the formation of various Me-PC complexes, or thirdly, Pb uptake leads to the displacement of Cu and Zn, which also induce PC synthesis. Further research is needed to investigate the function of PCs concerning Cu and Zn. To our knowledge, this is the first study that reports complex formation of PC with other than the inducing metal. Considering the elution of PC2 and PC3 in a wide molecular weight range, Me-PC complexes of various stoichiometries may be assumed. In the M_r range 1000 – 1600 Da, molecular weight considerations indicate complexes with a general stoichiometry of one or two metals forming a complex with two PC2 (Me1−2(PC2)2). Depending on the metal bound, the molecular weight of these complexes is 1287 Da or 1494 Da for Pb1−2(PC2)2, 1143 Da or 1206 Da for Cu1−2(PC2)2 and 1145 Da or 1210 Da for Zn1−2(PC2)2. Similar assumptions for PC3 suggest complex stoichiometries of Me1−3(PC3)2 in the M_r range 1600 – 2300 Da. Possible PC3 complexes detected in the M_r range 1000 – 1600 Da are Pb2−3-PC3 complexes and the formation of complexes involving PC2 and PC3 (e.g. Me1−3-PC2-PC3). In contrast to other studies (Scarano and Morelli, 2002; Andra et al., 2009), PC2 was detected in molecular weights > 2000
Da. In this $M_r$ range formation of complexes involving PCs and cysteine (Andra et al., 2009) or incorporation of sulfide, as observed in plants and yeast exposed to Cd (Barbas et al., 1992; Mehra et al., 1994), is possible. The prevalent formation of Me-(PC$_2$)$_2$ or Me-PC$_2$-PC$_3$ complexes is suggested by the high $\text{SH(PCI)}_n / \sum\text{Me}$ ratios in some of the fractions and by considering that complex stability increases with increasing number of coordinating thiols (Alberich et al., 2007). Considering formation of Me-(PC$_2$)$_2$ or Me-PC$_2$-PC$_3$ complexes involving Cu, Zn and Pb, PC-SH is still in excess, which might indicate oxidation of PC-SH or metal dissociation from PCs. The proposed stoichiometries of the Me-PC complexes are tentative, as unambiguous identification of composition and stoichiometry of Me-PC complexes requires higher mass resolution, such as mass spectrometry (Yen et al., 1999; Andra et al., 2009).

This study indicates several roles of PCs in Pb exposed *C. reinhardtii*. Considering that 13% of total recovered Pb eluted in PC containing fractions, including also the fraction containing the unidentified thiol and 10% of total Pb, it seems that PCs have a minor role in Pb detoxification. In a previous study, no toxic effects of Pb were observed in short-term experiments but toxicity on photosynthesis and growth was observed upon exposure for several days (Chapter 2). Considering that upon short-term exposure no toxic effects were observed, although ROS formation in *C. reinhardtii* upon Pb exposure was reported (Szivak et al., 2009), suggests that toxicity may be prevented by antioxidant mechanisms. Toxicity observed upon long-term exposure indicates that neither PCs nor other protective mechanisms are able to prevent Pb toxicity. Additional research is needed to further examine Pb toxicity and to identify the protective mechanisms involved in Pb detoxification in *C. reinhardtii*. 
3.6 References


Chapter 3


Chapter 4

Characterization of standard lead-phytochelatin complexes by nano-electrospray ionization mass spectrometry
4.1 Abstract
The role of phytochelatins (PCs) in metal detoxification is assumed to result from immobilization of metals, preventing interaction with important biomolecules, followed by the transport of the complexes into the vacuole of the algal cell. Therefore the characterization of Me-PC complexes is important to predict the role of PCs in the detoxification of a specific metal. Several analytical methods were used to examine metal-phytochelatin complexes. Most of these methods do not provide exact molecular weights, stoichiometries and compositions of the Me-PC complexes. The aim of this study was to develop a nano-electrospray ionization mass spectrometry method which allows unambiguous identification of Me-PC complexes. Standard Pb-PC$_n$ complexes ($n = 2 – 4$) were used to develop a nano-ESI-MS method which allows their identification and characterization. Pb-PC$_n$ mass spectra indicated the presence of the [M+H]$^+$ peak of PC$_n$ and complexes with various stoichiometries. A peak at [M+H–2]$^+$ in the zoom spectra of PC$_n$ indicated the formation of intramolecular disulfide bonds. Standard Pb-PC$_2$ analysis allowed the identification of four different complexes observed at m/z 746.10, 952.06, 1285.24 and 1491.20, corresponding to the singly charged Pb-PC$_2$, Pb$_2$-PC$_2$, Pb-(PC$_2$)$_2$ and Pb$_2$-(PC$_2$)$_2$ complexes. Their m/z indicated coordination of Pb by PC$_2$ through the thiol groups of PC cysteine and the carboxyl groups of PC glutamic acid. For standard PC$_3$ and PC$_4$ two different complexes were observed corresponding to Pb-PC$_3$, Pb$_2$-PC$_3$, Pb-PC$_4$ and Pb$_2$-PC$_4$. The isotopic patterns for all complexes were identical to the theoretical isotopic patterns. Addition of Zn to previously formed Pb-PC$_2$ complexes showed the appearance of the singly charged Zn-PC$_2$ complex at m/z 602.06 and the decrease of the Pb-PC$_2$ peak. A general signal loss with increasing Zn concentration, most probably due to the increase of oxidized PC$_2$ was observed.

4.2 Introduction
Phytochelatins (PCs) are metal-binding oligopeptides with the general structure (γ-Glu-Cys)$_n$-Gly ($n = 2 – 11$) which are enzymatically produced from glutathione by the enzyme phytochelatin synthase. PC synthesis is induced as a response to various metals in plants (Rauser, 1995; Zenk, 1996) and algae (Gekeler et al., 1988; Ahner et al., 1995). Because of their high cysteine content, PCs are assumed to bind metals through thiolate coordination and might be involved in metal homeostasis and
detoxification (Grill et al., 1985; Rauser, 1995; Zenk, 1996; Clemens, 2001). The role of PCs in metal detoxification is assumed to result from immobilization of metals, preventing non-specific binding to important biomolecules, followed by the transport of the Me-PC complexes into the vacuoles. Therefore, Me-PC complexes need to be analyzed to investigate whether PCs form complexes with a specific metal and might be involved in its detoxification.

Several analytical methods such as chromatographic separation (gel filtration or HPLC) coupled with UV detection, flame atomic absorption spectrometry (AAS), radioactive labeling, differential pulse polarography and inductively coupled plasma mass spectrometry (ICP-MS) have been used to analyze PCs and metal-phytochelatin complexes (Me-PC) (Grill et al., 1985; Maitani et al., 1996; Leopold and Günther, 1997; Leopold et al., 1999; Vacchina et al., 1999; Schmoger et al., 2000; Vacchina et al., 2000; Scarano and Morelli, 2002; Cruz et al., 2005; Kobayashi and Yoshimura, 2006). These methods, however, do not provide exact molecular weights, stoichiometries or compositions of Me-PC\textsubscript{n} complexes. In most \textit{in vivo} studies focusing on Me-PC complex characterization, Me-PC complexes are isolated from the cell homogenate by gel filtration and the collected eluate fractions are further analyzed for PC and metal content. PC detection often involves acidification and derivatization, which leads to dissociation of the Me-PC complexes, followed by HPLC analysis. Based on the detected molecular weight range obtained from gel filtration and the PC oligomers detected by HPLC, assumptions on stoichiometry and composition of the Me-PC complexes can be made. However, unambiguous characterization of Me-PC complexes regarding stoichiometry and composition is not possible with these methods.

Considering that complex formation is assumed to be a key factor in metal tolerance and detoxification, a technique to precisely detect and characterize these complexes is required. Several studies reported identification of Cd-PC and As(III)-PC complexes using electrospray ionization mass spectrometry (ESI-MS) (Yen et al., 1999; Raab et al., 2005; Navaza et al., 2006; Chen et al., 2007; Bluemlein et al., 2008; Bluemlein et al., 2009), but analysis of Me-PC complexes formed with other metals are not reported.

In a previous study, induction of PC\textsubscript{2} – PC\textsubscript{4} synthesis by Pb was observed in the green alga \textit{C. reinhardtii}. Furthermore, PC\textsubscript{n} were shown to form complexes with Cu, Zn and Pb upon Pb exposure. Therefore, the aim of this study was to develop an ESI-MS
method to identify and characterize Me-PC complexes formed with Pb and to assess the competition with Cu and Zn. In a first step, sample composition and ESI-MS conditions to analyze standard Pb-PC\textsubscript{n} (n = 2 – 4) complexes were optimized. In a second part of the study, the impact of Zn and Cu addition in various concentrations to previously formed Pb-PC complexes was examined.

4.3 Material and Methods

4.3.1 Chemicals

Pb(NO\textsubscript{3})\textsubscript{2}, CuSO\textsubscript{4} and ZnSO\textsubscript{4} salts, ammonium acetate (NH\textsubscript{4}CH\textsubscript{3}COO), ammonium carbonate ((NH\textsubscript{4})\textsubscript{2}CO\textsubscript{3}; pH 7), polylysine, and 3-morpholinopropanesulfonic acid (MOPS) used in this study were analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). Phytochelatin standards (PC\textsubscript{2}, PC\textsubscript{3} and PC\textsubscript{4}) were obtained from Invitrogen (San Diego, CA, USA). Formic acid was a suprapure chemical obtained from Merck (Darmstadt, Germany). Ultrafree-MC centrifugal filters (0.45 µm cut-off) were ordered from Millipore AG (Zug, Switzerland).

4.3.2 Sample preparation

In preliminary experiments, the solvent mixture for the analysis of Pb-PC complexes was optimized. The following sample composition resulted in the highest signal intensities in nano-electrospray ionization mass spectrometry (nano-ESI-MS) analysis. PC\textsubscript{n} and Pb were mixed, resulting in final concentrations of 0.5 mM PC\textsubscript{n} and 0.25 mM Pb(NO\textsubscript{3})\textsubscript{2} in 100 mM NH\textsubscript{4}CH\textsubscript{3}COO and 50 mM (NH\textsubscript{4})\textsubscript{2}CO\textsubscript{3}. Complex formation was allowed for 15 min at room temperature. Prior to sample analysis formic acid was added to a final concentration of 0.1 % (pH 6), followed by sample filtration using an Ultrafree-MC centrifugal filter device with a 0.45 µm cut-off.

For competition experiments between Cu or Zn and Pb, standard Pb-PC\textsubscript{2} complexes were prepared as described and complex formation was allowed, followed by the addition of the Cu or Zn solution. Final concentrations of Cu and Zn were 0.125 mM, 0.25 mM, 0.5 mM and 5 mM. Acidification was done by addition formic acid to a final concentration of 0.1 %.
4.3.3 Nano-electrospray ionization mass spectrometry (nano-ESI-MS) analysis of metal-phytochelatin complexes

Solvent mixture optimization for nano-ESI-MS analysis of Pb-PCₙ (n = 2 – 4) was carried out on a API4000 triple quadrupole mass spectrometer (Applied Biosystems, Rotkreuz, Switzerland) with attached nanospray source (Sciex, NanoSpray® III Source, Zug, Switzerland).

Final analysis of Pb-PCₙ complexes was carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The nanospray capillary, a coated fused silica tubing (TSP-FS; o.d. 375 μm; i.d. 100 μm; BGB, USA), was pulled with a needle puller (Model P-2000, Sutter Instruments Co., Novato, CA, USA). The resulting capillary was used for nanospray injection and was connected to a 250 μl Hamilton gas tight syringe with a stainless steel needle (Hamilton Bonaduz AG, Bonaduz, Switzerland). The sample was loaded into the syringe and injected into the mass spectrometer by direct infusion. Between each measurement, the syringe was cleaned with MeOH and H₂O.

Prior to analysis of Pb-PCₙ complexes, the instrument performance and calibration was checked with polylysine. The flow rate for standard Pb-PCₙ complex analysis was 1 – 3 μl / min. The optimal settings of the mass spectrometer operated in positive electrospray ionization mode were: ion spray voltage, 1.5 kV; capillary temperature, 200 °C; capillary voltage, 11 V; tube lens, 130 V; resolution, 30’000; max. injection time, 100’000 ms; auto gain control FT, 1 x 10⁶. The spectra were acquired from m/z 300 – 1600 for Pb-PC₂ and from m/z 300 – 2000 for Pb-PC₃ and Pb-PC₄. Mass accuracy of the measurement was < 2 ppm for lower mass ions (< 1’000 Da).

Mass spectra were acquired using LTQ Tune Plus V.2.5.5 (Thermo Fisher Scientific, San Jose, CA, USA) and analyzed using the software Xcalibur V2.0.7 (Thermo Fisher Scientific, San Jose, CA, USA). The isotopic distributions for the positively charged molecular ions of Me-PC complexes were generated with the spectrum simulation software integrated in Xcalibur.
4.4 Results

4.4.1 Analysis of standard Pb-PC\textsubscript{n} complexes

The full-scan mass spectrum of the standard Pb-PC\textsubscript{2} complexes (Fig. 4.1) was dominated by the singly charged $[\text{M+H}]^+$ ion of PC\textsubscript{2} at m/z 540.1422, which matches the elemental composition of protonated PC\textsubscript{2} ($\text{C}_{18}\text{H}_{30}\text{N}_{5}\text{O}_{10}\text{S}_{2}$) of 540.1429 with an error < 1.5 ppm (Fig. 4.1).

![Figure 4.1: Nano-ESI-MS full-scan spectrum (m/z 300 – 1500) of 0.5 mM PC\textsubscript{2} ($\text{C}_{18}\text{H}_{30}\text{N}_{5}\text{O}_{10}\text{S}_{2}$) and 0.25 mM Pb(NO\textsubscript{3})\textsubscript{2} in 100 mM NH\textsubscript{4}CH\textsubscript{3}COO, 50 mM (NH\textsubscript{4})\textsubscript{2}CO\textsubscript{3} and 0.1 % HCOOH (pH 6).](image)

Beside the protonated molecular ion, the zoom spectrum showed the expected isotopic distribution (Fig. 4.2a). It should be noted that the zoomed spectrum contained peaks at m/z 536.3 and 538.4 (Fig. 4.2a) that are not present in the theoretical spectrum (Fig. 4.2b).
The four peaks observed at m/z 746.1036, 952.0641, 1285.2378 and 1491.1962 correspond to the molecular weight of singly charged Pb-PC2, Pb2-PC2, Pb-(PC2)2 and Pb2-(PC2)2 complexes (Fig. 4.1). Pb-PC2 and Pb2-PC2 were detected in sufficient intensity to detect the Pb-specific isotopic pattern of both complexes. The measured isotopic pattern of Pb-PC2 is in the m/z range 740 – 752, as shown in Figure 4.3a. The relative intensity of the peaks at m/z 744.10, 745.10 and 746.10 were observed in a ratio that reflects the distribution of Pb isotopes ($^{204}$Pb 1.5 %; $^{206}$Pb 23.6 %; $^{207}$Pb 22.6 %; $^{208}$Pb 52.3 %). The measured isotopic pattern perfectly matched the theoretical pattern (Fig. 4.3), with a mass error of 0.3 ppm. Similarly, the isotopic pattern observed for Pb2-PC2 at m/z 944 – 958, including the isotopic pattern of two Pb ions, matched the calculated spectra (Fig. 4.4), with a mass error of 0.7 ppm. The signal intensities of the complexes involving one or two Pb ions and two PC2 molecules (Pb$_1$ – 2-(PC$_2$)$_2$) were too low to detect their isotopic pattern.
Chapter 4

Analysis of Pb-PC$_3$ samples showed that the [M+H]$^+$ peak for PC$_3$ at m/z 772.1946, which matches the compositions of protonated PC$_3$ (C$_{26}$H$_{42}$N$_7$O$_{12}$S$_3$) of 771.1946 (Fig. 4.5, mass error 1.0 ppm). Similar to PC$_2$, a peak at [M+H–2]$^+$ (m/z 770.1790), not present in the theoretical spectra, was present at a high signal intensity (Fig. 4.6a).
Figure 4.5: Nano-ESI-MS full-scan spectrum (m/z 300 – 2000) of 0.5 mM PC₃ (C₂₆H₄₁N₇O₁₄S₃) and 0.25 mM Pb(NO₃)₂ in 100 mM NH₄CH₃COO, 50 mM (NH₄)₂CO₃ and 0.1 % HCOOH (pH 6).

Figure 4.6: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of singly protonated PC₃ (C₂₆H₄₂N₇O₁₄S₃) at m/z 760 – 785.

Two peaks at m/z 978.1560 and 1184.1161 were detected, corresponding to the molecular weight of singly charged Pb-PC₃ (mass error 0.4 ppm) and Pb₂-PC₃ (mass error 0.4 ppm). The measured isotopic pattern and the theoretical spectra were almost identical for both detected complexes (Fig. 4.7 and Fig. 4.8).
Figure 4.7: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of the singly charged Pb-PC₃ complex m/z 970 – 990. Isotopic distribution of Pb (²⁰⁶Pb 1.5 %; ²⁰⁸Pb 23.6 %; ²⁰⁷Pb 22.6 %; ²⁰⁹Pb 52.3 %) is identified in the peak distribution at m/z 976.15, 977.15 and 978.16.

Figure 4.8: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of the singly charged Pb₂-PC₃ complex m/z 1175 – 1190. Isotopic distribution of 2 Pb (²⁰⁶Pb 1.5 %; ²⁰⁸Pb 23.6 %; ²⁰⁷Pb 22.6 %; ²⁰⁹Pb 52.3 %) is identified in the peak distribution of the m/z 1180.11 – 1184.12.

Analysis of the Pb-PC₄ spectra showed the [M+H]⁺ peak for PC₄ at m/z 1004.2406, corresponding to the compositions of protonated PC₄ (C₃₄H₅₄N₉O₁₈S₄) of 1004.2464 (Fig. 4.9, mass error 5.7 ppm). Comparison of the [M+H]⁺ peak at 1004.2406 for PC₄ to the theoretical spectra shows an excellent match of the isotopic patterns (Fig. 4.10),
except the \([\text{M+H–2}]^+\) peaks (m/z 1002.2238), as observed for PC₂ and PC₃, and the \([\text{M+H–4}]^+\) peak (m/z 1000.2084).

Figure 4.9: Nano-ESI-MS full-scan spectrum (m/z 300 – 2000) of 0.5 mM PC₄ (C₃₄H₅₃N₉O₁₈S₄) and 0.25 mM Pb(NO₃)₂ in 100 mM NH₄CH₃COO, 50 mM (NH₄)₂CO₃ and 0.01 % HCOOH (pH 6).

Figure 4.10: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of singly protonated PC₄ (C₃₄H₅₄N₉O₁₈S₄) at m/z 990 – 1020.
Two peaks corresponding to the molecular weight of the singly charged PC₄ complexes, Pb-PC₄ and Pb₂-PC₄, were detected at m/z 1210.1986 (mass error 7.3 ppm) and 1416.1564 (mass error 8.5 ppm). The measured and theoretical isotopic patterns are shown in Figure 4.11 and Figure 4.12. In addition, a peak at m/z 605.6035 was observed, matching perfectly with the isotopic pattern of [Pb-PC₄]²⁺ (Fig. 4.13).

Figure 4.11: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of the singly charged Pb-PC₄ complex m/z 1200 – 1220. Isotopic distribution of Pb (²⁰⁴Pb 1.5 %; ²⁰⁶Pb 23.6 %; ²⁰⁷Pb 22.6 %; ²⁰⁸Pb 52.3 %) is identified in the peak distribution at m/z 1208.20, 1209.20 and 1210.20.

Figure 4.12: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of the singly charged Pb₂-PC₄ complex m/z 1405 – 1425. Isotopic distribution of 2 Pb (²⁰⁴Pb 1.5 %; ²⁰⁶Pb 23.6 %; ²⁰⁷Pb 22.6 %; ²⁰⁸Pb 52.3 %) is identified in the peak distribution of the m/z 1412.15 – 1416.16.
Figure 4.13: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of the twicly charged Pb-PC₄ complex m/z 480 – 500. Isotopic distribution of Pb (²⁰⁴Pb 1.5 %, ²⁰⁶Pb 23.6 %, ²⁰⁷Pb 22.6 %, ²⁰⁸Pb 52.3 %) is identified in the peak distribution of the m/z 604.60 – 605.60.

4.4.2 Competition between Cu or Zn and Pb for PC₂ binding

Addition of Zn to Pb-PC₂ complexes resulted in the appearance of the singly charged Zn-PC₂ peak at m/z 602.0547, already at the lowest Zn concentration (Fig. 4.14a). In addition, the Pb-PC₂ peak was observed to decrease with increasing Zn concentration (Fig. 4.14).

Figure 4.14: Nano-ESI-MS full scan spectrum of competition experiments between Pb and Zn with increasing Zn concentrations from a) – c). The concentrations are a) 0.125 mM Zn, b) 0.25 mM Zn and c) 0.5 mM Zn.
Increasing metal concentration led to a decrease of all PC signals and to an increase of the ratio between the [M+H]⁺ peak for PC₂ at m/z 540.14 and the [M+H–2]⁺ peak at m/z 538.14. The isotopic pattern of singly charged Zn-PC₂ matched with the theoretical distribution of Zn (⁶⁴Zn 50 %; ⁶⁶Zn 27 %; ⁶⁷Zn 4 %; ⁶⁸Zn 20 %; ⁷⁰Zn 0.6 %) (Fig. 4.15). At the highest Zn concentration and for all Cu concentrations, PC containing signals were not detected (data not shown).

Figure 4.15: a) Zoomed spectrum and b) theoretical spectrum of the isotopic pattern of the singly charged Zn-PC₂ complex at m/z 595 – 615. Isotopic pattern of Zn (⁶⁴Zn 50 %; ⁶⁶Zn 27 %; ⁶⁷Zn 4 %; ⁶⁸Zn 20 %; ⁷⁰Zn 0.6 %) and PC₂ is shown at m/z 602.05 – 609.05

4.5 Discussion

To test the applicability of nano-ESI-MS for the analysis of Me-PC complexes, standard Pb-PCₙ (n = 2 – 4) complexes were analyzed. A method for nano-ESI-MS was developed to characterize in vitro formed Me-PC complexes, which might also be used for characterization of in vivo Me-PCₙ complexes.

The mass spectra of standard Pb-PC were dominated by the [M+H]⁺ peak of the corresponding PCₙ, indicating that either not all PC was involved in complex formation, dissociation of Pb-PC complexes occurs during sample analysis, or that the complexes formed were neutral and, therefore, not visible in the nano-ESI-MS spectra.

PCₙ seems to occur mainly in charge state 1⁺ under the conditions used, as no signal was detected that corresponds to charge state 2⁺. The isotopic pattern of analyzed PCₙ
was completely resolved. For all PC\textsubscript{n}, the isotopic distribution matched the theoretical spectra of the corresponding elemental composition (Fig. 4.2 / 4.6 / 4.10) and mass accuracy was high (< 2 ppm for ions < 1'000 Da). The \([\text{M+H–2}]^+\) peaks observed for PC\textsubscript{2} – PC\textsubscript{4}, as well as the \([\text{M+H–4}]^+\) peak observed for PC\textsubscript{4}, indicate the formation of one or two intramolecular disulfide bonds between cysteine thiol groups within PC. The formation of a disulfide bond results in a loss of 2H\textsuperscript{+} and, therefore, a shift from \([\text{M+H}]^+\) to \([\text{M+H–2}]^+\), which was also observed in other studies (Yen et al., 1999; Navaza et al., 2006).

Analysis of Pb-PC complexes revealed the formation of Pb-PC\textsubscript{n} complexes with various stoichiometries and compositions. The m/z detected for the Pb-PC\textsubscript{2} complex allows two complexes, assuming Pb coordination through thiol groups of PC cysteine. Either Pb is coordinated by one thiol group whereas the other is present as reduced thiol group, or the Pb ion is coordinated by both thiol groups present in PC\textsubscript{2}. In the second case, additional protonation of the complex must occur to result in a singly charged complex detectable by nano-ESI-MS. For the Pb\textsubscript{2}-PC\textsubscript{2} complex, the detected m/z corresponds to \([\text{PC}_2+\text{Pb–3}]^+\), indicating a loss of two H\textsuperscript{+}. This observation suggests that besides the thiols from cysteines, the carboxylic groups of the glutamic acid must be involved in complex formation to obtain a singly charged Pb\textsubscript{2}-PC\textsubscript{2} complex. Coordination of Pb by thiol and carboxyl functional groups is assumed to result in increased complex stability. Further studies would be required to examine whether the complex formation between one Pb and PC\textsubscript{2} involves only thiol groups or if Pb is coordinated by one thiol and one carboxylic group. Similarly, another study observed the loss of 2H\textsuperscript{+} and 4H\textsuperscript{+} for the binding of two Cd ions to standard PC\textsubscript{5}. Binding of a third Cd ion to PC\textsubscript{5} was not accompanied with the loss of H\textsuperscript{+}. The authors suggested the formation of complexes that involve two thiol coordinated Cd and a Cd that is bound electrostatically to the Cd\textsubscript{2}-PC\textsubscript{5} complex (Yen et al., 1999). To investigate whether the coordination of metals by PC is dependent on the metal and / or the chain length of PC, more work needs to be conducted.

From m/z considerations, four different Pb-PC\textsubscript{2}, two Pb-PC\textsubscript{3} and two Pb-PC\textsubscript{4} complexes were identified. To prove that both, Pb and PC\textsubscript{n}, are present in detected signals, the measured isotopic pattern was compared to the theoretical isotopic pattern. The isotopic patterns of the Pb-PC complexes are complicated because of the isotopic pattern of PC coupled to that of one or more Pb ions (\(^{204}\text{Pb} \ 1.5 \%\); \(^{206}\text{Pb} \ 23.6 \%; \(^{207}\text{Pb} \ 22.6 \%, \(^{208}\text{Pb} \ 52.3 \%\)). Comparison of the isotopic pattern of
measured and theoretical Pb-PC$_2$ at m/z 740 – 752 is indicative of the presence of Pb in the complex, as its isotopic pattern can be identified. Furthermore, the zoomed spectrum shows the PC$_2$ specific isotopic distribution (Fig.4.3). The detection of the various complexes also indicates that the formed complexes are stable enough to detect them by nano-ESI-MS. Formation of Pb-PC$_2$ and Pb$_2$-PC$_2$ complexes was also observed using differential pulse polarography (Alberich et al., 2007). Similar observations were made for Pb-PC$_3$ and Pb-PC$_4$ complexes, showing a good match between measured and theoretical patterns and a loss of 2H$^+$ for each additionally bound Pb ion. Surprisingly, no complexes including three and four Pb ions were observed for PC$_3$ and PC$_4$. In addition, accurate mass measurements confirmed the proposed elemental compositions.

The singly charged Zn-PC$_2$ peak appearing after Zn addition to Pb-PC$_2$ complexes is indicative for complex formation between PC and Zn, which was confirmed by the isotopic pattern for Zn clearly visible in the zoomed spectrum (Fig. 4.15). The expected increase of the Zn-PC$_2$ peak with increasing Zn concentration was not observed, perhaps due to an increase of oxidized PC$_2$, indicated by the increase of the ratio between m/z 538.14 and 540.14 and leading to a loss of potential metal binding sites. This hypothesis could also explain the signal loss observed with increasing Zn concentration. Similar observations were made in a study with Cd, where a signal loss was observed at concentrations higher than 0.3 mM Cd (Yen et al., 1999).

A previous study using Pb exposed *C. reinhardtii* showed that formation of Me-PC complexes includes Cu, Zn and Pb. PC$_2$ and PC$_3$ complexes were detected in a molecular weight range between 700 – 5300 Da. PC$_2$ was mainly observed between 1000 – 1600 Da and results suggested complexes with Me$_{1-2}$(PC$_2$)$_2$ (Chapter 3). Most complexes detected in the present study have a stoichiometry of Pb-PC$_2$ – 4 or Pb$_2$-PC$_2$ – 4. The ratio of PC-SH to Pb was much higher in the gel filtration fractions than in the present study, which possibly leads to the formation of different complexes. Another possibility is that nano-ESI-MS, despite being a soft ionization technique, results in the dissociation of complexes.

*In vivo* complexes were not analyzed by nano-ESI-MS because the concentration of the complexes was not sufficient. Therefore, further research is needed to improve the sensitivity for Me-PC complexes by nano-ESI-MS. In addition, both *in vivo* and *in vitro* Me-PC complexes have to be further examined to investigate whether these complexes differ.
4.6 References


Chapter 5

Outlook
5.1 Outlook

This project showed that Pb is a potent inducer of phytochelatin synthesis in *C. reinhardtii*, highlighting the importance of exposure time and concentration on PC synthesis. The intracellular Pb concentration was observed to be similar after 6 h of Pb exposure to the concentrations $10^{-8}$ M and $10^{-9}$ M, indicating saturation of Pb accumulation. This saturation indicates regulation of Pb accumulation either by limitation of uptake or by excretion of accumulated metal. Despite the detection of similar intracellular concentrations, induction of PC synthesis was observable within minutes at $10^{-8}$ M, but only upon long-term exposure to $10^{-9}$ M, indicating the importance of the subcellular metal distribution for the induction of PC synthesis. We assume that regulation of Pb accumulation leads to a redistribution of metals, essential and non-essential, in the cell, which induces PC synthesis. Redistribution of all metals may also explain complex formation between PCs and essential metals, such as Cu and Zn. To explore whether metal redistribution is responsible for induction of PC synthesis, the subcellular distribution of metals could be examined as a function of time. A few experimental procedures for subcellular fractionation of algae were reported in recent publications, which might be used for this purpose (Wang and Rainbow, 2006; Wang and Wang, 2008; Lavoie et al., 2009a; Lavoie et al., 2009b). Subcellular fractionation might also give indications why toxic effects on photosynthesis and growth were only observed upon long-term exposure and not in short-term experiments.

This work further indicated that nano-electrospray ionization mass spectrometry is suitable for identification and characterization of lead-phytochelatin (Pb-PC) complexes. However, additional research using this method is required to investigate whether the stoichiometry of Pb-PC complexes is determined by the ratio of PC-SH to metal or if the composition of the surrounding medium has an influence on complex stoichiometry. Nano-ESI-MS also allows the investigation of whether coordination of metals by PCs is dependent on the metal, the chain length of the involved PC oligomers, or on both. Furthermore, improvement of the solvent mixture for metal competition experiments might allow additional research on competition of metals for PC binding, giving more information on the complex stability of Me-PC complexes formed with various metals.
At the environmentally relevant free Pb ion concentration, $10^{-9}$ M, Pb is accumulated in algal cells, reaching a high concentration close to saturation, after short exposure times. Furthermore, despite induction of PC synthesis, long-term exposure to this concentration caused photosynthetic and growth inhibition. This finding indicates that PCs are not protective against toxic effects of Pb upon long-term exposure. Because of these delayed toxic effects observed in *C. reinhardtii*, it is important to examine long-term exposures to assess the toxicity of Pb on organisms.

### 5.2 References


Acknowledgements
I would like to express my gratitude to my two advisors Laura Sigg and Renata Behra, who gave me the opportunity for this thesis, which introduced me into the various fields about metals, algae and detoxification. I would also like to gratefully acknowledge Prof. C. Fortin, who came from Canada to assist at my PhD defense, and Prof. B. Wehrli who accepted to be a member of my committee.

Special thanks go out to all my lab colleagues from whom I learned a lot during my thesis. First of all I am very thankful to Bettina Wagner for her help in the laboratory, for everything she taught me about algae and for the nice moments we had in the lab. Further thanks go to David Kistler for his help with the ICP-MS measurements, René Schönenberger and Marc Suter for their help with the nano-ESI-MS measurements and data analysis and to Adrian Amman for the helpful discussions on SEC. Thanks also go to Flavio, Danielle, Ilona, Irène, Thea, Miriam, Philippe and all other colleagues in UTOX and at Eawag who supported me in my work and mentally in moments of doubt.

I would also like to thank my family for their support and caring over these years. My immense gratitude goes to my girlfriend, Franziska, who supported me with her love and kindness. Her patience and help in the daily life gave me the spirit and power to finish my PhD.

Finally I also thank the Swiss National Science Foundation for their financial support.