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THE CHARACTERIZATION OF THE NOVEL GENE CIA7 USING PB EXPOSED WILD-TYPE AND MUTANT STRAINS OF CHLAMYDOMONAS REINHARDTII

A Thesis
by
JOSE ANGEL GUTIERREZ JR.

Submitted to Texas A&M International University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

December 2017

Major Subject: BIOLOGY
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December 2017

Major Subject:  Biology
ABSTRACT

The Characterization of the Novel Gene Cia7 Using Pb Exposed Wild-Type and Mutant Strains of *Chlamydomonas Reinhardtii* (December 2017)

Jose Angel Gutierrez Jr., B.S., Texas A&M International University;

Chair of Committee: Dr. Ruby A. Ynalvez

Industrialization has increased the risk of heavy metal contamination. This leads to contamination of the ecosystem surrounding microalga and other primary producers. *Chlamydomonas reinhardtii* is a unicellular alga that is of interest in toxicity studies due its detoxification pathways. This study aims to compare three common markers of cellular health and heavy metal homeostasis namely, levels of Pb bioaccumulation, chlorophyll fluorescence, and cell size between two strains of *C. reinhardtii*: a wild type (CC4425) and a mutant (CC5013). The mutant strain has a gene designated as Cia7 that has been disrupted by *Ble* insert. Cia7’s cysteine rich protein product (CIA7) is hypothesized to provide increased heavy metal tolerance to *C. reinhardtii*, due to its structural similarities to known thiol-peptides such as phytochelatins and metallothioneins.

Levels of bioaccumulated lead were analyzed using inductively couple plasma optical emission spectrometry. Differences in chlorophyll fluorescence were determined using flow cytometry, while changes in cell size were determined under scanning electron microscopy. The results of this study show that CC5013 accumulated more lead than CC4425 and exhibited marked reductions in both chlorophyll fluorescence and cell size across increasing
concentrations of lead. These results lend support to the hypothesis that Cia7’s cysteine rich protein product (CIA7) could provide increased heavy metal tolerance to *C. reinhardtii*.
Completing a thesis, though a critical milestone for oneself, cannot be accomplished entirely alone. There are many people I must thank for assisting me over the years. First and foremost I would like to thank my mentor and thesis committee chair, Dr. Ruby Ynalvez. Her seemingly endless dedication and patience towards her graduate students has been nothing short of inspiring. I thank her high standards, her guidance, and her ability to be equal parts strict and understanding for any improvements I’ve made over the years as a researcher. It is through her encouragement that I seek to obtain my doctorates degree.

I would like to thank my committee members: Dr. Michael Kidd, Dr. Monica Mendez, and Dr. Hari Mandal, for taking time from their hectic schedules to assist me over the years. Their teaching/presenting styles have no doubt influenced my own, and I am grateful for that. I’d like to give special thanks to Dr. Marcus Ynalvez, for providing (and explaining) the statistical analysis for this thesis.

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INTRODUCTION

The effect of heavy metal contamination on environmental systems has drawn attention over the past few decades (Pawlik-Skowronska 2000; Sheng et al. 2004; Cao et al. 2015). Increased industrialization has increased the synthesis of chemical by-products containing toxic heavy metals (Pinto et al. 2003; Su et al. 2014 Sharma and Dubey 2005; Gisbert et al. 2003; Hsu and Guo 2002). Hyperaccumulation of heavy metals such as lead, cadmium, and mercury can induce notable dysfunction throughout a wide range of biota via disruption of essential biochemical molecules and pathways (Fargasova 1994; Pinto 2003; Su et al. 2014). Furthermore, heavy metal ions cannot be broken down or digested once ingested by an organism. These ions can be transmitted from lower to higher strata of various ecological food chains via exponential bioaccumulation from primary producers to consumers (Walker et al. 2006).

Heavy metals are defined by their atomic mass (>5 g cm$^{-3}$) and can enter an ecosystem as a range of chemical species (free and bound ions, complexes and compounds) (Alloway 2012). Lead (Pb) is listed as a toxic contaminating agent of high priority by the Environmental Protection Agency (EPA), and the Agency for Toxic Substances & Disease Registry (ATSDR) (ATSDR 2015; EPA 2015). This is attributed to lead’s persistent bioavailability in the environment and its deleterious effects on organismal health at both acute and chronic levels of exposure. Lead is a staple in heavy metal toxicity studies and its toxicity has been recognized for millenia (Needleman). Lead compounds are synthesized as by-products of certain industrial processes (paint, gasoline and cosmetics) and can find their way into the environment via mine drainage or improper disposal of reaction by-products (Cao et al. 2015; Pinto et al. 2003; Su et al. 2014). Excessive exposure to Pb can induce an increased risk of cardiovascular disease and organ failure in human beings and developmental damages in most other organisms (Occupational Safety & Hazards Administration).

This thesis follows the model of Biometals.
*Chlamydomonas reinhardtii* is a unicellular alga that is of interest in toxicity studies due to its detoxification pathways. An initial resistance is imparted by a cell wall rich in hydroxyl-proline containing numerous glycoproteins. Heavy metal ions that filter through its cell wall encounter a range of biochemical pathways that end with either the sequestration of the damaging metal ion, or its removal out of the cell via efflux (Hossain et al. 2012; Hanikenne 2003). Sequestration of metal ions is accomplished via the action of two classes of cysteine rich chelating proteins: metallothioneins (MT) and phytochelatins (PC) (Grill et al. 1987). Although well studied, there remain numerous MT and PC like proteins with functions that have not been fully elucidated. Knowledge on metallothioneins and phytochelatins could prove highly beneficial in bioremediation studies.

This study aims to compare three common markers of cellular health and heavy metal homeostasis namely, 1) levels of Pb bioaccumulation, 2) chlorophyll fluorescence, and 3) cell size between two strains of *C. reinhardtii*: a wild type (CC4425) and a mutant (CC5013). The mutant strain has a gene, Cia7, which has been disrupted by *Ble* insert. Cia7’s cysteine rich protein product (CIA7) is hypothesized to provide increased heavy metal tolerance to *C. reinhardtii*. Cysteine residues are known in thiol-peptides such as phytochelatins and metallothioneins. It is hypothesized that when treated with increasing concentrations of lead, CC4425 will cope better with the metal stress compared to CC5013. The primary hypothesis is that there will be a difference in lead bioaccumulation, chlorophyll fluorescence readings and cell size between CC4425 and CC5013. It is the goal of this research to demonstrate what role, if any, Cia7 plays in allowing *C. reinhardtii* to resist the damaging effects of Pb.
LITERATURE REVIEW

Toxic and essential heavy metals

Organisms of varying ecosystems exhibit different nutritional requirements. Nutritional requirements can be divided into two broad categories: macromolecular and micronutrient requirements. Essential heavy metals are thus defined as metals (typically transition) which are required in minute dosages by an organism to achieve continued growth and reproduction (Walker and Hopkin 2006). Toxic heavy metals serve no particular biological function, as they are neither generated nor utilized by living organisms. Toxic heavy metals can accumulate within cells and exert a persistent negative effect as the accumulated concentration increases over time. Presence of toxic heavy metals within an organism typically serves as a marker for increased levels of heavy metals within their environment. (Chowdhury and Chandra 1987). Essential dietary elements include iron, nickel, copper and zinc. Prevalent toxic nonessential metals include mercury, lead, cadmium and arsenic (Chowdhury and Chandra 1987; Walker and Hopkin 2006; Chang and Cockerham 1994).

Anthropogenic sources of lead in the environment

Heavy metals can exist in multiple forms within an ecosystem. These forms can include ions (free or bound), but most metals are found as part of multi-element complexes (typically Cl₂ containing salts) (Abel 1996). Increased industrialization has provided an avenue for the production of heavy metal containing by-products, which can seep into local habitats through intentional or negligent clean up practices. Chief among these anthropogenically produced heavy metals are lead and cadmium (U.S. Environmental Protection Agency; Occupational Safety & Hazards Administration). As of 2012, it is estimated that 1.6 million metric tons of lead are produced within the United States alone, typically from processes involving the manufacturing of
paints, pipes, ammunition, and gasoline (Occupational Safety & Hazards Administration). The following represent resent examples of lead contamination from anthropogenic sources.

Recent analysis of untreated wastewater within the Mezquital valley near Mexico City revealed elevated concentrations of lead (0.16 ± 0.05 mg/1). This is of concern due to Mezquital valley’s function as an irrigation source for some 900 km² of nearby agriculture soil (Guedron et al 2014). Contamination of water sources by lead presents major ecological challenges due to the waters being used as sources for irrigation (Abel 1996). Like in the Mezquital valley, waters of the Coimbatore sewage disposal system are utilized to irrigate crops. Improperly treated sewage represents another avenue by which lead can enter an ecosystem. Sewage disposal areas along the Coimbatore district of Tamil Nadu, India have recently tested positive for high concentrations of cadmium (Radha et al 2014).

Metal smelting and gasoline production plants provide a source of lead contaminants that can easily leech into nearby soil and waterbeds (U.S. Environmental Protection Agency; Abel 1996). Lead can leech into the environment via improper disposal of chemical waste resulting from alloy synthesis and the manufacturing of paints and gasoline (U.S. Environmental Protection Agency; Walker and Hopkin 2006).

The damaging effects of lead

Toxicity studies involving various taxa of higher plants and algae species suggests that Pb exerts its damage in somewhat similar ways across photosynthetic organisms (Pinto et al. 2003; Sharma and Dubey 2005; Clemens 2006; Shakya et al 2008; Hossain et al. 2012; Alves et al. 2014). Preliminary analysis of maize (Zea mays) exposed to increased concentrations of Pb showed a notable decrease in the rate of photosynthetic activity (Hossain et al 2012). A similar effect was noted in the Cuban Bulrush, Oxyarea cubense. O. cubense exposed to excess
concentrations of lead ions exhibited extensive damage at the chloroplast level (Alves et al. 2014).

Damage to photosynthetic organelles can be partially attributed to an increase in reactive oxygen species produced by the uncontrolled nonspecific binding of lead ions to enzymatically important biomolecules with the cell. These reactive oxygen species eventually lead to thylakoid damage and to the reduction/degradation of photosynthetic compounds such as chlorophyll \textit{a} and \textit{b} (Pinto et al. 2003; Shakya et al 2008; Alves et al 2014).

Morphological studies conducted by Islam et al. (2007) on \textit{Elsholtzia splendens} (a member of the mint plants) revealed extensive damage to the plant’s root systems leading to a notable decrease in growth in treatment groups receiving higher levels of lead ions. More than a decade earlier, Fargosova (1994) demonstrated a similar degradation in the root systems of \textit{Sinapis alba} grown on lead contaminated soil. Studies of several algal species across multiple genera reveal a similar decrease in cell size, density, and chlorophyll content when exposed to lead ions (Stauber and Florence 1989; Pawlik-Skowronska 2000; Pawlik-Skowronska 2002; Pinto et al. 2003; Dao and Beardall 2016). In algal species, lead may exert its damage by preventing the entrance of essential metals into the cell (Marin et al 2014). In \textit{Chlamydomonas reinhardtii}, lead ions can directly compete with essential enzyme cofactors such as copper for entry into the cell (Marin et al 2014). This decrease in copper can then damage or reduce the efficiency of \textit{C. reinhardtii}’s photosynthetic biochemical pathways, as several key enzymes utilize copper as a co-factor (Marin et al. 2014).

Effects of lead on photosynthetic pigment content and functionality

The ability of Pb ions to bind indiscriminately to moieties of nitrogen, oxygen, and sulfur contributes greatly to its phytotoxic properties (Prasad and Prasad 1987; Belatik et al. 2013;
Concentrations of Pb ranging from 125 to 500 µM were found to disrupt the α-helix and β-pleated sheets structures in submembranes extracted from spinach (Spinacia aleracea) chloroplasts (Belatik et al. 2013). Deregulation of the delicate photosynthetic systems can adversely affect the development of most photobionts via nutrient loss and production of reactive oxygen species (Xion et al. 2013).

Chlorophyll is the primary light-harvesting molecule in all photosynthetic organisms. Changes to the structure, function or quantity of chlorophyll has been utilized as a metric of cell health for decades (Chettri et al. 1998). The dose dependent loss of chlorophyll content due to Pb exposure, from the downregulation of genes associated with its productions, to the total extracted volume of chlorophyll itself, has been observed in multiple photosynthetic organisms (Prasad and Prasad 1987; Chettri et al. 1998; Dudkowiak et al. 2011; Fischer et al. 2014; Kumar and Prasad 2015). It is theorized that Pb can directly compete with metal ion cofactors important for maintaining the transfer of electrons through photosystems I and II. This, coupled with Pb’s ability to cause alterations to the α-helix and β-pleated sheets, leads to an overall reduction in photosynthetic efficiency and an increase in cellular respiration (Belatike et al. 2013.)

Heavy metal ions exist in a state of flux within natural systems. Due to interactions with other molecular ions and the formation of complexes, a heavy metal’s bioavailability is often varied and rarely simple to study in nature (Stauber and Florence 1989). Lead species do not typically exist as pure ions, rather, they can be found as complexed salts with moieties of other ions such as chlorine and nitrogen. Hydrogen ions (measured as pH) also have an effect on a heavy metal’s bioavailability (Macfie et al. 1994). Studies by Macfie et al. (1994) on the influence of pH on heavy metal uptake by the unicellular green algae, Chlamydomonas
reinhardtii found that an increase in [H+] correlated with an increase in cell viability and size in the presence of cadmium, copper and cobalt. Macfie et al. (1994) suggests a similar mechanism for lead ions although research is lacking.

Higher plants and chlorophytes employ a myriad of physical and biochemical responses to maintain heavy metal homeostasis (Macfie et al. 1994). The cell wall of higher plants and algal species can act as a potent primary defense mechanism against nonessential and potentially damaging heavy metal ions (Macfie et al. 1994; Sharma and Dubey 2005; Clemens 2006). Research by Andrade et al. (2010) on the brown algae Padina gymnospora found a high degree of Pb ion accumulated along the cell wall relative to internal organelles. Andrade et al. (2010) demonstrated that an increase in cell-wall biomolecules (listed as polysaccharides, nitrogen containing acids, sulfate dense molecules, and other ligand sugars such as fucose, mannose and galactose) was directly correlated with increased Pb ion exposure. Past research by Macfie et al. (1994) showed an increased survivorship of cell-walled versus non-cell walled C. reinhardtii that were exposed to Cd, Cu, and Co. Heavy metal ions also directly compete with other molecules or atoms vying for entry into the cell (Andrade et al. 2010).

While the cell wall and its ligands may provide a powerful first line of defense against heavy metal ions, a point exists where these defenses are weakened or otherwise negated. Heavy metals may bypass extracellular cell-wall ligands by complexing with cysteine rich molecules that directly compete with micronutrients into the cell, passing into the cell via passive transport (Pinto et al. 2003). Furthermore, there exists a different saturation point for each heavy metal, whereby the cell wall’s defenses are reduced with sufficiently high heavy metal ion concentration (Macfie et al. 1994; Pinto et al. 2003; Andrade et al. 2010). Algae and higher
plants (chief among them, the hyperaccumulators) employ a range of internal defenses that can sequester and eventually expel heavy metal ions prior to the onset of any cellular damage.

Heavy metal tolerance in Chlamydomonas reinhardtii

*Chlamydomonas reinhardtii* is a diploid, unicellular, soil dwelling alga with a worldwide distribution (Harris et al. 2008). Like other members of the genus *Chlamydomonas*, *C. reinhardtii* possesses a glycoprotein based cell wall rich in hydroxyl-proline moieties, as well as basal chloroplast, two anterior flagella for movement, a nucleus and several vacuoles (Harris et al. 2008). *C. reinhardtii* has been a model organism for the study of heavy metal tolerance and homeostasis for the past 30 years (Howe and Merchant 1992; Rubinelli and Siripornadulsil 2002; Hanikenne 2003; Blaby-Haas and Merchant 2012). As with other plant and algal organisms, exposure of *C. reinhardtii* to excess concentrations of heavy metals (particularly Cd$^{2+}$, Ag$^+$, Bi$^{3+}$, Pb, Zn$^{2+}$, Cu$^{2+}$, Hg$^{2+}$ and Au$^{2+}$) can cause extensive cellular organelle damage that eventually results in decreased biomass, decreased rates of photosynthesis, decreased rates of cell viability and cell death (Collard and Matagne 1990; Nagel and Voigt 1995; Nagel et al. 1996; Rubinelli and Siripornadulsi 2002; Cai et al. 2006; Dominguez et al. 2003; Elbaz et al. 2010; Saison et al. 2010; Scheidegger et al. 2010). Each of the aforementioned heavy metals can bypass *C. reinhardtii*’s cell wall once a sufficiently high concentration has been reached. While the cell wall can bind a significant amount of heavy metal ions at first contact, eventually a saturation point is reached. Past this point of saturation, the ions enter through the various protein gated channels and passive transport systems that dot the outer surface of the cell wall (Hanikenne et al. 2005; Blaby-Haas and Merchant 2012).

Entrance of heavy metals into the cytoplasm of *C. reinhardtii* triggers a rapid (often in a matter of minutes) cascade of gene upregulation leading to the synthesis of compounds that
sequester heavy metal ions before they damage or disrupt the cell’s biochemical processes (Howe and Merchant 1992; Lemaire et al. 1999; Hanikenne et al. 2001; Rubinelli and Siripornaduls 2002; Dominguez et al. 2003; Perales-Vela et al. 2006; Cai et al. 2006; Scheidegger et al. 2011; Flouty and Estephane 2012). To this end, each heavy metal exerts a similar general cytotoxicity (IE decrease in photosynthesis, growth, and viability) on *C. reinhardtii*. However, the underlying mechanism behind these cytotoxic effects varies depending on the heavy metal ion in question. The rise of anthropogenic sources of Pb, Cd, Cu and Ag into the environment has thrust these metals into the forefront of bioremediation research, which often times finds a degree of intersectionality with the study of *C. reinhardtii’s* ability to survive in metal contaminated environments (Hanikenne 2003).

Effects of heavy metals and responses by Chlamydomonas reinhardtii

Lead: Although lead is of high interest in bioremediation research, the mechanisms by which it damages *C. reinhardtii* are not as well understood as that of either mercury or cadmium. Research delving into *C. reinhardtii’s* defenses to Pb ion accumulation is also lacking. Exposure of *C. reinhardtii* to dosages of Pb (1, 5, and 20 µM) caused a reduction of chlorophyll *a* values (26%, 33%, and 53%) after 48h of exposition, as well as notable damage to organelles such as the thylakoid and mitochondria (Irmer et al. 1986). As a photosynthetic organism, *C. reinhardtii* is presumably damaged by Pb in much the same manner as most other photoautotrophs. However, research delving into the exact mechanisms behind Pb’s toxicity towards *C. reinhardtii* are relatively unexplored, at least in comparison to other heavy metals such as cadmium and mercury. As is the case in other alga and higher plants, Pb increases the production of ROS via non-specific interactions with the charged sites of proteins and lipids (Scheidegger et al. 2011). Scheidegger et al. (2011) also noted the linear increase of phytochelatin production in *C.*
*Chlamydomonas reinhardtii* cultures exposed to increasing concentrations of Pb. As the concentration and exposure time of Pb increased, so did the concentration and polymerization levels of phytochelatin proteins (Scheidegger et al. 2011). These findings indicate that the study of Pb toxicity in *Chlamydomonas reinhardtii* is comparable to studies involving other photobionts.

Cadmium: The effects of cadmium on *C. reinhardtii* are well researched, as the metal is the one most often chosen in comparative growth and bioaccumulation studies (Hanikenne 2003). Cd\(^{2+}\) is quick to enter the cell via the macrophage proteins (Nramp) transporters DMT1-2 (Rosakis and Koster 2005; Brautigam et al. 2011). DMT1-2 is responsible for transporting magnesium, copper, and iron into the cell (Rosakis and Koster 2005). Once inside the cell, cadmium accumulates in the cytoplasm and chloroplasts (Collard and Matagne 1990; Nagel et al. 1996; Dominguez et al. 2003). Cadmium ions exhibit a high degree of nonselective affinity towards the charged groups of proteins and lipids. Nonspecific binding by Cd\(^{2+}\) can lead to the production of hydroxyl radicals that exert oxidative stress upon the cell and can potentially cause the loss of photosynthetic activity within the chloroplast via damage to photosystem II’s electron transport chain (Collard and Matagne 1990; Nagel and Voigt: 1995; Lemaire et al. 1999; Hanikenne et al. 2001; Cai et al. 2006). Dominguez et al. (2003) found that an accumulation of Cd\(^{2+}\) at levels exceeding 300 µM in solution lead to a 54% reduction in biomass, as well as a reduction in overall carbon (3.2%) and nitrogen (7.5%). A similar decrease in growth rate and chlorophyll content was previously noted by Collard and Matange (1990). Strains of *C. reinhardtii* that are lacking cell walls exhibit a marked sensitivity to cadmium. Exposure of a cell wall-deficient strain of *C. reinhardtii* (CW15) by Nagel and Voigt (1995) showed a 50% decrease in biomass at just 30 uM.
Cadmium triggers a wide range of enzymatic and physiological changes at elevated concentrations. Exposure of *C. reinhardtii* to 100 uM of Cd\(^{2+}\) triggers a rapid reduction in the activity of nitrate reductase (NR), which is integral in the catalysis of cytosolic nitrate to nitrite (Dominguez et al. 2003). Activity of glutamate dehydrogenase (GDH, involved in glutamate synthesis), serine-acetyltransferase (SAT), and O-acetyl-L-serine (thiol)lyase (OASTL, catalyzes the reaction between sulfide and OAS to form cysteine) are increased by roughly 75\%, 110\% and 25\% respectively, following a 24h exposure of *C. reinhardtii* to 150 uM of Cd\(^{2+}\) (Dominguez et al. 2003). Cadmium ions cause the upregulation of genes involved in chlorophyll biosynthesis (*Crd1, CHLL*), S-adenosylmethionine (SAM) synthesis (*CHRSAMS*), and iron uptake (*H43*) (Rubinelli et al. 2002; Hanikenne 2003). Hanikenne et al. (2005) noted the upregulation of the *Cds1* gene (whose protein product, CrCds1 is a member of the half size ABC transporters that transport phytochelatin complexes to and from the vacuole) in the presence of cadmium. GDH, SAT, OASTL, and SAM all play a role in the eventual biosynthesis of two classes of metal-sequestering thiol-peptides, the phytochelatins (PC) and metallothioneins (MT) (Nagel et al. 1995; Hanikenne et al. 2001; Dominguez et al. 2003; Hanikenne et al. 2005; Cai et al. 2006; Perales-Vela et al. 2006; Scheiegger et al. 2010; Brautigam et al. 2011).

Mercury: Mercury is among the most phytotoxic of the nonessential heavy metals (Elbaz et al. 2010). Mercury ions can exert extensive cytotoxicity at levels <5uM (Wei et al. 2011). Mercury ions trigger severe oxidative stress via non-specific, irreversible covalent bonding with the catalytic sites of enzymes and the charged sites of proteins and lipids (Elbaz et al. 2010; Wei et al. 2011). Exposure of *C. reinhardtii* cultures to just 4 uM of Hg for a period of 96 hours was enough to cause a 56\% reduction in growth rate relative to a control (Elbaz et al. 2010). *C. reinhardtii* subjected to similar concentrations of mercury also displayed a marked increase in
lipid peroxidation, measured in the production of excess reactive oxygen species (Elbaz et al. 2010; Wei et al. 2011).

The introduction of Hg triggers the upregulation of genes encoding the enzyme glutathione synthetase, which in turn increases the production of glutathione. The –SH binding sites of glutathione have a marked ability to sequester Hg ions (Howe and Merchant 1992). Hg acts as an important promoter of genes involved in the production of antioxidant enzymes (Elbaz et al. 2010). The elevation of Hg$^{2+}$ ions and the subsequent flood of ROS activates the genes coding Mn-SOD (Superoxide dismutase, a known antioxidant), CAT (Catalase) and APX (Ascorbate peroxidase) (Elbaz et al. 2010). P5CS (Pyrroline-5-carboxylate synthase) is also upregulated by incremental dosages of mercury up until a concentration of 8 uM, where it begins to decrease below the levels seen in the control (Elbaz et al. 2010). Hg also upregulates genes and enzymes involved in the production of PCs and MTs, as well as the production of endogenous CO, which can sequester free radicals, via the expression of Heme-Oxygenase (Elbaz et al. 2010; Wei et al. 2011). Mercury was previously known to upregulate genes involved in thioredoxin production (Lemaire et al. 1999). Sequestered mercury is typically transported to the vacuole or out of the cell in a complexed form (Elbaz et al. 2012; Wei et al. 2011).

Phytochelatins and metallothioneins

Heavy metals such as lead, cadmium, and mercury can induce the transcription of enzymes that synthesize phytochelatins (PCs) and metallothioneins (MTs) (Pawlik-Skowronska 2001; Pawlik-Skowronska 2003; Clemens 2006; Cao et al. 2015). Both of these polypeptides are cysteine rich, with varying concentrations of cysteine residues correlating to which heavy metal is present within the cell. These findings suggest that the elevated presence of cysteine within
these PCs and MTs directly contribute to their function as metal ion sequestrers/chelators. C. reinhardtii was discovered to have an unannotated gene region designated as “Cia7”, whose protein product fits the motif of many common phytochelatins and metallothioneins (Csy-X_{2}\text{-Cys-X}_{19}\text{-Cys-X}_3\text{-Cys}) (Ynalvez and Moroney 2008). Comparing the differences in levels of pb bioaccumulation, chlorophyll fluorescence, and morphology between a CIA7 encoding wild type (CC4425) versus a Cia7-inactivated mutant (CC5013) could contribute to knowledge of the biochemical pathways involved in C. reinhardtii’s metal detoxification. The common pathways of lead toxicity in plants and algae, as well as the highly conserved nature of Cia7’s protein product (CIA7) indicates that this research has implicit ties to a wide breadth of studies involving metal ion stress, bioaccumulation of heavy metals, and bioremediation amongst hyperaccumulating species of plants and algae.
MATERIALS AND METHODS

Cell cultures and growth

*C. reinhardtii* strains CC4425 (wild type) and CC5013 (mutant with *Ble* insert in *Cia7*) were obtained from the Chlamydomonas Resource Center at the University of Minnesota. *C. reinhardtii* cells were maintained in Tris Acetate Phosphate (TAP) agar (1.5%) plates at 23°C while cells were grown in 50 mL of liquid TAP medium. Culture flasks were then placed on an orbital shaker (VWR DS2-500-1) at 130 rpm at 23°C with constant fluorescent lighting. Culture flasks were kept on the shaker for a period of one to two weeks, with a change in TAP media every 4 days until an OD$_{650}$ of 0.800 ± 0.05 was obtained. Absorbance readings were obtained using a Spectronic Genesys 8 spectrophotometer. Cell cultures with an OD$_{650}$ above or below 0.800 ± 0.05 were equilibrated by diluting the cultures with TAP medium or by concentrating the cultures via centrifugation (10 minutes at 3,500 RPM) and resuspension. Cell cultures equilibrated to 0.8 OD$_{650}$ were used for treatment flask inoculation.

Table 1. Overview of cell culture and treatment groups for bioaccumulation, cell size, and chlorophyll fluorescence studies.

<table>
<thead>
<tr>
<th>Experimental Comparative Analysis</th>
<th>Strains</th>
<th>Treatment Groups</th>
<th>Incubation Time (Hours)</th>
<th>Cell Culture Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioaccumulation</td>
<td>CC4425, CC5013</td>
<td>0µM and 150 µM Pb</td>
<td>96</td>
<td>150</td>
</tr>
<tr>
<td>Cell Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll Fluorescence</td>
<td>0µM, 150 µM and 250µM Pb</td>
<td>48</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis

The purpose of this experimental design was to compare the total levels of bioaccumulated Pb between two strains of *C. reinhardtii*: a wild type (CC4425) with a functioning *Cia7* gene, and a *Cia7*-silenced mutant (CC5013). Two treatment groups (0µM and
150 µM Pb) were prepared for each strain of *C. reinhardtii* (Table 1). Two hundred and eighty mL of culture cells equilibrated to 0.800 ± 0.05 ABS at 650 nM were used to inoculate treatment flasks. The remaining 120 mL consisted of either Millipore H₂O (negative control) or PbCl₂ solution (1,000 µM) for a final concentration of 150 µM Pb. Treatment groups were allowed to incubate for long term exposure, 4 days (Hu et al. 2001; Franklin et al. 2002; Jamers et al. 2009; Dao and Beardall 2015) on a VWR DS2-500-1 orbital shaker at 130 RPM under constant lighting. After the 4 day incubation period, cells were equilibrated to 0.800 ± 0.05 ABS at 650 nM.

One hundred and fifty mL of cells from each treatment group were pelleted using a Beckman GS-15R Centrifuge (23°C, 3,500 RPM). Once the supernatant was removed, pellets were desiccated on a heat block (100°C) for 1 hour. The mass pellets were measured and pellets were pretreated with 5 mL of concentrated nitric acid (Amresco CAS#7697-37-2) and left to incubate under a laminar flow hood for 20 min. Pretreated cells were then fully digested using the Mars 6 One touch microwave at “Plant Cell” settings. The volume of each of the digested cells were adjusted to 50 mL using Millipore H₂O prior to measurement of lead content using a Varian 720-ES ICP-OES. Statistical analysis of recorded Pb concentration values was conducted utilizing a 2X2 Factorial model with randomized complete block design.

Flow cytometry analysis

Cell culture preparation: Liquid cultures were prepared by inoculating 50 mL of TAP media with 2 inoculating loopfuls of either CC4425 or CC5013. The cells were from cultures grown no more than a week on 1.5% TAP-agarose plates. The liquid cultures were maintained in 250 mL Erlenmeyer flasks under constant 24 h illumination by fluorescent lighting to either an
ABS of 0.400-0.800 ± 0.050 at 650nM. This absorbance correlates with the log phase of *C. reinhardtii* which allowed a high population of cells for flow cytometry analysis.

Treatment flask preparation and incubation: Treatment flasks consisted of Pb solutions at 3 working concentrations: 0 µM (control), and two sublethal concentrations of PbCl₂ (150 µM, and 250 µM). Treated cells were incubated under constant lighting and shaking (130 RPM) for a total of 4 days (96 h). Previous Pb toxicity experiments noted 24 hours as short term exposure and ≥ 4 days as long term exposure (Hu et al. 2001; Franklin et al. 2002; Jamers et al. 2009; Dao and Beardall 2015). In an effort to obtain data from both short and long term exposure without risking cellular acclimation to the heavy metals, 4 days was deemed a cut off point for sample analysis. Every 24 hours for 4 days, a sample of 300 µL per trial (3 trials total) was drawn and analyzed via a flow cytometer. In order to prevent the loss of metal concentrations, a series of duplicates grown simultaneously with the treatment flasks were used to supplement the total volume removed for analysis. Three hundred µL of samples were diluted with 1 mL of neutral pH saline prior to flow cytometry analysis. Samples were analyzed using a Cytek DxP8 FACSCalibur™ Flow Cytometer (488/561/405 nm solid-state lasers) under the following fluorescent laser settings: P2 Coarse Gain: 1X, P3 PMT Gain: 25, P4 BLUFL1: 450, P5 BLUL2: 400, P6 BLUFL3: 300 and P7 BluFL4: 350. A total of 10,000 cells were analyzed from each sample aliquot. Measurements were repeated in triplicate and geomean values were recorded. Statistical analysis of BluFL1-4 fluorescence geomean values was conducted utilizing a 2x3 factorial design model with repeated measures.

Scanning electron microscope analysis

Sample preparation: Two treatment groups, 0 µM and 150 µM Pb were prepared for each of strain of *C. reinhardtii* (Table 1; recipes are provided in the appendix). Thirty mL of treated

...
cells were allowed to incubate for 48 hours on a VWR DS2-500-1 orbital shaker at 130 RPM under constant lighting. Aliquots of 1-1.5 mL were drawn every 24 hours. Aliquots were pelleted and the supernatants were replaced with 1% glutaraldehyde (Sigma-Aldrich CAS Number 111-30-8). Cells were incubated in 1% glutaraldehyde for a period of at least 72 hours, after which cells were resuspended in roughly 5 mL of Millipore H2O and collected through Whatman Nucleopore Track Etched 0.2µm, 13mm polycarbonate filters (VWR). Polycarbonate filters with cells were then dehydrated via an ethanol gradient using the following concentrations: 25%, 50%, 75%, 95%, and 100%. Polycarbonate filters with cells were left for 15 minutes in each ethanol concentration to ensure thorough dehydration. Samples were then mounted on SEM analysis stubs and critical point dried.

SEM Settings: Critical point dried samples were analyzed under low vacuum using a JEOL JSM-6610 Scanning Electron Microscope. Samples were analyzed at 800X magnification using the LSEI setting of the microscope. Parameters such as working distance, spotsize and pressure were adjusted to until an image of acceptable quality was produced.

SEM Measurements: In order to have unbiased measurements, once an image was properly resolved, a 3X3 grid was overlaid on top of the micrograph using the measurement tools provided by the SEM software. Each section of the grid was assigned a number between 1 and 9. A random number generator was then used to pick a block from among the 9 total blocks in the grid. Individual cells within this block were then measured using the polygonal area tool provided by the SEM software. A maximum of 15 cells were measured per block. This process continued until a total of 50 cells were measured per sample. Statistical analysis of recorded cell area measurements followed a factorial experiment with random complete block design (RCBD).
RESULTS AND DISCUSSION

This study describes the insertional mutant, CC5013 (previously designated as Cia7), generated by insertional mutagenesis approach. This mutant grows normally under high CO₂ conditions, but grows poorly in a low CO₂ environment, thus, the gene disrupted by the insert has been designated as Cia7 (inorganic carbon acquisition). This gene encodes a protein of unknown function, and has high similarity to conserved bacterial proteins. Protein-protein Basic Local Alignment Search Tool (BLAST) and domain searches showed the presence of a conserved domain found in more than 100 hypothetical proteins from bacteria, and one from the marine alga, Ostreococcus tauri. This implies that this protein performs an essential function. All of the proteins have the sequence motif Cys-X₂-Cys-X₁₉-Cys-X₃-Cys, with the presence of four conserved cysteines indicating that CIA7 is a putative metal-binding protein (Ynalvez and Moroney, 2008).

Increased Pb Bioaccumulation in CC5013

The objective of this experiment was to compare the bioaccumulation of lead between the wild type (CC4425) and cia7-mutant (CC5013). Both strains were exposed to Pb at sublethal concentrations of 150 µM for 4 days. The results of this study aimed to demonstrate whether the mutation of Cia7 in CC5013 affects the ability of C. reinhardtii to efflux bioaccumulated Pb ions; higher levels of bioaccumulated Pb could indicate a dysfunction in CC5013’s ability to maintain heavy metal homeostasis.

Bioaccumulation analysis was carried out using Inductively Couple Plasma Optical Emission Spectrometry (ICP-OES). This is a method for detection of individual or multiple elements within a sample. In ICP-OES, plasma energy (generated by an argon-fed plasma torch) is used to push an element’s protons into a higher energy state. Movement from a high to low energy state by the proton causes the release of emission rays which are correlated back to a
specific element by the ICP-OES’s software (Hitachi 2017). High specificity, relative ease of use, and the ability to be operated without the use of hazardous chemicals has made ICP-OES analysis the current standard for heavy metal content analysis in biotic and abiotic samples, including algae (Holcome 1989; Romani et al. 2009; Nascimento et al. 2010).

ICP-OES analysis of Pb ions indicated that the mutant strain CC5013 bioaccumulated an average of 17.07 ± 1.97 ppm Pb (P < 0.001) when treated with 150 µM Pb over a period of 4 days. This is in contrast to the wild type CC4425, which only accumulated an average of 6.35 ppm ± 1.97 ppm (P < 0.05 between replications) (Fig. 1; Table 1). On the other hand, there was no significant difference in Pb bioaccumulation, 0.10 ppm and 0.11 ppm of Pb (P > 0.9) at 0 µM Pb for the mutant and the wild-type respectively (Table 2). The concentration of Pb present in 0 µM may be due to trace concentrations (.2ppm) of heavy metals present in the concentrated nitric acid (Amresco CAS#7697-37-2). Significant differences were noted when comparing Pb treated strains to one another (Table 2). There is a significant difference between 0 µM and 150 µM treatments of the mutant strain (P < 0.001) (Table 2).

![Fig. 1 Pb bioaccumulation between wild type (CC4425) and mutant (CC5013) following 4 days of incubation under 24 h illumination. Mean values are displayed with error bars representing a standard error of 1.97 ppm.](image)

On the other hand, statistical analysis revealed no significant difference between the 0 µM and 150 µM of the wild-type (P > .005) (Table 1). Direct comparisons between CC4425 (wild-type)
and CC5013 (mutant) showed that CC5013 accumulated 2.7 times more Pb than CC4425 exposed to 150µMs of Pb (P<0.01) (Fig. 1).

**Table 2.** Correlation analysis utilizing mean concentration as the dependent variable.

| Pb Treatment | Strain | Mean Concentration (ppm) | Standard Error | Pr > |t| | MEAN Number |
|-------------|--------|--------------------------|----------------|-------|---|----------------|
| 0 µM        | CC5013 | 0.10                     | 1.97           | 0.9605| 1 |
| 0 µM        | CC4425 | 0.11                     | 1.97           | 0.9557| 2 |
| 150µM       | CC5013 | 17.07                    | 1.97           | 0.0001| 3 |
| 150µM       | CC4425 | 6.36                     | 1.97           | 0.0179| 4 |

**Pairwise plot of Least Squared Means**

<table>
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<th>3</th>
<th>4</th>
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<td>0.0009</td>
<td>0.0655</td>
</tr>
<tr>
<td>2</td>
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<td>-</td>
<td>0.0009</td>
<td>0.0659</td>
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<td>0.0009</td>
<td>0.0009</td>
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<tr>
<td>4</td>
<td>0.0655</td>
<td>0.0659</td>
<td>0.0085</td>
<td>-</td>
</tr>
</tbody>
</table>

ANOVA analysis revealed the following: 1) variation was not due to the blocking of the experiment and 2) factorial analysis utilizing values for cell strain and concentration was determined as being the most significant source of variation between trials (Table 3). This analysis supports the findings presented by the pairwise correlation analysis.

**Table 3.** ANOVA table of results from a 2x2 factorial experiment with a randomized complete block design.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>P Value</th>
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<td>Treatment (T)</td>
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<td>34.80</td>
<td>0.0011</td>
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<tr>
<td>Strain (S)</td>
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<td>85.80</td>
<td>7.39</td>
<td>0.0347</td>
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<tr>
<td>T*S</td>
<td>1</td>
<td>86</td>
<td>86.20</td>
<td>7.43</td>
<td>0.0344</td>
</tr>
</tbody>
</table>

Heavy metals enter algal cells by active transport or by endocytosis through chelating proteins. The synthesis of sulfhydryl containing binding proteins, exclusion of metals from cells by ion-selective transporters, and excretion or compartmentalization have been suggested with
regard to reducing heavy metal toxicity (Arunakumara and Xuecheng, 2008). *C. reinhardtii* exhibited the ability to bioaccumulate Pb (Flouty and Estephane, 2012). Differences in Pb bioaccumulation between strains may be attributed to multiple factors. The absence of a key metal sequestering protein could perhaps explain the noted increase in bioaccumulated Pb in CC5013.

Although the role of Cia7 has not been fully elucidated, prior research by De Llano (2014) has demonstrated that the strain is sensitive to Pb exposure, more so than its wild type counterpart, CC4425. While gene silencing experiments focusing on Pb bioaccumulation in *C. reinhardtii* are rare, similar studies have been conducted on other heavy metals. Fujiwara et al (2000) demonstrated how arsenate sensitive insertional mutants of *C. reinhardtii*, bioaccumulated higher concentrations of lead relative to the wild type (CC4425). The disruption of the gene Cia7 by a Ble insert resulted in a non-functional protein thus influencing the difference in bioaccumulation between CC4425 and CC5013. It is possible that the increased bioaccumulation of Pb ions in CC5013 may be due to a dysfunction in a key metal sequestering/efflux system. It can be proposed that damage to these systems may lead to a retention of Pb ions within CC5013 cells. Research by Belatik et al. (2013) indicates that Pb ions may bind to the internal and external linings of the chloroplasts in photoautotrophs. Pb ions may be binding directly to the chloroplasts more readily in CC5013 than in CC4425. CC4425, with its functioning metal sequestering or metal efflux system (provided by the CIA7 protein), has the ability to remove Pb ions from its cytoplasm. On the other hand, in CC5013, Pb ions linger within its internal milieu. Since these Pb ions are bound to the chloroplasts, they stay within CC5013 until such time that apoptosis occurs, potentially explaining the increased levels of
bioaccumulation observed in the mutant strain. Cell fractionation followed by ICP-OES analysis of isolated chloroplasts may yield more support for this proposal.

Pb induced fluorescent quenching in CC5013

Changes to intracellular concentration, structure, or function of chlorophyll are typical signs of metabolic dysfunction and can be lethal to chlorophytes should the photosynthetic system become sufficiently impaired. As such, chlorophyll content and fluorescence have been prime metrics for assessing the health of photobionts for decades (Prasad and Prasad 1986; Krause 1991; Maxwell and Johnson 2000; Arunakamura et al. 2008; Carfagna et al. 2013; Saleh 2016; Jio et al. 2017). However, very few experiments have combined a flow cytometric approach for detecting the effects of Pb ions on chlorophyll intensity in unicellular alga. It was the goal of this experiment to detect what difference, if any, occurred in the chlorophyll fluorescent intensities between two strains CC4425 and CC5013 (WT and mutant respectively) of *C. reinhardtii* exposed to two sublethal concentrations of Pb over the period of 4 days. To determine which combination of strains, treatment and exposure time had the greatest effects (if any) on the two strains of *C. reinhardtii*, a 2x3 factorial design with repeated measures analysis of variance model was utilized to detail the factors resulting in statistically significant changes. This experimental design allowed for the determination of the statistical significance of combinations of variables, as opposed to individual ones.

Effects of Pb on forward scatter signal (FSC)

Forward Scatter (FSC) values are roughly linearly correlated with cell size (Jamers et al. 2006). Figure 2 displays the FSC values of CC4425 and CC5013 across days (24-96 Hours) and metal concentrations (0 µM- 250 µM Pb). FSC values for the wild type at 0µM, 150 µM and 250µM remained constant throughout the 96 h incubation period (Fig.2).
Fig. 2 Histograms Displaying FSC Values Across Strains and Time. Histograms separated into 4, 2X3 plots based on sample points (A) 24h, (B) 48h, (C) 72h, and (D) 96h. Within plots (A-D), histograms are separated by strains (CC4425 vs CC5013, top to bottom) and Pb concentration (left to right: 0µM, 150µM, 250µM).

FSC values for CC5013 were notably different. FSC values for CC5013 samples at 24h, 0 µM Pb were significantly (P<0.005) larger than those of CC4425. This may indicate the following: 1) CC5013 cells are naturally larger than the wild type, even in relatively stress-free conditions or 2) CC5013 cells display some form of clumping not seen in CC4425. Although a flow cytometer can separate cells quite efficiently, past observations have shown that CC5013 can display clumps of 3-4 cells even in 1X TAP media without xenobiotics. Such a trend was also observed by Dellano (2014), who previously worked with the CC5013 strain. These clumps could potentially exhibit elevated fluorescence peaks, as a “single cell” counted by the flow cytometer would actually display 3-4 cells worth of fluorescent output. However, even with the probability of false positives, the FSC values for CC5013 decreased significantly (P<.0001) when treated with 150 µM and 250µM of Pb.
FSC values in CC5013 decreased in a dose dependent manner with increasing Pb concentrations (Figure 2). Relative to the negative control group (0 µM), sublethal concentrations of 150µM Pb induced a 50% reduction in FSC values, while the elevated sub-lethal concentration (250µM) caused a decrease of 65%. These results indicate a relative reduction in cell size in the mutant strain exposed to elevated concentrations of Pb.

Table 4. ANOVA table of results from a 2x3 factorial experiment with repeated measures for forward scatter (FSC) values.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>11,945,830</td>
<td>0.20</td>
<td>0.6586</td>
</tr>
<tr>
<td>Strain</td>
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<td>354,888,181</td>
<td>354,888,181</td>
<td>5.98</td>
<td>0.0239</td>
</tr>
<tr>
<td>Concentration</td>
<td>2</td>
<td>8,738,798,317</td>
<td>4,369,399,159</td>
<td>73.59</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Strain * Concentration</td>
<td>2</td>
<td>7,583,538,875</td>
<td>3,791,769,438</td>
<td>63.86</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Days</td>
<td>3</td>
<td>390,623,539</td>
<td>130,207,846</td>
<td>2.19</td>
<td>0.1205</td>
</tr>
<tr>
<td>Strains * Days</td>
<td>3</td>
<td>1,525,017,423</td>
<td>508,339,141</td>
<td>8.56</td>
<td>0.0007</td>
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<tr>
<td>Concentration * Days</td>
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<td>207,762,299</td>
<td>34,627,050</td>
<td>0.58</td>
<td>0.7396</td>
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<tr>
<td>Strain * Concentration * Days</td>
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<td>124,729,550</td>
<td>20,788,258</td>
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</table>

Research comparing FSC values of *Chlamydomonas reinhardtii* treated with heavy metals is scarce. Research that applies these flow cytometry techniques to Pb treated *C. reinhardtii* are non-existent. Although, Jamers et al. (2009) conducted a similar experiment but using cadmium at 5 µM and 100 µM. Concentrations as low as 5 µM were enough to induce a change in cellular size in *C. reinhardtii*, with cells treated with 5 µM of cadmium registering FSC values much larger than those of the 0 µM control (Jamers et al. 2009). Cells exposed to 200 µM, while larger than those of the control, displayed a reduced size compared to cells exposed to 5 µM of cadmium (Jamers et al. 2009). Jamers (2009)’s results are similar with the findings of this study. Jamers and colleagues’ research reported that heavy metals can induce changes in cellular size which can be quantified rapidly and efficiently using a flow cytometer.
Researchers have reported changes to cell size, cellular area and growth of microalgae exposed to Pb (Carfagna et al. 2013; Putri 2017). Carfagna et al. (2013) found that exposing *Chlorella sorokiana* (a single celled freshwater algae) to concentrations of Pb (as lead nitrate) at 250 µM for 24 hours caused a decrease in cell growth measured as packed cell volumes) of 30% and decrease in total cell soluble protein content of 61%. As photosynthetic rates are decreased by the effects of Pb and the ROS the cells produce, microalgae like *Chlorella sorokiana* and *Chlamydomonas reinhardtii* may undergo a process in which they catabolize structural proteins to replenish the nitrogen and sulfur utilized in the production of phytochelatins. This scavenging processes may ensure adequate reproduction at the cost of a shortened carbon skeleton (Carfagna et al. 2013). Putri et al. (2017) measured the length and width of individual *Spirulina plantensis* (a microalga) exposed to concentrations of Pb ranging from 0 to 4 ppm over a period of 10 days. Following an incubation period of 10 days, cells exposed to 4 ppm of Pb exhibited a reduction in total cellular area of 22%, going from an average of 25.98 µm² to 19.89 µm² (Putri et al. 2017).

While some researchers were able to report a change in cell size due to Pb exposure, others noted little to no change in cell size in their specimen of choice. Shanab et al. (2012) noted no change in the cell size of three microalgal species (*Pseudochlorococcum typicum, Pseudochlorococcum typicum* and *Scendesmus quadricauda*) exposed to Pb concentrations of 100 mg/L. These contrasting results indicate that changes to cellular size may be species dependent, with some species being able to better tolerate Pb than others. This is the first report on a study of the effect of Pb on *C. reinhardtii* strains using flow cytometry analysis. Based on the results of this experiment, it showed that CC5013 cells are generally less tolerant of xenobiotic stress compared to CC4425, as evidenced by 50% reduction in FSC values at 150µM Pb and a 65% reduction in FSC values at 250µM Pb. CC4425 cells, in turn, had no significant
change in FSC values across metal concentrations and time points. These results indicate a relative reduction in cell size in the mutant strain exposed to elevated concentrations of Pb as compared to wild type.

Effects of Pb on chlorophyll fluorescence signals

Chlorophyll fluorescence measurements were conducted using a Cytek DxP8 FACSCalibur™ Flow Cytometer (488/561/405 nm solid-state lasers). Two blue light filters (BluFl3 and BluFL4) were chosen for analysis due to their usage in detecting green fluorescent protein (GFP) in microbial populations. The wavelengths associated with Blu-FL3 and BluFl4 were previously found to correlate with those required to induce chlorophyll fluorescence.

Tables 3 and 4 show the ANOVA for BluFL3 and BluFL4 fluorescence measurements. Exposure of CC5013 (the mutant strain) to concentrations of Pb of 150 µM and 250 µM resulted in a statistically significant decrease in BluFl3 and BluFl4 fluorescence (Tables 4 and 5). BluFL3 and BluFL4 values in Pb treated CC5013 cells decreased in a dose-dependent manner with Pb concentrations. BluFL3 values in CC5013 cells treated with 150 µM and 250 µM of Pb decreased by 73% and 91% respectively at the end of the 96 h incubation period (P<.0001). BluFL3 and BluFL4 values in CC4425 demonstrated no statistically significant change in geomean fluorescence values across Pb concentrations. Heavy metals such as lead and cadmium are known inducers of chlorosis in photosynthetic organisms (Belatik et al. 2013). Although the exact mechanisms are still being elucidated, Pb ions appear to disrupt photosynthesis at photosystems I and II by breaking down the α-helix /β-pleated sheets that comprise the protein structures of photosystem 1 (Belatik et al. 2013).
Fig. 3 Histograms Displaying BluFL3 Values Across Strains and Time. Histograms separated into 4, 2X3 plots based on sample points (A) 24h, (B) 48h, (C) 72h, (D) 96h. Within plots (A-D), histograms are separated by strains (CC4425 vs CC5013, top to bottom) and Pb concentration (left to right: 0µM, 150µM, 250µM).

Fig. 4 Histograms Displaying BluFL4 Values Across Strains and Time. Histograms separated into 4, 2X3 plots based on sample points (A) 24h, (B) 48h, (C) 72h, (D) 96h. Within plots (A-D), histograms are separated by strain (CC4425 vs CC5013, top to bottom) and Pb concentration (left to right: 0µM, 150µM, 250µM).
Table 5. ANOVA Table of results from a 2x3 factorial experiment with repeated measures for BluFL3 values.

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<td>Days</td>
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<td>6,081,142</td>
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<td>2.27</td>
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<td>Strains * Days</td>
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<td>Concentration * Days</td>
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Table 6. ANOVA Table of results from a 2x3 factorial experiment with repeated measures for BluFL4 Values.

<table>
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Despite its advantages over traditional chlorophyll measurement techniques such as acetone extractions or pulse amplitude measurements (PAM), flow cytometry has mainly been used to classify oceanic microalga based on chlorophyll content (Jamers et al. 2009).

Research utilizing flow cytometry to analyze differences in chlorophyll fluorescence output in microalgae has been limited, thus no comparisons from previous studies in C. reinhardtii will be used in this discussion. However, there were studies reported on photobionts including microalgae and some higher plants. Chlorophyll extract studies conducted on photobionts have shown a trend of increased chlorosis (loss/destruction of chlorophyll) along
increasing concentrations Pb (De Filippis 1979; Clijsters and Assche 1985; Arunakumara et al. 2007; Baumann 2009; Dellano 2014). Spinach (*Spinacia oleracea*) cells exposed to concentrations of 200 µM of Pb exhibited a decrease in chlorophyll *a* fluorescence of 51% (Belatik et al. 2013).

A similar trend was reported by Kumar and Prasad (2015) a few years later. Kumar and Prasad (2015)’s study on the spinach *Talinum triangulare* reported a dose dependent decrease in various metrics of chlorophyll fluorescence, including decreases in quantum yields, maximum fluorescence yields, steady-state fluorescence and maximal fluorescence yields. Pb has been shown to induce chlorosis in several species of lichens, including *Cladonia convoluta* and *Cladonia rangiformis* (Chettri et al. 1998). Chettri et al. (1998) reported a reduction of 10-15% in the ratio of chlorophyll *a/b* in *C. convoluta* and *C. rangiformis* exposed to 20 µg/g (dry weight) of Pb. Microalga have demonstrated a similar trend in Pb induced chlorosis. *Spirulina platensis* experienced a decrease in chlorophyll content of 15% when exposed to 5 mg/l of Pb² (Arunakumara et al. 2007). Similarly, Dellano (2014) reported a loss of chlorophyll content in *C. reinhardtii* of 59% and 69% in cells treated with 200 µM and 100 µM respectively. These findings indicate that levels of chlorophyll damage vary depending on the concentration of Pb, incubation time, and the type of photobiont. However, a trend is readily observable. Organisms that are less tolerant to Pb exhibit increased rates of chlorosis that may be measurable using either fluorescent readings or direct chlorophyll extraction. The results of this experiment show a similar trend. CC5013 is hypothesized to be more susceptible to heavy metal stress due to the absence of the Cia7 gene product. This gene product shares several structural similarities with known phytochelatinins. Flow cytometric analysis of BluFL3 and BluFL4 fluorescence suggests
that CC4425, with its functional Cia7 gene, can tolerate the chlorotic effects of Pb more so than CC5013.

Pb induced morphological changes in CC5013

Cells tolerant to xenobiotics such as heavy metals or phytotoxic agents typically resist changes to their ultrastructure and size when treated with said metals/phytotoxic agents (Irmer et al. 1986; Haider et al. 2006; Carfagna et al. 2013). Scanning Electron Microscopes (SEM) and Transmission Electron Microscopes (TEM) have been employed for decades to observe and categorize fine surface and internal details in a large swath of organisms, encompassing all manner of prokaryotic and eukaryotic cells (Ma et al. 2006). Research into the morphological changes caused by Pb in microalgal cells is scarce. Most research on the subject typically focuses on cell growth as a measure of population density. Furthermore, few heavy metal toxicity studies involving mutant strains utilize SEM imaging, which has great potential to show morphological difference between mutant and wild type cells. The goal of this experiment is to compare what changes, if any, occurred in the cell size (measured as cellular area in µm²) of two strains of C. reinhardtii (CC4425, wild type and CC5013, mutant). Cells from both strains were exposed to two concentrations of PbCl₂ (0 µM and 150 µM) for a period of 48 hours. Samples were drawn every 24 hours and manually measured utilizing the polygon area tool provided by the JEOL JSM-6610 Scanning Electron Microscope.

CC5013 exhibited reduction of cell area when exposed to Pb

CC5013 cells exposed to 150µM of PbCl₂ showed a statistically significant reduction in cell size; an average area of 11.72 µm² in the 0 µM treatment groups compared to 7.93 µm² in the 150 µM PbCl₂ treatment groups(P<0.0001) (Table 7). These results indicate that on average,
CC5013 cells exposed to 150 µM of PbCl₂ experienced a 32% reduction in size relative to the 0 µM control groups.

**Fig. 5** SEM Micrographs of CC4425 Cells. Treatment groups are as follows: (A) 24 h, 0 µM, (B) 24 h, 150 µM PbCl₂, (C) 48 h 0 µM and (D) 48 h 150 µM PbCl₂.

**Fig. 6** SEM Micrographs of CC5013 Cells. Treatment groups are as follows: (A) 24 h, 0 µM, (B) 24 h, 150 µM PbCl₂, (C) 48 h, 0 µM and (D) 48 h, 150 µM PbCl₂.
CC4425 wild type cells showed no significant difference in cell size between metal treatment groups (Table 7). No significant difference was recorded between 24 h and 48 h treatments for either strain (Table 7). CC5013 cells in 0 µM PbCl₂ conditions start off larger than CC4425 cells (11.715 µM² vs 9.6072 µM² respectively) but significantly decrease in size when exposed to 150 µM of PbCl₂ (7.9329 µm² in CC5013 vs 9.2329 µm² in CC4425) (Figure 7). Interestingly, the results of this experiment correlate with the Forward Scatter Values (FSC) values obtained in the flow cytometry experiment.

Pb has been shown to cause decreases in cell size and induce ultrastructural changes in several other photobionts. Haider et al. (2006) demonstrated how the leaf cells of Phaseolus mungo and Lens culinaris (two common Asian crops) decreased in size by roughly 50% when exposed to concentrations of 250 ppm Pb. Similarly, TEM analysis of Chlorella sorokiniana, a unicellular freshwater microalga) exposed to 250 µM Pb (as lead acetate) revealed extensive damage to internal structures such as the chloroplast (Carfagna et al. 2013). While these studies are not directly related to CC5013, they nonetheless demonstrate Pb’s ability to induce observable changes in the structure and morphology of Pb sensitive cells.

**Fig. 7** Least Square Means of Cell Area (µm²) Measurements of 0µM and 150µM Pb Treated Wild Type (CC4425) and Mutant (CC5013) C. reinhardtii.
Table 7. Correlation analysis utilizing mean area as the dependent variable.

| Strain   | Metal | Mean Area (µm²) | Standard Error | Pr > |t| | MEAN Number |
|----------|-------|-----------------|----------------|------|---|----------------|
| CC4425   | 0 µM Pb | 9.61            | 0.29           | <.0001 | 1 | |
| CC4425   | 150 µM Pb | 9.24           | 0.29           | <.0001 | 2 | |
| CC5013   | 0 µM Pb | 11.72          | 0.29           | <.0001 | 3 | |
| CC5013   | 150 µM Pb | 7.93          | 0.29           | <.0001 | 4 | |

Pairwise plot of Least Squared Means

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Table 8. ANOVA from a 2x3 factorial experiment with random complete block design.

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The results of this experiment indicate that Pb induces visible changes in cell size only in CC5013 cells. This observation could suggest that CC5013 is more sensitive and less tolerant of Pb stress relative to CC4425.
CONCLUSIONS AND RECOMMENDATIONS

Photoautotrophs have evolved a myriad of ways to resist the damaging effects of naturally occurring heavy metals. These photosynthetic organisms maintain heavy metal homeostasis via the production of cysteine rich proteins that can sequester, and eventually remove, the heavy metal ions before they can damage the cell’s delicate photochemical systems. These proteins appear to be expressed in a dose-dependent manner, increasing in quantity as the concentration of heavy metal ions increases within the cell. The unicellular microalga *Chlamydomonas reinhardtii* has served as a model organism for the study of heavy metal stress and bioremediation for decades. Although research relating to *C. reinhardtii*’s ability to survive sublethal concentrations of heavy metals is abundant, the exact mechanisms have yet to be fully elucidated. Cia7 is an unannotated gene thought to play a role in *C. reinhardtii*’s ability to tolerate heavy metal stress. The determination of effects of Pb on the bioaccumulation, chlorophyll fluorescence and cell size of two strains of *C. reinhardtii* (CC4425, wild type vs CC5013, mutant) was done in an effort to shed light on the role of the Cia7 gene.

The results of this study indicate that CC5013 is more sensitive to the damaging effects of Pb compared its wild type counterpart, CC4425. Metal bioaccumulation, chlorophyll fluorescence and cell size are all markers of cellular health. Cells that can properly maintain heavy metal homeostasis typically resist changes to these metrics over time. Compared to the Cia7-expressing CC4425, CC5013 showed increased levels of Pb bioaccumulation, decreased chlorophyll fluorescence, and decreased cell size when exposed to Pb. This supports the hypothesis that Cia7 plays a role in imparting tolerance of Pb to *C. reinhardtii*. This study also indicates a difference in morphology between CC4425 and CC5013 cells growing in nutritionally replete media.
Flow cytometer analysis and SEM measurements indicate that CC5013 cells are generally larger than CC4425 cells in non-metal treated, nutritionally replete media. The reason for this is not entirely understood. However, one possible reason for this increase in size could be an alteration or dysfunction in CC5013’s ability to undergo cytokinesis, resulting in larger, albeit fewer, cells. The results of the flow cytometry experiment also suggests that non-metal treated CC5013 cells express higher levels of chlorophyll fluorescence relative to non-metal treated CC4425 cells. This may be due to CC5013 containing more chloroplasts per cell than CC4425. CC5013 may be producing a higher degree of chloroplasts per cell in an effort to supplement some sort of nutritional deficiency. Due to the energetically costly nature of producing numerous chloroplasts, CC5013 cells may be left with insufficient nutrients to properly carry out cell division. However, this explanation has not been fully explored.

Further experiments are needed to fully elucidate the gene cia7’s role in *Chlamydomonas reinhardtii*. Expression of the cia7 gene should be compared at different concentrations of Pb to demonstrate whether or not its expression is upregulated or downregulated by exposure to Pb ions. Similarly, the protein product (CIA7)’s localization and concentration may be determined via fluorescent tagging and confocal microscopy/flow cytometry. Lastly, the morphological and ultrastructural differences between CC4425 and CC5013 could be explored by high-vacuum, high-magnification SEM analysis.
REFERENCES


Environmental Protection Agency. Learn about lead. www2.epa.gov/lead/learn-about-lead.pdf


VITA

Jose Angel Gutierrez Jr. was born in Laredo, Texas. He attended Early College High School from 2006-2010. It was after experiencing the quality of the biology department’s professors that he decided to pursue his bachelor’s degree in Biology at Texas A&M International University. It was during this time that he was given the opportunity to volunteer in Dr. Mandal’s organic chemistry lab, where he learned the basics of exploratory chemical research. He later engaged in research under the guidance of Dr. Michael Kidd and Dr. Ruby Ynalvez, the latter of whom inspired him to pursue his master’s degree at TAMIU. During this time, Jose worked as an undergraduate research assistant at the department of Biology and Chemistry. Jose completed his bachelors in the spring of 2014, and promptly registered for TAMIU’s master’s program in biology. At the start of his graduate program, Jose Angel worked at the University’s tutoring center as a biology tutor. Afterwards he was awarded two consecutive assistantships: first the GREAT assistantship, then the LBV assistantship. These assistantships provided him with the opportunity to teach multiple labs ranging from Survey of Life science, Cell Biology and Zoology. Jose plans to use this experience to pursue a doctorates degree in Mycology from the University of Texas at San Antonio (UTSA) with the goal of eventually returning to TAMIU as faculty. Jose has presented his research at multiple conferences around the United States, including the 2017 ASBMB conference in Chicago, and the 2016 NSRF conference in Galveston, Texas.