Development of Cadmium Bioassays using the Green Alga *Chlamydomonas reinhardtii*

Madhav L. Soowamber

A Thesis

In

The Department

Of Biology

Presented in the Partial Fulfillment of the Requirements

for the Degree of Masters of Science (Biology) at

Concordia University

Montreal, Quebec, Canada

November 2011

CONCORDIA UNIVERSITY

School of Graduate Studies

This is to certify that the thesis prepared

By: Madhav L. Soowamber

Entitled: Development of Cadmium bioassays using the green alga *Chlamydomonas reinhardtii*

Master of Science (Biology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final Examining Committee:

_____Chair
Dr. S. Dayanandan

_____External examiner
Dr. L. Varin

_____Examiner
Dr. P. Gulick

_____Examiner
Dr. R. Storms

_____Supervisor
Dr. W. Zerges

Approved by _____
Chair of Department or Graduate Program director

December 05, 2011 _____
Dean of Faculty

ABSTRACT

Development of Cadmium bioassays using the green alga *Chlamydomonas reinhardtii*

Madhav L. Soowamber

Toxic trace metals are major environmental pollutants due to their high toxicity at low concentrations, non-biodegradability, and accumulation in the food chain. Metals are important pollutants from metal mining, a major industry in Canada. Therefore, effective means of toxic trace metal quantification in natural water and soils are required for environmental risk assessment. Although analytical chemistry provides highly accurate and precise methods for quantifying metals, complete environmental risk assessment requires a determination of whether or not a metal is bioavailable, i.e. present in toxic or benign species. Of these toxic trace metals, our focus was on Cd as it is considered to be one of the most toxic heavy metals at sublethal concentrations. The absolute Cd concentration in natural waters does not necessarily reflect the concentration of a metal that is bioavailable. Therefore, analytical methods need to be complemented with bioassays (organisms that report the presence of bioavailable metal. This thesis contributes to the development of two types of cadmium (Cd) bioassays using the green alga *Chlamydomonas reinhardtii.* These bioassays are intended to detect trace amounts of the bioavailable species of cadmium (Cd). Previous global transcriptome studies by Simon et al. identified 10 Cd genes that are

induced by Cd^{2+}exposure at concentrations in micromolar range. We focused on a gene encoding a predicted amino acid transporter (*AOT4*) because it had the highest level of induction at low [Cd^{2+}] (1.2 µM). Reporter genes were constructed using the promoter region of this gene. Validation of the promoter region was based on Northern blot experiments. Another method used the technology of Panomics Inc. that allowed for direct quantitation of *AOT4* mRNA levels. I established 2 Panomics assays and optimized them so that they could be used with *C. reinhardtii*. The goal of these bioassays is to make them specific and respond to environmentally relevant Cd concentrations and allow use of sensitive, simple, portable equipment for *in situ* Cd measurements

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Zerges for his patience and constant guidance that have made this project and this thesis possible. I would also like to thank my committee members Dr Gulick and Dr Storms for their guidance.

I would also like to thank my colleagues, Jim Uniacke, Marco Schottkowski, Matthew Peters, James Dhaliwal and Zhe Yu for their support. A special thanks to Yu Zhan for helping in editing this thesis and for her constant support.

I would also like to thank my Guruji, my parents and my sister for their patience, prayers and constant support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AOT4:	Amino acid transporter
Cd^{2+}:	Free cadmium
Gsts:	Glutathione s-transferase
Gsx:	Glutathione peroxidase
HSK:	Housekeeping gene
MTs:	Metallothioneins
PC:	Phytochelatins
PCR-1:	Protochlorophyllde reductase 1

INTRODUCTION

1. Toxic trace metal pollution and toxicity

Metals are important environmental pollutants because they are not biodegradable, they are persistent, and they are toxic and accumulate in the food chain. Metals are released into the environment by diverse anthropogenic activities ranging from mining to textile production. Toxicological effects can be measured at levels of organization ranging from molecules to ecosystems.

Metals can be broadly divided into two classes: micronutrients (essential elements) and non-essential trace elements. The former includes: copper, manganese, iron, and zinc (for plants and animals); cobalt, chromium and selenium (animals); boron and (plants) (Jim Jian Wanga, 2004). Most of these are constituents of essential enzymes or proteins. Deficiencies occur when too little of these elements are obtained, whereas toxicity can arise above a certain threshold concentration. Iron and copper are essential but can have detrimental effects at high concentrations. For example Fe at low concentration is an enzymatic cofactor and a structural determinant in proteins (Yang et al., 2006), but at high concentrations it can induce lipid peroxidation (via formation of free radicals upon reaction with oxygen) in humans and has promoted ischemic myocardial injury in experimental animals (Salonen et al., 1992).

Metal speciation is also an important determinant of toxicity. Metals can occur in stable complexes by interacting with inorganic (*e.g*. sediments like $CaCO_3$) and organic ligands (*e.g*. proteins, humic acids), and particles (*e.g.* colloids, phytoplankton, bacteria).

The complexation of metals is dependent on physicochemical conditions such as pH, redox potential/oxidation state and the presence and concentration of complexing agents. These metal species may behave differently with respect to their mobility, lability and bioavailability and thus natural systems are under constant change and practically never reach true equilibrium (Simon et al., 2008). Computer programs have been designed to account for all these variables and are the only way one can predict what species are actually present in solution and how they are distributed.

2. Bioavailability

It is important to access both real and potential damage caused by metals to the environment (Fedorenkova et al., 2010). The total amount of metal in any sample can be precisely quantified by a host of methods including Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). These methods determine the total amount of metal but cannot determine the extent to which a metal is bioavailable. Determination of bioavailability must be made *in situ* (e.g. directly within a natural body of water) because of the above mentioned reasons i.e. metal speciation depends on the metal concentration, the presence of anions and chelators, pH/redox status and the presence of adsorbent sediments (Simon, 2009).

3. Desired features of a bioassay

The hallmarks of a bioassay should cater to the following, specificity to bioavailable metal and response to specific metal(s). The bioassay should not be

influenced by metals other than the one that it is targeted to measure. Sensitivity to environmentally relevant concentrations of the metals is another crucial facet of a bioassay. The bioassay should be able to measure environmentally relevant low and high metal concentration in a practical time frame. The bioassay should be practical to use in field situations for environmental monitoring. Since the ultimate goal of this project is measuring in situ levels of Cd^{2+}, the bioassay should usesimple, portable equipment and materials.

4. Problems with analytical methods of metal quantification

Analytical methods for quantifying metal speciation are well established. These include atomic absorption spectrometry (AAS), inductively coupled plasma-atomic electron spectrometry (ICP-AES), gas chromatography, X-ray absorption fine structure spectroscopy (XAFS), and sequential extraction procedure (SEP) (Caussy et al., 2003; Jain, 2000). These methods offer a powerful analysis when combined but require expensive, bulky equipment, high-grade analytical reagents, and specialized training (Caussy et al., 2003).

5. Biosensors

By definition, a biosensor is a device that uses the bioassay. A biosensor combines a biological component (the sensing element), which is responsible for the selectivity of the device, with a detection system (the transducer) for measuring the reaction of the biological component with the substance (analyte) being monitored

(Ramanathan, 1998). The biological component can be an enzyme, antibody, receptor or even whole cells; the biological reaction detected can be the activity of an enzyme, the binding of an analyte to an antibody or receptor, the induction of gene expression within cells or even cell death. Detection systems for measuring these biological events usually rely on electrochemical (Scott et al., 1997) or optical principles (Guzzo and DuBow, 1994; Si Shi-Hui, 1996).

Bacteria can be useful as biosensors for the detection of compounds of interest in the environment or other in samples (Yagi, 2007). The advantage of such biosensors over analytical methods e.g. atomic absorption spectroscopy may include high sensitivity, high specificity and measurement of the bioavailable portion of a sample as described above. As a result, bacterial detoxification systems have been used to produce some biosensors for use in the environmental area (Miller, 1999). In general, three types of bacterial biosensors may be distinguished. All three types use a reporter gene that is under the control of an appropriate promoter, which is what varies between the methods. The reporter gene produces a protein whose presence can be conveniently monitored. This protein could be an enzyme that produces a colored product (e.g chlorophyll, galactosidase), or a protein like luciferase that produces a flash of visible light. The presence of the signal from the protein produced by the reporter gene is an indication that transcription of the reporter gene has been turned on (Slauch and Silhavy, 1991).

If a reporter gene is expressed using a constitutive promoter in the absence of the toxin, the signal should increase exponentially express constitutively. However if a

toxin (e.g. metal) is added to the medium, the rate of increase should be reduced if cell growth and/or viability are reduced.

Another type of biosensor is one in which the reporter gene is controlled by a non-specific control element (Ramanathan et al., 1997). This could, for example, be a promoter that responds to heat shock or oxidative stress. Both of these responses can be elicited by exposure to heavy metals as well as organic contaminants, so increased expression of the reporter gene is a general indicator of stress rather than response to a specific compound.

Finally, the reporter gene may be put under the control of a very specific promoter that responds to only one or few compounds. An example of such a biosensor/bioassay places the firefly luciferase gene as a reporter under the control of the mercury-inducible *mer* promoter (Virta, 1995). The *mer* operon encodes resistance to mercury, which is a nonessential and toxic metal for bacteria. Firefly luciferase converts ATP, oxygen and luciferin to AMP, pyrophosphate, carbon dioxide, oxyferin and light. The latter can be detected using a luminometer or liquid scintillation counter (i.e. small signals can be detected). In the absence of mercuric ion (Hg^{2+}), *MerR* (Mercury Resistance Operon Repressor) bound to the *mer* promoter prevents transcription of the luciferase gene. In the presence of Hg^{2+}, MerR undergoes a conformational change that allows transcription to occur, so light is detected from luciferase activity. A signal is observed at 1 $x10^{-16}$ M Hg^{2+}, and a more or less linear response is observed up to about 10^{-6} M Hg^{2+}. Cadmium ion also produces a response but only above 1 µM (Virta, 1995).

This is a sensitive and specific biosensor that can be used for the detection of mercuric ion at low concentrations. Once again, in a sample like soil, only free Hg^{2+} would be detected, not bound Hg^{2+}.

6. Review of ecotoxicogenomics

General:

Ecotoxicogenomics is the study of gene and protein expression integrating transcriptomics, proteomics, and metabolomics into ecotoxicology (Snape et al., 2004). This area of study helps solving open questions that cannot be answered by standard ecotoxicity tests currently used in environmental risk assessment. A transcriptomic profile is a snapshot of the relative mRNA expression levels of a gene set, which can range from a few relevant genes to the entire genome (Neumann and Galvez, 2002). In a transcriptomic bioassay, a test organism is exposed to a contaminant solution and a select set of biomarker genes are monitored in order to quantify the effects of a toxicant of interest (Hutchins et al., 2010).

Advantages:

Ecotoxicogenomics can identify early responses i.e. before toxicity occurs and monitor bioavailable toxicant rather than its nominal concentration (Fedorenkova et al., 2010). Another principal advantage of transciptomic bioassays is that they can provide some insight into the test organism's physiological response(s) to stress (Hutchins et al., 2010) This is an advantage because it can provide information regarding the type of

stress that the organism is experiencing and, hence, assist in the identification of the toxicant.

7. General heavy metal tolerance mechanisms:

Active transport of metal out of the cell

Organisms use active transport mechanisms to export toxic metals across their cell membranes. Non-essential metals usually enter the cell through nutrient transport systems but their export is rapid. Such export systems can be ATPase or non-ATPase driven and are specific to the cation or anion being exported (Gaballa and Helmann, 2003). For example, Cd resistance is encoded by the *cad* operon in *S. aureus* and its export is ATPase dependent (Rensing et al., 1998).

Intracellular and Extracellular sequestration

An example of Intracellular sequestration is the accumulation of metals within cystene-rich proteins within the cytoplasm. This prevents exposure to essential cellular components. Metals that are subject to intracellular sequestration are usually Cd, Cu and Zn to mention a few (Clemens et al., 2002). For example Cd sequestration in *S. putida* produced low molecular weight cysteine-rich proteins (Trevors et al., 1986).

In plants, algae, bacteria, fungi and some members of the Archaea, the cell wall and biological membranes have important roles to play in regulating the toxicity of metals (Simon, 2009). The plasma membrane contains transporters for essential micronutrient metals but which also transport toxic trace metals into the cell and thereby play a role in metal toxicity. The cell wall of plants including that of algae has

the capacity to bind metal ions (Simon et al., 2008). For example, the cell wall of *C. reinhardtii* contains several soluble glycoproteins, especially hydroxyproline, different types of sugars (arabinose, manose, galactose and glucose) and a number of insoluble glycoproteins (Macfie and Welbourn, 2000; Simon et al., 2008) are capable of binding metal ions. Macfie and Welbourn (2000) showed that a mutant strain with a defective cell wall (*CW15*) is apparently more sensitive than the wild-type strain to Cd, Co, Cu and Ni suggesting that the cell wall plays an important role in metal tolerance (Macfie and Welbourn, 2000). On the other hand, Kola et al. showed that Cd internalization was not significantly different among the wild type and cell wall-less strain of *C. reinhardtii* (Kola and Wilkinson, 2005; Macfie and Welbourn, 2000).

Detoxification

One of the methods of metal detoxification that is relevant to this project is via complexation in vacuoles under essentially three forms: phytochelatins, polyphosphate bodies and transformed products through glutathione conjugation (Simon et al., 2008).

Glutathione S-transferases (GSTs) are most often thought of as detoxification enzymes since they catalyze the conjugation of glutathione to a variety of hydrophobic electrophilic and usually cytotoxic substrates. In phase I, a detoxification pathway is observed for electrophilic compounds in which enzymes such as cytochrome P450 are involved (Simon et al., 2008). In phase II, enzymes such as UDP:glucosyl transferase and GST use a functional group as a site for further conjugation that usually results in a less toxic and more soluble compound. Finally, in phase III (compartmentalisation),

ATP-dependent membrane pumps recognize and transfer the conjugates (*e.g.* herbicides and heavy metals) across membranes to be stored in vacuoles (Simon et al., 2008). Transport is selective for oxidized glutathione-conjugates (Lu et al., 1997).

8. General introduction of *Chlamydomonas reinhardtii*

C. reinhardtii is a unicellular eukaryotic alga and like most green alga, s in the *Plantae* phylum which included vascular plants but not many other photosynthetic organisms. For example red algae, glaucophyte algae, and many algal groups founded by secondary endosymbiosis) http://users.ugent.be/~fleliaer/publications/crps_2011 _html/evolution_green_algae.htm). *C. reinhardtii* is an organism of choice for us because it can be grown heterotrophically using acetate (as a carbon source) or phototrophically. Not only are these green algae easily grown in huge amounts on pure mineral medium but they harbor complex machinery for post-translational modifications like complex glycosylations, similar to higher plants (Fuhrmann et al., 1999). Also, it offers a system that is easily amenable to molecular manipulations and its genome has been sequenced and annotated (Merchant et al., 2007). Furthermore, *C. reinhardtii* is endogenous to fresh water bodies where heavy metal pollution is to be monitored.

9. Related heavy metal studies in *C. reinhardtii*

The effects of heavy metals on *C. reinhardtii* are well documented. For example, the growth, photosynthetic activity and chlorophyll content of *C. reinhardtii* have been

shown to be affected by cadmium and copper (Chugh L.K., 1999). It has also been shown that these changes could be due to the cell wall of the organism. In fact, the cell wall of *C. reinhardtii* displays a high affinity for such metallic cations (Macfie and Welbourn, 2000). For example, cell wall deficient strains are consistently more sensitive to cadmium, copper, nickel and cobalt than are walled strains (Boswell et al., 2002; Collard and Matagne, 1990). As a side note, Cd, Cu and Zn inhibit nitrate uptake by the cells (Devriese M, 2001).

Gekeler *et al*. first demonstrated the occurrence of phytochelatins (PC) in *C. reinhardtii* (Gekeler W, 1989). Howe & Merchant, and Hu *et al*. both showed that PC are the major intracellular metal-chelators and they are induced upon cadmium treatments. PC complexes can sequester up to 70% of the total cadmium found in cadmium-treated cells (Howe and Merchant, 1992; Hu S, 2001). Glutathione (GSH) was also shown to be the principal compound induced after exposure of cells to mercury (Howe and Merchant, 1992).

Hu *et al*. further characterized PCs. They found two types of PC-Cd complexes namely the acid labile sulfide-containing high molecular weight (HMW) complexes and the low molecular weight (LMW) complexes (Hu S, 2001). LMW complexes are rapidly converted in HMW complexes that accumulate into the cells and contribute to a stable cadmium sequestration. LMW complexes only accumulate after prolonged cadmium exposures.

Two thioredoxin genes (TRX), encoding isoforms *m* and *h*, located in the chloroplast and the cytosol, respectively have been characterized (Rochaix et al., 1998).

The transcriptional expression of both genes is stimulated by cadmium and mercury, but in a different manner. Relevant *cis*-acting elements and protein accumulation are only observed for TRX h. Moreover, heavy metals inactivate TRXs, presumably by binding to their dithiol active site. Lemaire et al. also suggested that thioredoxins (TRXs) may contribute to heavy metal detoxification in *C. reinhardtii* via ROS detoxification. These studies suggest a possible implication of TRXs in heavy metal detoxification with a different regulation pattern for each TRX (m and h) (Lemaire et al., 2002).

10. Potential of using *C. reinhardtii* as a biosensor for toxic trace metals

C. reinhardtii is endogenous to fresh water where heavy metal pollution is to be monitored. It has high robustness and tolerance to environmental conditions, a high growth rate and the potential to be cultured easily at a low cost (Fletcher et al., 2007). The low number of cells used in the context of a bioassay would not detoxify the medium.

11. Reporter genes used in *C. reinhardtii*

Methods used to circumvent silencing

Although this alga is easily transformable, the low efficiency of transgene expression from the *C. reinhardtii* nuclear genome has severely held back functional genomics research. For example, poor transgene expression is held responsible for the lack of sensitive reporter genes to monitor gene expression *in vivo*, analyze subcellular protein localization or study protein–protein interactions (Shao and Bock, 2008).

The molecular reasons for the very low efficiency of transgene expression in *C. reinhardtii* are not understood. Possible mechanisms include the presence of non-conventional epigenetic suppression activities and/or an exceptionally compact chromatin structure that does not permit active transcription of transgenes (Neupert et al., 2009).

In an effort to find ways to improve foreign gene expression in *C. reinhardtii*, Klein et al showed that there seem to be enhancer like sequences in the region between the promoter and the protein coding region of the exogenous genes that helped in the expression of these foreign gene (bacterial uidA (GUS) gene) in *C. reinhardtii*, hence the use of the 5' UTR (Klein et al., 1994). Addition of the 5' UTR allowed the foreign gene to be expressed at the same levels as an exogenous gene. Klein et al. demonstrated this effect using the *rbcL* gene promoter fused to the coding sequence of the bacterial *uidA* gene, encoding 3-glucuronidase. This result was further supported by Salvador et al who showed that regions in the 5' UTR affect mRNA stability positively and their absence (verified by deletion mutants) affected the half-life of mRNA (Salvador et al., 2004). A research article that complements to some extent these prior findings is one by Barnes that uses 5 different constructs (5 promoters of chloroplast genes of *C. reinhardtii*) harboring GFP (Barnes et al., 2005). Results of his experiments showed that even though tweaking the 5' UTR helped in the expression of the foreign gene, the levels of expression was not as high as endogenous gene expression. The 3' UTR did not play a significant role in foreign gene expression.

There have been several experimental attempts to reduce silencing of transgenes. Codon bias was changed in a foreign gene to favor codons found in the *C. reinhardtii* nuclear genome. Work done by Fuhrmann showed that making a GFP (green fluorescent protein) gene that matched the *C. reinhardtii* nuclear codon bias (GC rich), increased GFP expression levels over forms of GFP that did not use a codon bias (Fuhrmann, 2004). Another way of countering silencing of exogenous genes is by the introduction of introns in the foreign gene sequence (Lumbreras, 1998). Lumbreras et al showed that by including a short region (200-300 base pairs) upstream of a promoter of RBCS2 in addition to the first intron of RBCS2 within his gene of interest*, ble*, increased expression of the latter. The presence or absence of the 3' UTR of RBCS2 did not seem to affect expression of *ble*. Nevertheless, there are only a couple of research articles describing the use of a reporter gene in *C. reinhardtii*, even though many claim to have developed one.

The bias of special codons (Fuhrmann et al., 1999), methylation of exogenous genes and potent RNA interference have been stumbling blocks in the way towards the expression of exogenous genes in *C. reinhardtii*. Wu JX et al. point to the fact that study on expressing exogenous genes in *C. reinhardtii* is still in its early stages (Wu JX, 2008).

12. Development of methods for the expression of GFP (green fluorescence protein) has been used as a reporter gene in *C. reinhardtii*.

Fuhrman and Hegemann synthesized a gene encoding green fluorescent protein for expression in *C. reinhardtii* (*cgfp)* (Fuhrmann et al., 1999). After fusion of *cgfp* with

ble (confers resistance to the drug zeomycin), the Ble-GFP fusion protein accumulated in nucleus. This finding indicates that that GFP may be a useful reporter for the analysis on gene expression in *C. reinhardtii*.

13. Specific aims of this thesis

Development of a bioassay for bioavailable Cd

Previous global transcriptome analysis of *C. reinhardtii* exposed to Cd^{2+} identified 10 genes that are induced by Cd^{2+} concentrations ranging from 7.8 nM to 9.0 µM (Simon et al.). These concentrations were shown previously to have slight, but measurable effects on the growth rate of *C. reinhardtii* in culture and to be more than two orders of magnitude below lethal concentrations (Macfie and Welbourn, 2000). Of these 10 genes, we focused on the *AOT4* gene encoding a putative amino acid transporter because it had a level of induction at 42 fold, the highest induction recorded among the aforementioned 10 genes.

14. Construction of reporter genes with the *AOT-4* promoter fused to genes encoding luciferase and protochlorophyllide reductase (*pcr-1*)

The *C. reinhardtii* genome database was used to locate the 5' flanking region of *AOT4.* This region is likely to contain the *AOT4* promoter region. A previous construct harboring the luciferase gene driven by a tandem promoter of Hsp70/Rbcs2 was used as a backbone to generate our *AOT4-luc* construct. The goal was to use the promoter region of *AOT4* to replace the original tandem promoter so that the luciferase gene

would be under the control of a Cd inducible promoter. Transformant strains with this reporter construct integrated in the genome are expected to increase luciferase expression and bioluminescence in response to exposure to Cd. Bioluminescence should therefore serve as an indicator of bioavailable Cd. However certain disadvantages might be expected. First, luciferase expression must be measured on the basis of its bioluminescence soon after it synthesis as there is possibility of having the signal degrade over time. In field tests of potentially contaminated water, this activity would be difficult to quantify *in situ* or later in the laboratory due to the possible short half life of the bioluminescent signal. Therefore it was necessary to explore other reporter genes.

The gene encoding an enzyme in chlorophyll biosynthesis, protochlorophylide oxidoreductase (POR), *PCR-1*, is expected to have a few advantages over luciferase. First off, it is an endogenous gene so should be able to escape silencing. Second, chlorophyll is relatively stable and therefore it can serve a signal that accumulates during a bioassay, survive during transport back to the lab, and it can be easily quantified using spectrophotometry or fluorimetry. Once chlorophyll is produced it can be preserved and easily measured by spectrophotometric or fluorescence methods. There may be some disadvantages however. First, there may not be a linear relationship between the expression level of *PCR-1* and the synthesis of chlorophyll. For example, a trace amount of the enzyme may be sufficient to give a wild-type phenotype. Validation of *PCR-1* would be necessary to establish it as a useful reporter gene.

It should be noted that there are two POR enzymes in *C. reinhardtii*. The POR encoded by *PCR-1* has a light-dependent activity. There also exists a light independent POR, as is the case in many plants (e.g. pine trees). In chlorophyll biosynthesis, protochlorophyllide (PchI) needs to be reduced to chlorophyllide (ChI) through the light dependent protochlorophyllide oxidoreductase (LDPOR) or the light independent protochlorphyllide oxidoreductase (LIPOR). In *C. reinhardtii* LDPOR and LIPOR can reduce PchI to ChI. In *C. reinhardtii* the pc-1 phenotype results in non-functional LDPOR. Without LDPOR, *C. reinhardtii* is sensitive to light (Si Shi-Hui, 1996). Also the LIPOR needs to be absent, so that the effects of deficiency for the LDPOR can be detected. LIPOR has the ability to reduce PchI to chlorophyll in the dark. This assay will therefore require the use of double mutant strains for both enzymes. These strains were generated by previous members of the laboratory, and carry the *pc-1* mutation as well as the mutation *y7* which abolishes LIPOR (the light independent POR). Transforming *C. reinhardtii* that has these mutations (*pc-1, y7* and *CW 15*) with the *PCR-1* gene results in complementation of the POR enzyme. In other words, Cd^{2+} should induce the AOT4-PCR1 expression and therefore chlorophyll synthesis should be enhanced in the presence of the metal.

15. *AOT4* mRNA quantification with state-of-the-art assays from Afimetrix (Quantigene)

We also tried another bioassay using the Affymetrix Quantigene approach that allows for direct quantification of the mRNA levels of *AOT4* after Cd exposure. This method is similar to an ELISA in the sense that the mRNA of interest is anchored in a

96-well plate using specific probes and quantified using luminescence generated form an enzyme-substrate (bound to our mRNA of interest) reaction (http://www.panomics.com/index.php?id=product_1). This method is robust as it requires minimal sample preparation i.e. *C. reinhardtii* cells are lysed and the crude extract containing a population of mRNAs is directly added to the assay plate (no RNA purification, reverse transcription or enzymatic steps in amplification are needed). As opposed to the luciferase signal, the response is preserved because mRNAs in this homogenization buffer are stable and can be quantified at any time back in the lab. In other words, transcripts are stable in the lysis buffer and can serve as a stable record. This assay is also easy to optimize as a set of internal controls facilitate data analysis. The manufacturers claim that differences in as small as 5% can be quantified and that Quantigene probes sets give a >99% success rate on the first design (http://www.panomics.com/index.php?id=product_1). This hold true in our case as the all the probes ordered for AOT4 gave quantifiable results. This system is also amenable to high throughput analyses, which would be useful for processing a large number of samples and studying more than one gene expression pattern.

The main disadvantage of the Affymetrix Quantigene approach is the cost. Based on our experience with this system, the cost of each plate easily exceeds $500 and the probes specific to our mRNA of interest cost in the excess of $200 per gene. It also would be difficult to immediately obtain results while still in the field because a great deal of laboratory equipment is required for sample processing.

MATERIALS AND METHODS

1. *C. reinhardtii* strains and culture conditions

C. reinhardtii strains were cultured on Tris-acetate-phosphate medium (TAP) (Gorman and Levine) under dim light (50 µE cm^{-2} sec^{-1}) with orbital shaking. Cultures were prepared for Cd exposure by centrifuging at 3,300 *g* for 7 min prior to resuspending the cells in a 0.25X TAP medium without trace metals. This was performed three times. Following the third sedimentation by centrifugation, the cell pellet was resuspended in 0.25X TAP medium without trace metals to a density of approximately 6.4 X 10^5 cells/ml (cell density was calculated using a hemacytometer). Cadmium nitrate was then added to a final concentration of 11.6 µM (after time 0) and aliquots taken at times 0, 1, 2 and 4 hours.

2. Cadmium concentrations and speciation

Glassware and other vessels were washed in 0.1 M HCl to avoid metal contamination. To ensure a stable concentration of free metal i.e. to avoid metal depletion by uptake by the organism or metal complexation by exudate production (Simon, 2008), exposure solutions were buffered with 5 $X10^{-4}$ M citrate. The concentration of cadmium nitrate used was 11.6 µM and that correlated to a free Cd concentration of 1.2 µM (Simon, 2008). This calculation was done using the program MINTEQ which is a free chemical equilibrium software for calculation of metal speciation.

3. RNA and Poly(A)$^+$ RNA Extraction from *C. reinhardtii*

The wild-type strain (C137) was cultured to a density of 5-6 $\times 10^6$ cells/ml and diluted to 6.4 X 10^5 cell/ml after washing as described above. Aliquots of 1 litre (from a 4 litre culture) were taken at times 0, 1, 2 and 4 hours. The aliquots were centrifuged at 3500 g for 5 minutes. Cell pellets were suspended in 1 ml TE (10 mM Tris-Cl pH8, 1 mM EDTA pH8) and transferred to 1.5 ml microfuge tubes. Cells were pelleted again by centrifugation at 2500 g for 5 min and stored at -80 °C. To isolate the RNA, 0.5-0.6 ml Tri-Reagent (Sigma) was added to the frozen pellet of cells and the resulting mixture was vortexed 2 times for 15 s on the highest setting. Sterile glass beads (equivalent to 70-80 mg, 0.5mm in size) were added to this mixture, which was then vortexed for 15 s, followed by incubation at room temperature for 5 min. Samples were then centrifuged at 18500 g for 5 min and maintained at room temperature for an additional 5 min. To each sample, 0.1 ml chloroform was added. Samples were vortexed and maintained at room temperature for 5 min. Samples were then centrifuged at 14 k rpm for 5 minutes and aqueous phase was extracted with a mixture of phenol-chloroform-isoamyl alcohol (24:24:1) and then extracted twice with chloroform-isoamyl alcohol (24:1). Sodium acetate was added to a final concentration between 0.3-0.5 M to the aqueous phase,. One volume of isopropanol was added to the samples. The samples were vortexed and maintained at -80℃ for 30 min. They were then centrifuged at 18500 g for 10 min and the RNA pellet was washed with 75% ethanol. The RNA pellet was resuspended in 20 µl of DEPC-treated HPLC grade H_2O and stored at -80°C. Total RNA concentrations were

determined by measuring absorbance at 260 nm (Sambrook and Russell, 2001). Poly(A)$^+$ RNA was isolated from total cellular RNA using the streptavidin-biotin-magnetic-bead technique provided in the Promega PolyAtract kit (Madison, Wis.) according to the manufacturer's instructions. RNA quality and quantity were estimated from the measured absorbance at 280 and 260 nm and by analysis of the intactness of rRNA species with an Agilent 2100 BioAnalyzer (Agilent Technologies, Foster City, CA, USA).

4. Northern Blot Hybridization

Poly(A)$^+$ RNA (10 μg) in sample buffer (16.5% formaldehyde, 46.2% ultrapure formamide) was loaded into each lane of a 1% agarose gel containing 16.5% formaldehyde in 50 mM HEPES [pH7.5] and 10mM EDTA [pH8.0] and electrophoresed. The RNA was transferred to positive charged nylon membrane (Zeta-Probe Blotting Membranes, Bio-Rad) using upward capillary transfer method (Sambrook and Russel, 2001) and fixed on the membranes using a UV crosslinker (Stratlinker). For the probe for the specific mRNAs, purified DNA fragment complementary to mRNAs were labeled with [α-^{32}P] dCTP (Perkin Elmer) using the random priming method and purified from unincorporated [α-^{32}P] dCTP using a G-50 spin-column (Sambrook and Russel, 2001). The Northern blot membranes were pre-hybridized for 1-1.5 h in pre-hybridization buffer (50% formamide, 7% SDS, 0.12M Na_2HPO_4, 0.25M NaCl) at 65°C and hybridized with the ^{32}P-labeled probes for 16-24 h at 42°C. After hybridization, the blots were washed twice (10 min each) in 2X Saline Sodium Citrate (SSC) with 1% Sodium Dodecyl Sulfate (SDS) at room temperature then washed twice (10 minutes each) with 0.2X SSC

and 0.1% SDS at 65°C. The membranes were exposed to a phosphor-imager screen (GE Healthcare) for at least 4 h and the autoradiographs were revealed with the Typhoon phosphor-image analyzer (GE Healthcare).

5. Construction of the Luciferase (*luc)* reporter plasmid

The plasmid pRbcBRL (HSP196) was obtained from Dr Christoph Beck (University of Frieburg). To generate our *luc* plasmid, the Hsp70A promoter of pRbcBRL(HSP196) was excised by digestion with the flanking enzymes *Sac* I and *Xho* I and replaced by the *AOT4* promoter region flanked by the same restriction sites. The *AOT4* promoter region was generated from the BAC BTQ7679 using the primers fwd *Sac* I-AOT4: 5`CCGGAGCTC GCGCTCTACTACCACCAAATG3` and rev *Xho* I-AOT4:5'AAACTCGAGTCCGTCAGTCATTT GTAAAAC 3' by PCR. PCR amplification (GeneAmp 2400 PCR machine, Perkin Elmer) was performed using the following temperature cycles: 2 min at 94^{o}C; 1 min at 94^{o}C, 60 s at 52^{o}C, and 60 s at 72^{o}C for 23 cycles; and 7 min at 72^{o}C.

6. Construction of the *PCR-1* fusion plasmid

The promoter region of the protochlorophyllide gene (*PCR-1*) and part of its coding region was excised using the enzymes *Sac* I and *Sgf* I from a plasmid (B4) containing full length *PCR-1*. The promoter region of *Aot4* and part of the *PCR-1* coding region that would be used to replace the excised fragment were generated using fusion PCR as follows. The promoter region of *AOT4* was generated using the primers fwd *Sac* I-AOT4: 5`CCGGAGCTCGCGCTCTACTACCACCAAATG3' and Rev pcr1-ATG-Aot4: 5'TGGTGAGGGC CATTTGTAAAACAGTGCAAATAAGCGTGA 3' and the same BAC as above as template. This

gave a PCR product containing the promoter region of *AOT4* with a 10 bp overhang complementary to 10 bp after the ATG of the *PCR1* gene. PCR Amplification (GeneAmp 2400 PCR machine, Perkin Elmer) was performed using the following temperature cycles: 2 min at 94^{o}C; 1 min at 94^{o}C, 60 s at 52^{o}C, and 60 s at 72^{o}C for 23 cycles; and 7 min at 72^{o}C.

Another PCR reaction using the following primers fwd Aot4-ATG-Pcr1: 5'TG TTTTACAAATGGCCCTCACCATGTCCGCC3' and Rev *EcoR*I-*Sgf*I-Pcr1: 5'CCGGAATTCGCGAT CGCGTTGGGCTTGAA3' and the B4 plasmid as template generated a PCR product that had part of the coding region of pcr-1 with a 10 bp overhang before its ATG that was complementary to 10 bp in the *AOT4* promoter region. PCR Amplification (GeneAmp 2400 PCR machine, Perkin Elmer) was performed using the following temperature cycles: 5 min at 94^{o}C; 30 s at 94^{o}C, 30 s at 61^{o}C, 45 s at 72^{o}C for 23 cycles; and 7 min at 72^{o}C. 8% DMSO in the reaction mix was crucial for the reaction to occur.

Using these two individual fragments as template, another PCR reaction was performed to fuse them using the end primers; fwd *Sac*I-AOT4: 5`CCGGAGCTCGC GCTCTACTACCACCAAATG3' and Rev *EcoR*I-*Sgf*I-Pcr1: 5'CCGGAATTCGCGATCGCGTTGGG CTTGAA3'. PCR Amplification (GeneAmp 2400 PCR machine, Perkin Elmer) was performed using the following temperature cycles: 3 min at 94^{o}C; 30 s at 94^{o}C, 45 s at 56^{o}C and 45 s at 72^{o}C for 23 cycles; and 7 min at 72^{o}C.

7. *C. reinhardtii* transformation with *luc* plasmid and *PCR-1* fusion plasmid

C. reinhardtii strain, CC 503 (cw92 cell wall mutant) transformations were performed using the glass bead method (Kindle). For transformation with the *luc* plasmid, the aforementioned *C. reinhardtii* strain was used and for the *PCR-1* plasmid, the *C. reinhardtii* mutant strain #6 (*pc1: y7: cw15: arg7)* was used. In both cases, cells were cultured to 1-2 X 10^6 cells/ml and pelleted at 2500 g for 5 minutes and centrifugation was stopped without braking. The pellet was gently resuspended for 20 minutes in TAP+R+2% D-sorbitol to a concentration of 5 X 10^8 cell/ml. 300 µl of the resuspended cells were added in a tube containing 300 mg of 0.5 mm diameter sterile (sterilized by baking for 4-6 hours) glass beads along with 100 µl of 20% PEG and 1.5 µg of linearized plasmid. The tube was vortexed immediately at high speed for 15 seconds. 2.5 ml of in TAP+R+2% D-sorbitol were added to the transformed cells and placed in the 24^{o}C incubator with shaking (140 rpm) for 5 hours. For strain #6, this incubation step was done in the dark. The cells were pelleted at 2500 g for 10 min. Most of the supernatant was discarded, with a remaining 300 µl used to resuspend the pellet for plating on a TAP-R medium in a petri plate with the selectable antibiotic zeocin (Invitrogen) and incubated at 24^{o}C. For strain #6, this incubation step was done in the dark (i.e plates were wrapped in aluminum foil). The concentration of zeocin was titrated (ranging from 2.5 µg zeocin/ml to 10 µg zeocin/ml). The ideal concentration of zeocin (a concentration that was enough for selection but not lethal) was found to be 5 µg/ml.

8. Luminescence Assay

The luciferase assay was performed using the *Renilla* Luciferase Assay System (Promega, cat. E2820) according to the manufacturer's protocol. Frozen cell pellets (from Cd exposed cultures) were resuspended in 40 µl of 1X lysis buffer, incubated at room temperature for 15 min on a rotary shaker (750 rpm) and then incubated on ice until the assay. Since luciferase activity decays during the assay, with a half-life of approximately 3 min, the assay was performed on 2 samples at a time. 5 µl of each lysate was added to 25 µl of assay buffer in Optiplate 96-well plates (Perkin-Elmer cat. 6007290), supplemented with 1X coelenterazine (Gold biotechnology), at room temperature, mixed for 3 s and luminescence was recorded for 2 s using a Wallac Victor2 1420 Multilabel Microplate Reader (Perkin-Elmer). Two biological replicates were performed using independent cultures.

9. Panomics Quantigene Assay

After Cd^{2+} exposure, cells were pelleted at 14,000 rpm for 10 min and resuspended in 300 µL of QuantiGene Homogenizing Solution (Panomics, cat#QG0516) and frozen at -80^{o}C. Cell lysis was performed using a slow freeze-thaw method i.e. freeze to -80^{o}C and thaw to room temperature (repeated twice for a total of three times). 3 µl of Proteinase K (50 ug/µL, Panomics, Cat# QS0511) was added to each thawed sample and incubated at 65^{o}C for 30 min. Every 10 min during this incubation, the sample was vortexed at maximum speed for 15 seconds. After this lysis step, the lysis mixture was centrifuged and the supernatant was used for the subsequent assay or stored at -80^{o}C. The assay was performed according to the manufacturer’s protocol.

RESULTS

1. Localization of the promoter region of *AOT4*:

Two goals of this long-term project were to develop a new reporter gene for use in *C. reinhardtii* and to use this reporter gene for the development of a bioassay for toxic trace metals in the environment. I opted to use the promoter region of the *AOT4* gene because; AOT4 is the gene that is most strongly upregulated following exposure to Cd at concentrations in the micromolar range and *AOT4* expression responds to environmentally relevant Cd concentrations (Simon et al). Figure 1 shows the gene model for *AOT4* on the genomic DNA sequence in the JGI Version 3 database of *C. reinhardtii*. *AOT4* is the putative amino acid transporter-based on the results of Blast searches by annotators of the database (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). In the database, it was found that *AOT4* has an expressed sequence tag (EST) that also includes an upstream gene (name not given on database). I tentatively named this gene *AOT4-1* and the originally annotated gene *AOT4-2*. Since the basis of this research lies in knowing the regulatory region of *AOT4*, it is important to know the location of its promoter i.e. whether *AOT4* actually includes the upstream *AOT4-1* gene, or not. Therefore, I carried out sequence comparisons and Northern blot experiments .

One of our reasons for hypothesizing that *AOT4-1* should be considered as part of the *AOT4* gene is based on sequence analyses of its predicted polypeptide product. It has been found that *AOT4-2* is most similar to two membrane-spanning amino acid

transporters in *Saccharomyces cerevisiae* (*AVT1* and *AVT4). AVT1* is required for the vacuolar uptake of large neutral amino acids including tyrosine, glutamine, asparagine, isoleucine, and leucine. *AVT4* is involved in amino acid efflux from the vacuole and is one of the first (along with *AVT3* and *AVT6*) neutral amino acid transporter to be shown to transport compounds from the lumen of an acidic intracellular organelle (Russnak et al., 2001). *AOT4-1* has sequence similarity to the amino terminal region of these (*AVT1*, *AVT3*, *AVT4* and *AVT6*) particular yeast transporters. For example, *AOT4-2* has 10 transmembrane domains like *AVT1* (and all transporters of this class); however, the AVT transporter family also has extended N-terminal regions lacking trans-membrane domains. Although similarity between *AOT4-1* and the N-terminal domain of the *AVT* family of proteins was not identified in BLAST searches, alignments with ClustalW revealed several regions of similar amino acid sequences (Simon, 2009). These regions of similarity support the case for *AOT4-1* encoding the N-terminal portion of the *AOT4* protein and therefore suggest that the *AOT4* promoter region is located upsteam of the AOT4-1 expressed sequence tag (EST). Additional evidence is provided in the next subsection.

A third reason that strengthens our choice of location for the promoter region of *AOT4* is that both *AOT4-1* and *AOT4-2* are metal regulated. This is described below where the induction of these genes is monitored via Northern blot analysis and Panomics assays.

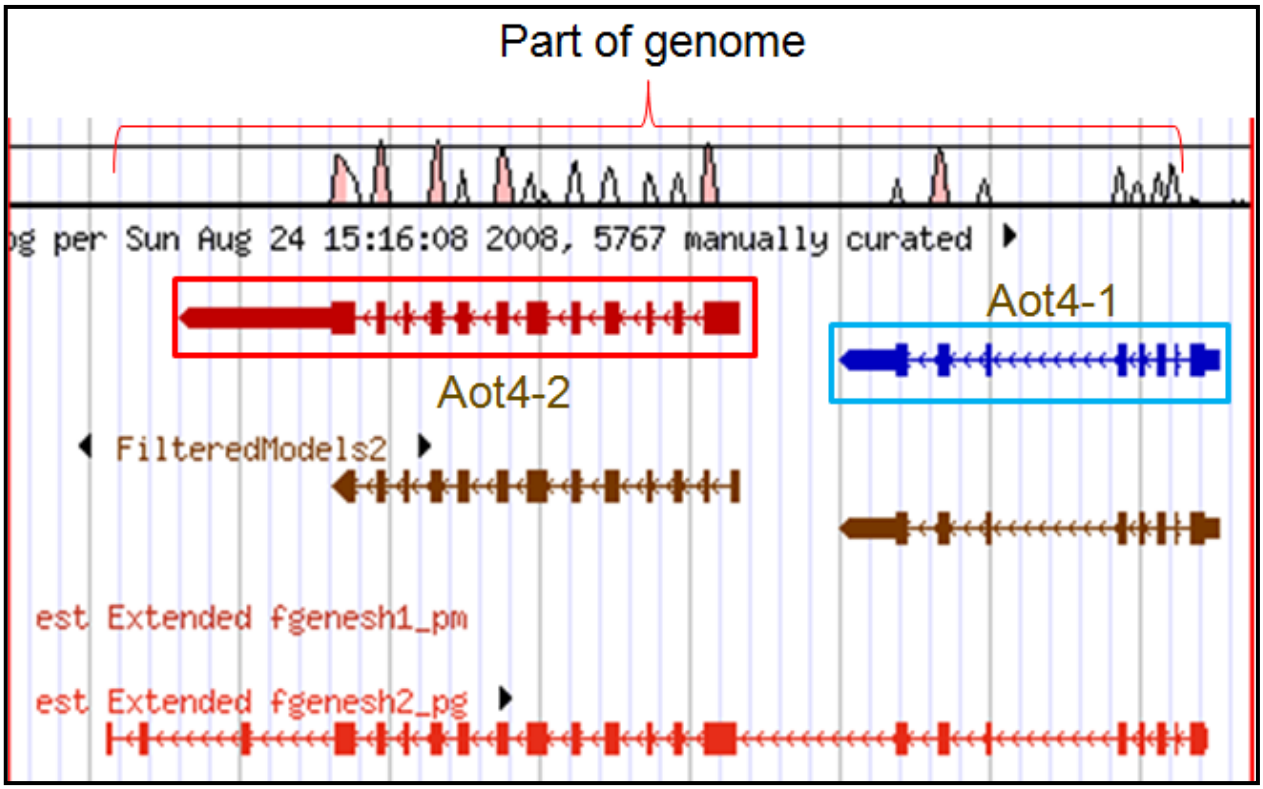

Figure 1. Gene model taken from the *C. reinhardtii* database showing the location of the components of *AOT4* (*AOT4-1 in red box* and *AOT4-2 in blue box*) and the EST that links them. The peaks at the top represent possible location of exons on the 2 genes. The distance between the darker grey vertical lines is 10000 base positions.

2. Northern blot analysis of *AOT4* transcripts:

Northern blot analyses were carried out in order to address two questions regarding *AOT4* which are important for its use as a Cd biomarker. The first question was if a common transcript is encoded by *AOT4-1* and *AOT4-2* in support of their constituting one gene as described in the previous subsection. In this case at least one transcript with the approximate size of their predicted transcripts combined should be detected. Second, transcripts encoded by *AOT-2* and any other transcripts encoded by *AOT4-1* should be induced by exposure to Cd. Simon et al. had found that the [Cd^{2+}] that showed the highest *AOT4* induction was 1.2 μM free Cd^{2+}, this metal concentration was used to perform a time course on a culture of *C. reinhardtii.* Poly $(A)^{+}$ RNA was subjected to Northern blot analyses and then probed separately with DNA corresponding to either *AOT4-1* or *AOT4-2*. As it can be seen in Figure 2, two transcript species were detected by both probes. The size of the largest transcript is approximately the expected size of a single transcription unit encoded by a single *AOT4* transcription unit, i.e. from both *AOT4-1* and *AOT4-2.* This corresponds to 2200 base pairs determined by the addition of the predicted transcript sizes of *AOT4-1* and *AOT4-2* obtained from the *C. reinhardtii* database (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). Therefore, the presence of large transcript species suggests that *AOT4* is encoded by the entire region that I have been presenting as *AOT4-1* and *AOT4-2*.

On the other hand, smaller transcript species were also detected with both the *AOT4-1* and *AOT4-2* probes. The smaller transcripts of varying lengths detected using

the *AOT4-1* probe could be due to differential splicing or the existence of transcripts from orthlogous genes whose mRNAs have complementarity to the probes used here. The *AOT4-2* probe picks up some transcripts but these do not precisely comigrate with those transcribed from *AOT4-1*.

The second prediction made by my hypothesis that *AOT4-1* and *-2* constitute a single transcription unit is that one or more transcripts detected with the *AOT4-1* probe should increase in abundance in response to exposure to Cd^{2+}. Transcripts levels from the *AOT4-1* gene are induced by exposure of cells to Cd^{2+} and this supports the hypothesis that AOT4-1 and AOT4-2 are regulated by the same control region. It is therefore possible that AOT4 encompasses the AOT4-1 and AOT4-2 regions but further work is required to further verify if my hypothesis is correct.

As transcriptional regulatory elements are generally in 5' flanking regions of genes, I used the intergenic region between *AOT4-1* and the neighbouring gene as the Cd-induced promoter in the reporter gene constructs that are described in below.

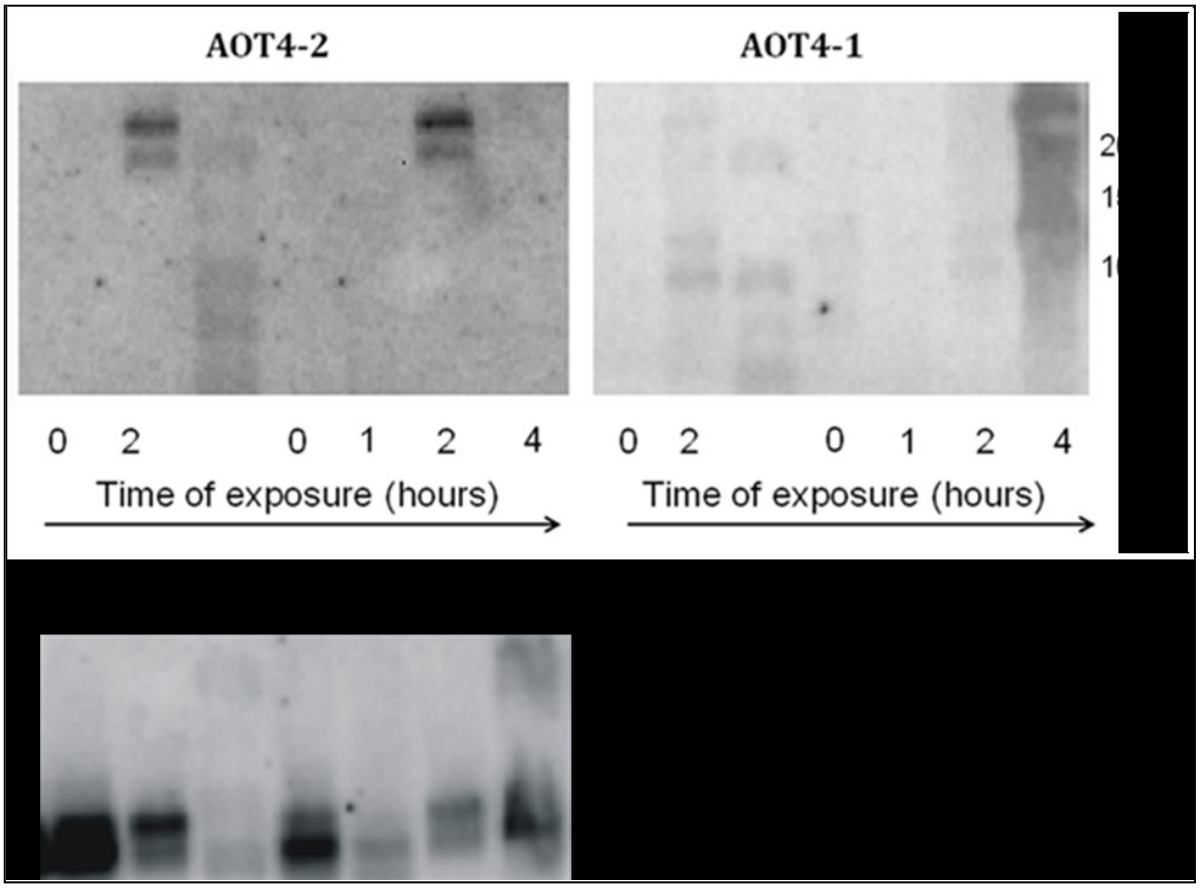

Figure 2. Panels show poly(A)$^+$ RNA isolated from a *C. reinhardtii* culture exposed to free 1.2 µM Cd^{2+} and the poly(A)$^+$ RNA for either 0, 1, 2 or 4 h. The panel on the left shows the results of probing with a genomic fragment corresponding to *AOT4-2* and the panel on the right shows the results of probing with a fragment corresponding to *AOT4-1*. On the right side of right panel, there are size markers i.e 2000 bases 1500 bases and 1000 bases. The panel on the bottom is an RNA loading control. The level of the LHC (light harvesting complex) transcript was used as that control.

3. The Quantigene Multiplex assay of *AOT4*

Another metal bioassay using *C. reinhardtii* could be based on measurements of the levels of endogenous mRNAs that are upregulated by metal exposure. This would have several advantages over reporter genes for in situ measurements (In exotoxicology, "in situ" means that the measurement is carried out in the environment and not in the laboratory). First, in contrast to the metal-regulated reporter gene assay, which measures one promoter's response, this *in situ* system can measure expression levels of a set of genes that have been selected to give the most accurate transcriptomic signature pattern for a particular metal. Second, in contrast to reporter gene responses, which require monitoring the activity of the protein product soon after exposure (to avoid errors via protein degradation), the mRNA of a Cd-induced gene in such an *in situ* bioassay is chemically stable (due to the homogenization buffer it is stored in) and therefore obtained in the field and then measured later in the laboratory. In other words, Luciferase or GFP can by precisely quantified but it requires monitoring in cells or fresh lysates under controlled laboratory conditions. Their activity is labile in lysates and transport storage conditions. Also more time is required to enhance the level of a protein than an mRNA. Third, transgenic *C. reinhardtii* strains are not required for a transcritomics-based bioassay and therefore there is no issue of their introduction into the environment. This assay also requires very few cells. This is advantageous because the algal cells can change the concentration of metal, either by adsorption or uptake, and thereby introduce inaccuracy (Simon et al., 2008). Use of a very low cell density could therefore be used in the test exposures, and yet provide sufficient total RNA for

analyses. Finally, the steps for extraction of the RNA for this assay are simple; one simply resuspends cells in a homogenization buffer sold by Affimetrix and after a few centrifugation, vortexting and freeze-thaw steps, the RNA is obtained. Unfortunately, the composition of this buffer is not given by the company and, therefore, could not be provided here. The principle of the Quantigene assay is similar to an ELISA (Enzyme-linked immunosorbent assay). I will describe how the singleplex assay works and then the multiplex assay. For the singleplex assay, Quanitgene2, a capture plate and oligos are used to isolate our mRNA of interest. The oligos are made up of two regions, one that recognizes our target mRNA from a complex pool of mRNA and the other that hybridizes to the inner surface of the capture plate. After an overnight incubation period and a few washes to remove unbound mRNAs, signal amplification is performed via sequential hybridization of 3 probes. The last hybridized probe has an enzyme attached to it. Subsequent addition of a substrate generates a luminescent signal that is proportional to the amount of target mRNA present in all the cells in each well. The signal is measured and quantified by a luminometer. In contrast to qPCR, which uses an enzymatic amplification, this assay amplifies the signal by labeling the analyte mRNA with a "Christmas tree" of inter-hybridized oligonucleotides attached to fluorescent dye molecules. This gives a signal that is directly proportional to the amount of the analyte mRNA.

The basis remains the same for the multiplex assay (Quantigene 1) but instead of using one probe per well; this system utilizes fluorescent microspheres as a support to capture the mRNA species. In other words, each microsphere recognizes and anchors a

different target mRNA based on the specific probes attached to its surface. This is then visualized through the addition of Streptavidin-conjugated Phycoerythrin and the resulting fluorescence signal is proportional to the amount of mRNA captured on the bead surface by the target-specific probe set. The mRNA signal is quantified in a machine that is essentially a Fluorescence-activated cell-sorting machine (FACS). The mRNA associated with each microsphere being analyzed is based on the signature ratio of fluorescent dyes within it.

The first encounter we had with the Quantigene 1 assay was via an experiment performed independently (and double blind) by the Affymetrix Company as shown in Figure 4 below. I carried out the treatments and analyses of the results. *C. reinhardtii* was exposed to 3 different [Cd^{2+}] over 6 hours and aliquots of exposed cells were taken at every hour and subjected to the multiplex protocol. The probes used targeted *AOT4-1* and *AOT4-2*. As it can be seen below, at 1 µM of free Cd^{2+}, before the 2 hour mark, the highest fold of gene expression is reached for both genes under study. The internal controls used and the machine calibration help in assaying if the maximal binding capacity if the beads has been reached. A 1.5 fold increase for *AOT4-1* and 12 fold increase for *AOT4-2*. In both cases, at and after 4 hours, the fold induction tapers off to slightly below the 1 fold mark. Once again this is additional evidence that both genes are metal regulated and peak expression occurs at around 2 hours, which is consistent with the Northern blot results. This positive result was further verified by using the Quantigene 2 assay (see Figure 5). This would serve as validation of the link between *AOT4-1* and *AOT4-2.*

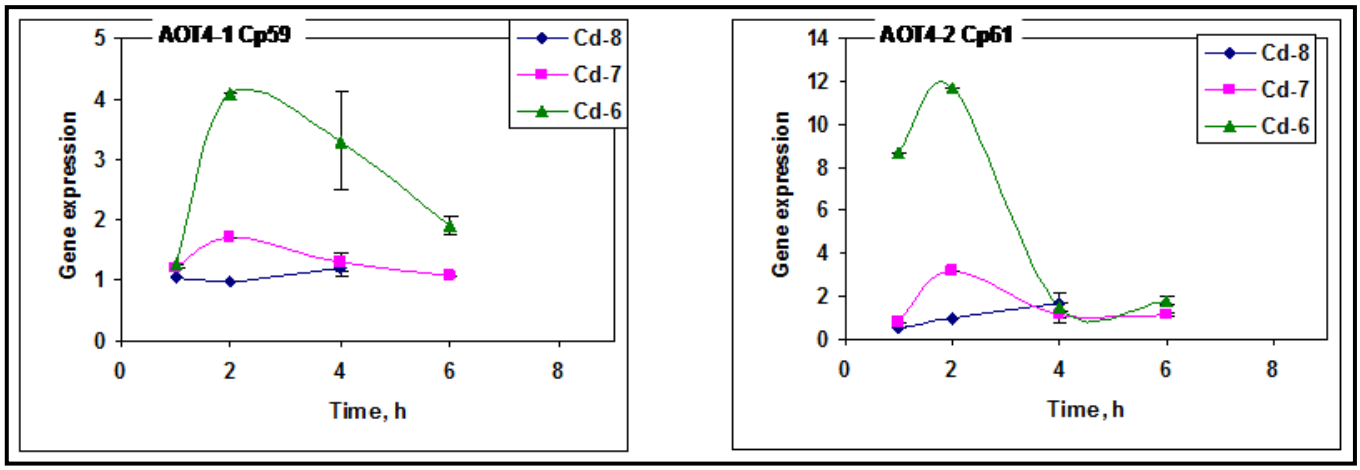

Figure 3. *AOT4-1* (right) and *AOT4-2* (left) expression levels were assayed using the Quantigene 1 assay on *C. reinhardtii* cells exposed to 3 different Cd^{2+}concentrations ; 1 µM, 0.1 µM and 0.01 µM. The line in green (triangle symbol) shows the expression profile of *C. reinhardtii* at 10^{-8} M of Cd^{2+}. The purple line (square symbol) shows the expression profile for 10^{-7}M of Cd^{2+} . The blue line shows the expression profile at 10^{-6} M of Cd^{2+}

4. The Quantigene singleplex assay of *AOT4 expression*

The Quantigene 2 singleplex assay was performed in the lab after the encouraging results obtained from Figure 3. However, the Quantigene 2 system had not been optimized yet for studying *C. reinhardtii*. A part of the project was devoted to fine-tuning this system to make it a valid platform for our choice organism. In other words, we set out to find the minimum amount of cells that could be used for this assay. This is actually one of the hallmarks of this bioassay (see above). In this system, when we talk about the amount of cells assayed, we use the term cell equivalents. Cell equivalents simply refer to an amount of cell homogenate obtained on a per cell basis. For example, if I were to have lysed 10^6 cells and then taken 1% of that homogenate, that aliquot would contain 10^4 cell equivalents.

The ribosomal housekeeping gene S26 was established as a control RNA for the amount of RNA in each sample. It is important to know the range of amount of material that can be used in an assay that gives linear relationship to the signal in order for the assay to be quantitative. It was necessary to determine the range of linearity of the signal with respect to the amount of cell-equivalents. I tested varying amounts of cell-equivalents and measured the response. These data are shown in Figure 4.

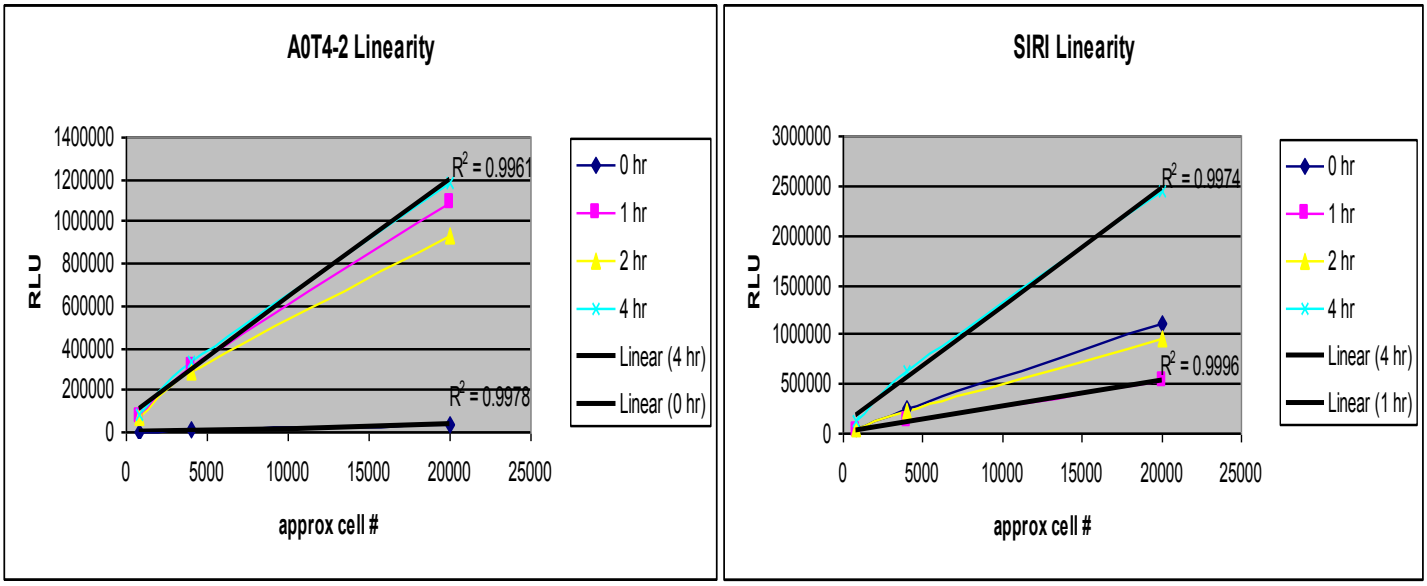

Figure 4. Using *AOT4-2* and *SIRI* determining the range of linearity of the system. Different cells equivalents were assayed over a period of 4 hours (4 time points) and the signal was linear when using the equivalent of 5000 cells.

It was found that the maximum number of cell that could be used in order to obtain results within the linear range of the signal was less than 5000 cells. Also using more than 10^4 cells there was signal saturation and consequently linearity was lost. However, during other tests, using 3000 cells was deemed too low as the signals were just above background. A number of cells between 3000 and 5000 is optimal for quantitative results.

Figure 5 shows the end results of the Quantigene 2 (singleplex) experiment. The main difference between the two assays is that for the multiplex assay, as many as 35 different mRNAs can be quantified. For example an aliquot of cells taken at the time point of 2 hours can be used for detecting a few genes of interest (see Materials and Methods for experimental details of the Quantigene assay) whereas in the Quantigene 2 one gene of interest can be assayed per aliquot. In this simplified assay (Quantigene 2), 3 different genes were studied, notably *AOT4-1*, *AOT4-2* and *SIR1*. The latter was also one of the genes Simon et al. had found through their global transcriptomic studies of Cd^{2+} exposed *C. reinhardtii*. In that study, *SIR1* expression did show induction but not as drastic as *AOT4*. In this case, one could say that the purpose of including SIR1 in this assay was to serve as a control.

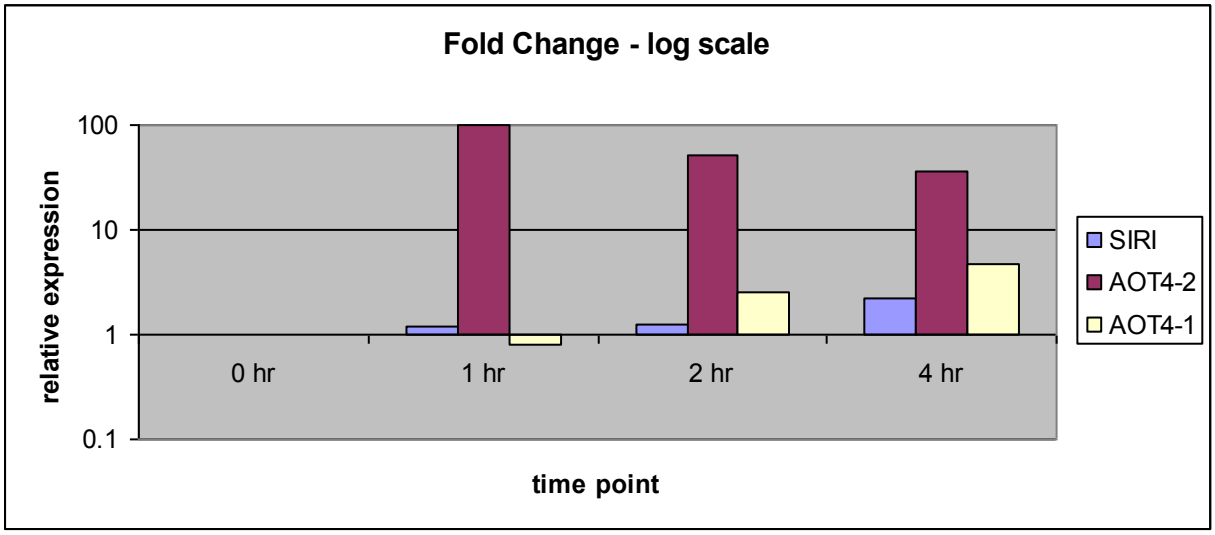

Figure 5 Quantigene 2 time course assay on *C. reinhardtii* using [Cd^{2+}] at 1 µM. *AOT4-2* expression is more than 10-fold. *AOT4-1* expression is seen at the 2 hour mark and increases by the 4 hour mark.

Transcripts detected with probes from each region were increased during exposure to Cd^{2+}. However, the induction was greater and earlier with the AOT4-2 probe set than with the AOT4-1 probe set (Fig 5). A possible explanation for this incongruity of the timing of these responses is suggested by the temporal pattern of expression of the large 2.2 kb transcript detected with both probe sets, and the smaller transcripts detected with only the AOT4-1 probe set. The AOT4-2 probe set may detect only the 2.2 kb transcript which is elevated after 2 h of exposure. The delay induction of transcripts detected by the AOT4-1 probe set may reflect the appearance of the smaller transcript species detected by Northern blot only with probe from this region. Above, I postulated that these different AOT4 transcript species result from differential splicing.

However, the way *AOT4-1* is expressed over time does not match with the one if Figure 3. *AOT4-2* on the other hand, does show more than 10 fold induction at 2 hours which does match the fold induction shown by the Quantigene 1 in Figure 3. For SIR1, relative expression thought almost inexistent in the first hour seems to increase after 2 hours.

5. The Luciferase reporter system

My initial experiments had been to develop a reporter gene based on two different published reporter genes for *C. reinhardtii* encoding the chemiluminescent protein luciferase. Two genes encoding luciferase from different organisms have reported to be expressed and generate high levels of luminescence in *C. reinhardtii* transformants. *C. reinhardtii* would be transformed with a luciferase construct where

the latter would be under the control of a Cd^{2+}inducible promoter. This assay had its advantages. In-situ readouts of bioluminescence could be performed using portable instruments within an acceptable amount of time (2 hours). The luciferase construct had also been codon optimized for *C. reinhardtii* to avoid potential gene silencing, which effectively prevents the expression of most foreign genes (see Introduction). Figure 6 shows a schematic representation of this reporter system.

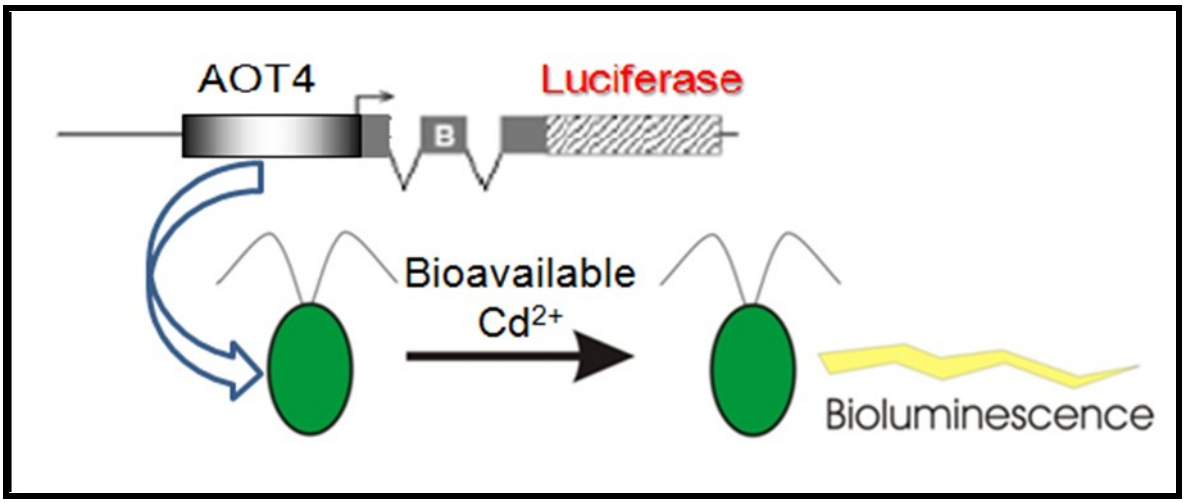

Figure 6. Rationale behind the luciferase reporter system: The *AOT4* promoter region would be fused to the luciferase reporter gene and upon successful transformation, *C. reinhardtii* with this construct would produce quantifiable luciferase in the presence of Cd^{2+}. The region, B, between the luciferase and the *AOT4* region is for Bleomycin, the selectable marker used for selecting for positive transformants. The green circles are a schematic representation of *C. reinhardtii.*

I made some of the steps towards construction of an *AOT4*-luc reporter gene. The region identified as containing the *AOT4* promoter was cloned into a luciferase construct that had its regulatory region excised (see Materials and Methods). The new construct, termed p*AOT4-luc*, was transformed into wild type *C. reinhardtii.* This transformant then was expected to express luciferase in response to exposure to Cd^{2+}. Unfortunately, I was unable to detect any luciferase activity. Hundreds of colonies were screened by PCR to amplify DNA sequences specific to the reporter gene in order to identify those with a successful integration of the *luc* construct. Ten transformant strains (strains 1-4, 14, 15, 17, 21, 32, 34) showed proper integration without rearrangement (pcr data not shown).

The positive transformants were exposed to 1 µM Cd^{2+} for 2 hours, lysed and then the extracts were tested for bioluminescence produced by the luciferase with a microplate reader. In a few trials, only strain 15 showed a slight induction meaning that the reporter gene was expressed. However, this result was not reproducible. In other words, strain 15 would give signals no higher than background more often than showing a significant induction. Figure 7 gives an overview of the results mentioned above.

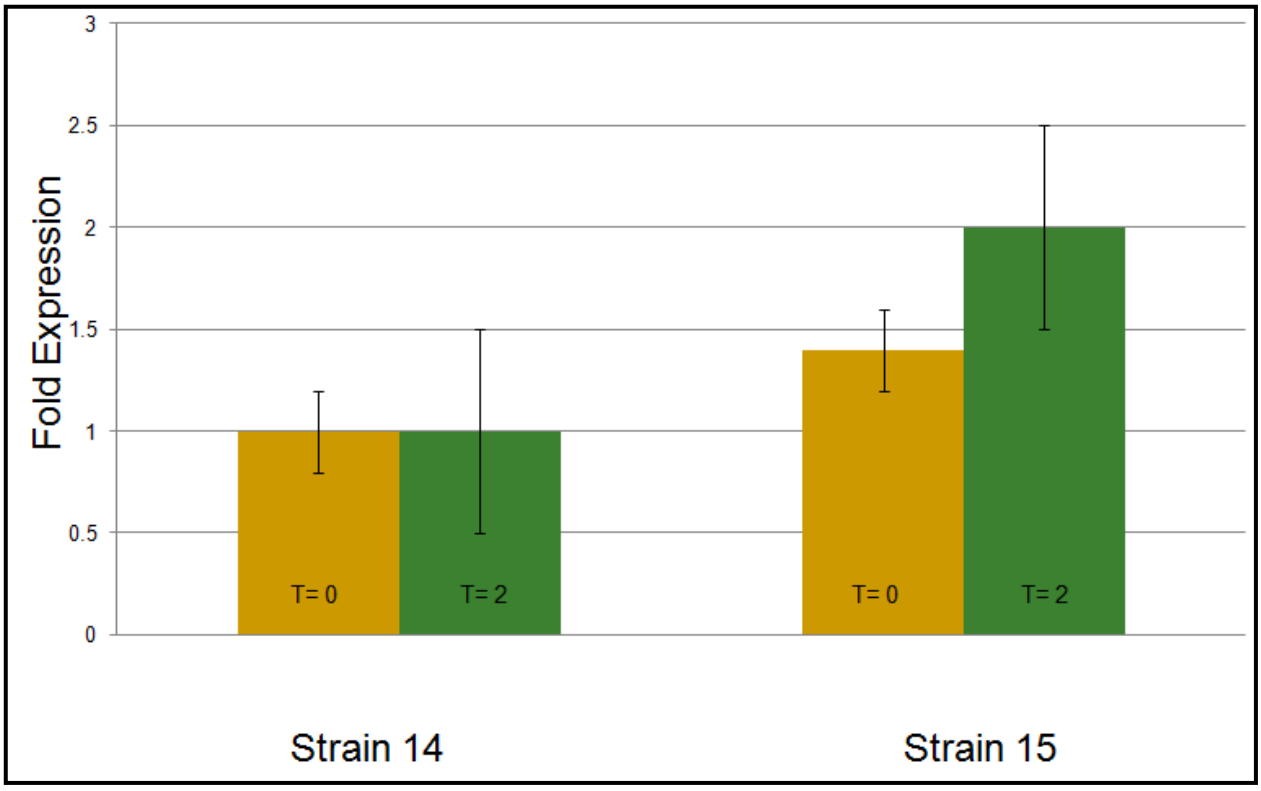

Figure 7. There was no significant induction of the bioluminescence signals from luciferase in the transformant strains with the AOT4-lucg construct after 2 hours Cd^{2+} exposure. The fold expression is based over the fold expression of control un transformed cells. There were 3 biological replicates for each strain.

6. Quantigene 1 assay using ROS genes

Although the oxidative stress genes are not specific, they might serve this function as a potential bioassay. For example, from what can be gathered so far, *AOT4* is induced at a threshold of 1 uM Cd^{2+}. This is at the very upper limit of concentrations found in the environment (Simon et al., 2011). Therefore, through the aforementioned experiments, I have determined the minimum concentration that induces these genes.

Because the luciferase reporter system showed very weak or no expression, other reporter systems were tested. After all, the goal of this project was to develop bioassays for measuring Cd^{2+}so we were not going to limit ourselves to only one bioassay. Oxidative stress through reactive oxygen species (ROS) has been a subject of attention in our lab and there are markers for showing the effects of such stress. The idea now was to see if Cd^{2+} was inducing oxidative stress at micromolar and nanomolar concentrations of Cd^{2+}using *AOT4-2* and a couple of other markers, Gsx and Gsts. As far as we know, this is a first study of its kind. Simon et al, who were also using the Quantigene 2 method used a different housekeeping gene to avoid the saturation of the signal described above (Simon et al., 2011). This transcript was encoded by the HSK gene. This was thus an opportunity to test a more reliable control. However, this transcript was found to be marginally induced by Cd^{2+} exposure by the group of Dr. Wilkinson (personal communication). This issue is addressed further in below. The 2 other oxidative stress markers used were:

(1) Gsx (glutathione peroxidase) which is involved in the detoxification of hydrogen peroxide and organic hydroperoxides by reducing them to water and the corresponding alcohol

(2) Gsts (glutathione s-transferase) which catalyses the conjugation of reduced glutathione via a sulfhydrl group to electrophilic centers on a wide variety of substrates. This activity detoxifies endogenous compounds such as peroxidised lipids.

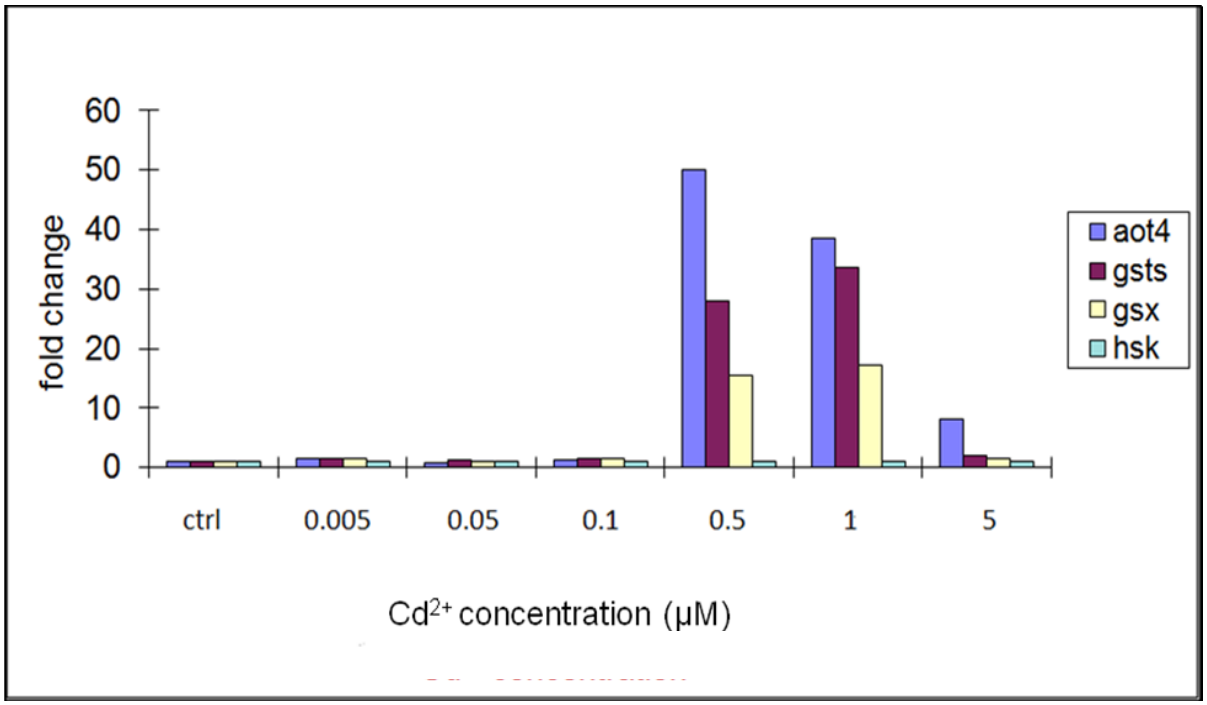

Figure 8. Evaluating if free Cd^{2+} induces oxidative stress at nanomolar and micromolar concentrations using the new housekeeping gene *HSK*, *AOT4-2*, *GSTS* and *GSX*. Ctrl is expression in the absence of Cd^{2+}. At each [Cd^{2+}], the Quantigene 2 was used to measure the transcript levels of AOT4, Gsts, Gsx or HSK. The exposure time to Cd^{2+} was for 2 hours in all cases.

At about 0.5 uM and down to a few nM, there is no significant expression of any of the marker genes. However at and above 0.5 µM, the fold change increased drastically. The *AOT4-2* signal increases 50 fold, GSTS 30 fold and GSX 15 fold. However at 5 µM, there is a drastic drop in gene expression possibly due to cell death, because this concentration is known to affect viability (Macfie and Welbourn, 2000).

It was brought to our attention that the second housekeeping gene HSK, is actually slightly induced by Cd exposure. This induction biased the fold-changes determined for the genes whose differential expression was under analysis (Drs. Dana Simon and Kevin Wilkinson, personal communication). Therefore, I measured the protein concentration of the lysates using the BCA assay (Walker, 1994) and used the mass amounts of protein as a standard The change made here was that the amount of protein was measured in each sample (by comparing with a known protein standard) and a known amount loaded into the Quantigene 2 system for analysis. The results obtained shown in Figure 9 show a striking induction of the ROS genes and *AOT4.* These results also demonstrate the importance of ROS genes in metal response and tolerance.

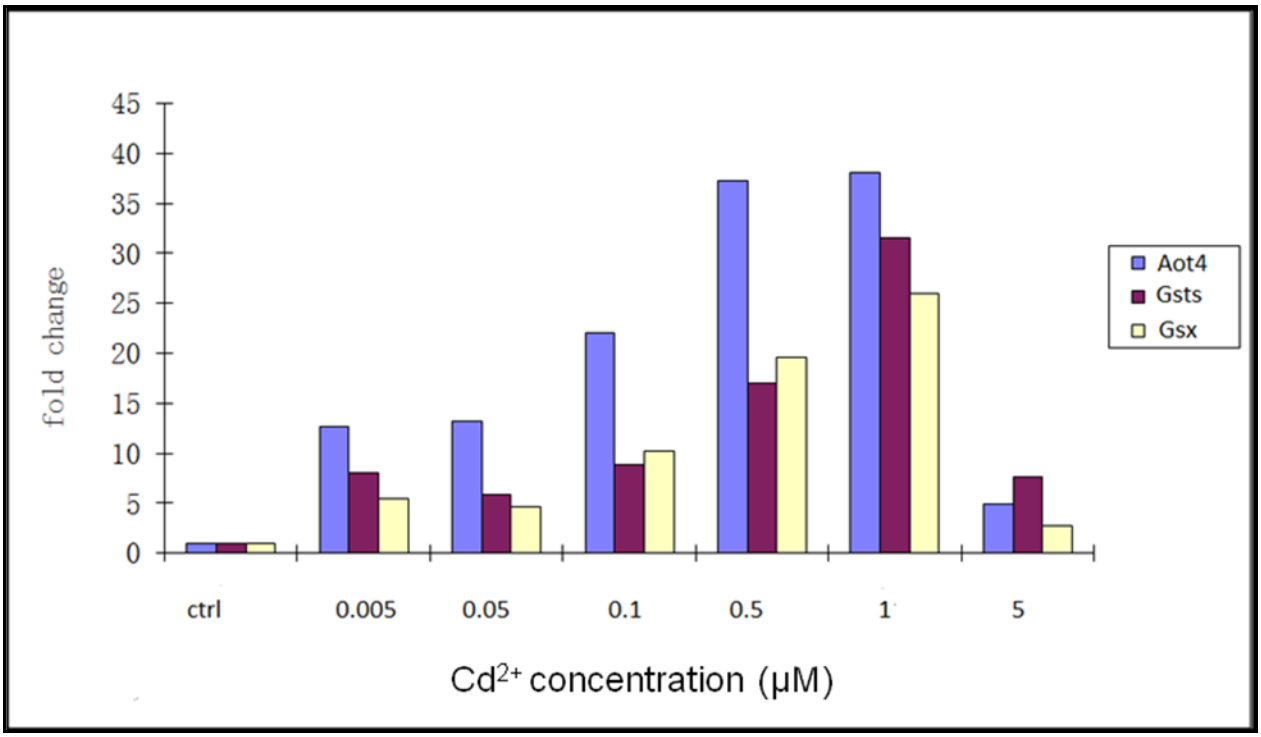

Figure 9. Evaluating if free Cd^{2+} induces oxidative stress at nanomolar and micromolar concentrations. A protein standard was used instead of HSK. Ctrl is expression in the absence of Cd^{2+}. At each [Cd^{2+}], the Quantigene 2 was used to measure the transcript levels of AOT4, Gsts and Gsx. The exposure time to Cd^{2+} was for 2 hours.

At 5 nM Cd^{2+}, the lowest concentration used, *AOT4-2* transcript increased 15 times, GSTS increased at almost 10 ftimes and GPX increased 5 fold. There is a constant increase in transcript levels until 1 µM. At 5 µM, as seen in figure 8, there is a sharp drop in expression levels of all the 3 transcripts. Therefore, these oxidative stress marker genes are induced at much lower concentrations of Cd^{2+} than were known to induce oxidative stress responses.

DISCUSSION

In recent years, new classes of aquatic pollutants have received attention from environmentalists, scientists, and regulators due to their introduction into the environment, unforeseen effects associated with the pollutants, or enhanced analytical techniques presently capable of detecting them (Helen C. Poynton, 2009). Because of the scope of the problem of emerging contaminants, genome-based approaches may aid in understanding how these chemicals interact with aquatic organisms. Ecotoxicology has in recent years embraced the genomic technologies to create the rapidly growing field of ecotoxicogenomics Genomic tools monitor the molecular responses of the organism to pollutants and provide an illustrative picture suggestive of the toxic effects experienced by the organism (Snape et al., 2004). Monitoring those responses can serve as an indicator of environmental effects and ecotoxicological end points (Fedorenkova et al., 2010). In the same vein, the goal of this thesis is to make some important contributions to the ongoing collaborative project (with Dr. Wilkinson at University of Montreal) to develop bioassays for the detection of the bioavailable species of toxic trace metals (aquatic pollutants) using gene expression responses in *C. reinhardtii*.

Importance of ROS detoxifying genes: induction at nanomolar concentrations of Cd^{2+}.

The most important finding of this research has been the indication that genes involved in ROS tolerance are also induced by exposure to nanomolar concentrations of Cd^{2+}. This finding is novel, as previous studies have not observed ROS gene inductions at

such low concentration. It should be noted that while this work was in progress similar results were published (Stoiber et al., 2010, 2011).

This finding could be an insight into the evolution of *C. reinhardtii*, and specifically, whether or not it could have evolved tolerance mechanisms against these toxic trace metals. Previous studies revealed little or no effect at concentrations in the micromolar range (Macfie and Welbourn, 2000). In this case, there would be no selective pressure to favour the evolution of tolerance mechanisms that are specific to particular toxic trace metals. In other words, it suggests that there may be an acclimation response, i.e. that low concentrations induce a response in anticipation of a more severe exposure. During evolution, *C. reinhardtii* could have evolved to protect itself against ROS by preparing for exposure at higher concentrations. For example, being in a nanomolar environment of Cd^{2+} (Simon et al., 2011) could be a signal that exposure to higher concentrations is pending; so the organism anticipates such a possibility and increases the production of enzymes that are involved in ROS detoxification.

The panomics multiplex system as the future

The Quantigene 2 (singleplex assay) has quite a few advantages. As I have shown, little material is required; crude lysate from 8000 cells is sufficient. This is advantageous because cells can alter metal concentration by binding and uptake. Therefore we want to use a few cells numbers. Being able to use few cells might even allow the transcritomic analyses of endogenous *C. reinhardtii* in the body of water being

sampled. A new approach in ecotoxicogenomics is the identification of toxicant-specific patterns of gene expression in fish and other aquatic organisms (Pierron et al., 2011). As *C. reinhardtii* is endogenous to freshwater, it might be possible to develop a *C. reihardtii* system for ecotoxicogenomics since it may have some advantages over use of organisms that are higher on the food chain.

A contribution of this work was the validation of new methods of mRNA quantification for use in toxic trace metal bioassays. First, conditions were established in regards to the amount of cell equivalents for the gene(s) of interest. An appropriate control gene for standardization of the amount of RNA loaded was also determined (see Results).

Global transcriptome profiling by the collaborating groups involved in this project revealed that metal-specific biomarker genes do not exist (Simon et al., 2011). However, they did identify specific patterns of gene expression in response to exposure to various toxic trace metals. Based on the results presented, it seems unlikely that only one gene is induced by Cd^{2+} and by no other metal or abiotic condition; it makes sense that a battery of genes can be used as a fingerprint of the presence of a specific bioavailable metal species. Since this Quantigene 2 system can target up to 36 genes per sample, we can speculate that the response of *C. reinhardtii* to a selected set of responsive genes will be different for different metals at different concentrations. In other words, the expression pattern of a small subset of genes, numbering less than 36 genes, could be diagnostic of one particular metal. The key component of this system

would be choosing the right palette of genes to assure the correct representation of susceptible gene transcripts.

Attempts to use luciferase encoding reporter genes in a toxic trace metal bioassay.

The idea behind these two reporter genes was simple i.e. to have a Cd^{2+} inducible promoter fused to a gene whose product could be easily quantified. The major problem encountered was the inability to detect luciferase activity in extracts of transformants. Figure 7 showed that even bonafide transformants were not stable in their expression of the luciferase gene. It came to our attention that there are only three reports of the use of luciferase in *C. reinhardtii* since the original publications that claimed to establish them in 2004/2005. Therefore, it would seem that this system does not work because, like all exogenous/foreign genes, *C. reinhardtii* recognizes these genes are foreign and silences their expression using its potent RNA interference system (Schroda, 2006).

This research has shown us that the holy grail of cadmium promoters does not exist. In other words, there is not one cadmium inducible promoter that is going to be exclusive for the metal alone. RNAseq work performed by Simon et al. has shown that *AOT4* is also inducible by silver and a recent study revealed that it is among a limited set of genes induced by sulphur depravation (Gonzalez-Ballester et al., 2010). It was also found that there are a couple better gene candidates that are induced by Cadmium. A way to circumvent to test candidate promoters (based on the candidate genes) would be to have a “plug and play type” reporter system i.e. to be able to excise the promoter

regions of the luciferase/*PCR-1* genes and replace it with possibly better candidate promoters. A cre/lox system has been used to that specific aim but has not been explored entirely (Heitzer and Zschoernig, 2007).

Another improvement that could be made is to have the reporter gene product (luciferase) be quantified inside the cell. For this project, the cells that had supposedly synthesized luciferase had to be lysed and the breakdown product of a luciferase substrate which gave a bioluminescent signal was quantified. If this lysis step could be avoided, I believe that the signal obtained in the presence of Cd^{2+} would have been more sensitive but this has yet to be tried.

Development of a new reporter gene for the *PCR-1* assay and future directions

The *PCR-1* system still remains a viable project. It offers some advantages over the luciferase system. For example, *PCR-1* is an endogenous gene so it can escape silencing. Once chlorophyll is produced it can be conserved and easily measured. Chlorophyll can be measured by spectrophotometric methods and it can be stored after in methanol. The only stumble block was the actual making of the gene construct that would be used for transforming mutant *C. reinhardtii*. The *PCR-1* fusion construct was hard to generate due to its size. PCR stitching reactions eventually did give some encouraging results in terms of generating pieces of the fusion construct. Maybe generating the fusion construct via a yeast system (Shao and Zhao, 2009) could be the way to go in this case.

A major aim of ecotoxicology is to measure and predict the effects of contaminants on natural populations, communities and ecosystems (Pierron et al., 2011). Investigations on site, using multiple biomarkers have received sustained interest over the last 20 years (Zhou et al., 2008). Among other approaches, transcriptomic approaches have the potential to detect, characterize and assess the effects of pollutants at the transcriptional level (Pierron et al., 2011). On the other hand, despite the growing interest of ecotoxicologists in gene expression analysis, transcriptomic tools currently miss non-model aquatic species that are perhaps more interesting and more relevant from an ecological and toxicological viewpoint (Pierron et al., 2011). In this thesis, I first present a method where the goal was to identify directly the transcription levels of specific *C. reinhardtii* genes in response to environmentally relevant Cd^{2+} concentrations. For this, I attempted to use two indirect methods in which Cd^{2+}-regulated expression is measured via reporter genes. Although the take home message through my work and that of the collaborators is that one gene cannot be used to monitor the presence of one metal, there may be patterns of expression based on a subset of carefully selected genes that could help determine the concentration of bioavailable species of specific metals.

LITERATURE CITED

Barnes, D., Franklin, S., Schultz, J., Henry, R., Brown, E., Coragliotti, A., and Mayfield, S.P. (2005). Contribution of 5'- and 3'-untranslated regions of plastid mRNAs to the expression of Chlamydomonas reinhardtii chloroplast genes. Mol Genet Genomics *274*, 625-636.

Boswell, C., Sharma, N.C., and Sahi, S.V. (2002). Copper tolerance and accumulation potential of Chlamydomonas reinhardtii. Bull Environ Contam Toxicol *69*, 546-553.

Caussy, D., Gochfeld, M., Gurzau, E., Neagu, C., and Ruedel, H. (2003). Lessons from case studies of metals: investigating exposure, bioavailability, and risk. Ecotoxicol Environ Saf *56*, 45-51.

Chugh L.K., S.S.K. (1999). Photosynthetic activities of Pisum sativum seedlings grown in presence of cadmium. Plant Physiology and Biochemistry *37*, 7.

Clemens, S., Palmgren, M.G., and Kramer, U. (2002). A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci *7*, 309-315.

Collard, J.M., and Matagne, R.F. (1990). Isolation and genetic analysis of Chlamydomonas reinhardtii strains resistant to cadmium. Appl Environ Microbiol *56*, 2051-2055.

Devriese M, T.V., Garbayo I, Leon R, Vilchez C, Vigara J (2001). Effect of heavy metals on nitrate assimilation in the eukaryotic microalga Chlamydomonas reinhardtii. Plant Physiology and Biochemistry *39*.

Fedorenkova, A., Vonk, J.A., Lenders, H.J., Ouborg, N.J., Breure, A.M., and Hendriks, A.J. (2010). Ecotoxicogenomics: bridging the gap between genes and populations. Environ Sci Technol *44*, 4328-4333.

Fletcher, S.P., Muto, M., and Mayfield, S.P. (2007). Optimization of recombinant protein expression in the chloroplasts of green algae. Adv Exp Med Biol *616*, 90-98.

Fuhrmann, M. (2004). Production of antigens in Chlamydomonas reinhardtii: green microalgae as a novel source of recombinant proteins. Methods Mol Med *94*, 191-195.

Fuhrmann, M., Oertel, W., and Hegemann, P. (1999). A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in Chlamydomonas reinhardtii. Plant J *19*, 353-361.

Gaballa, A., and Helmann, J.D. (2003). Bacillus subtilis CPx-type ATPases: characterization of Cd, Zn, Co and Cu efflux systems. Biometals *16*, 497-505.

Gekeler W, G.E., Winnacker EL, Zenk MH (1989). Algae sequester heavy metals via synthesis of phytochelatin complexes. Archives of Microbiology *150*.

Gonzalez-Ballester, D., Casero, D., Cokus, S., Pellegrini, M., Merchant, S.S., and Grossman, A.R. (2010). RNA-seq analysis of sulfur-deprived Chlamydomonas cells reveals aspects of acclimation critical for cell survival. Plant Cell *22*, 2058-2084.

Guzzo, A., and DuBow, M.S. (1994). A luxAB transcriptional fusion to the cryptic celF gene of Escherichia coli displays increased luminescence in the presence of nickel. Mol Gen Genet *242*, 455-460.

Heitzer, M., and Zschoernig, B. (2007). Construction of modular tandem expression vectors for the green alga Chlamydomonas reinhardtii using the Cre/lox-system. Biotechniques *43*, 324, 326, 328 passim.

Helen C. Poynton, C.D.V. (2009). Ecotoxicogenomics: Emerging Technologies for Emerging Contaminants. Journal of the American Water Resources Association *45*, 14.

Howe, G., and Merchant, S. (1992). Heavy Metal-Activated Synthesis of Peptides in Chlamydomonas reinhardtii. Plant Physiol *98*, 127-136.

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

Hutchins, C.M., Simon, D.F., Zerges, W., and Wilkinson, K.J. (2010). Transcriptomic signatures in Chlamydomonas reinhardtii as Cd biomarkers in metal mixtures. Aquat Toxicol *100*, 120-127.

Jain, C.a.I.A. (2000). Arsenic: Occurrence, Toxicity, and Speciation Techniques. Wat Res *34:17*.

Jim Jian Wanga, D.L.H., Rodney E. Hendersona &Paul F. Bella (2004). Comparison of Soil-Test Extractants for Phosphorus, Potassium, Calcium, Magnesium, Sodium, Zinc, Copper, Manganese, and Iron in Louisiana Soils. Communications in Soil Science and Plant Analysis *35*, 16.

Klein, U., Salvador, M.L., and Bogorad, L. (1994). Activity of the Chlamydomonas chloroplast rbcL gene promoter is enhanced by a remote sequence element. Proc Natl Acad Sci U S A *91*, 10819-10823.

Kola, H., and Wilkinson, K.J. (2005). Cadmium uptake by a green alga can be predicted by equilibrium modelling. Environ Sci Technol *39*, 3040-3047.

Lemaire, S.D., Miginiac-Maslow, M., and Jacquot, J.P. (2002). Plant thioredoxin gene expression: control by light, circadian clock, and heavy metals. Methods Enzymol *347*, 412-421.

Lu, Y.P., Li, Z.S., and Rea, P.A. (1997). AtMRP1 gene of Arabidopsis encodes a glutathione S-conjugate pump: isolation and functional definition of a plant ATP-binding cassette transporter gene. Proc Natl Acad Sci U S A *94*, 8243-8248.

Lumbreras, V. (1998). Efficient foreign gene expression in Chlamydomonas reinhardtii mediated by an endogenous intron. Plant J *14*.

Macfie, S.M., and Welbourn, P.M. (2000). The cell wall as a barrier to uptake of metal ions in the unicellular green alga Chlamydomonas reinhardtii (Chlorophyceae). Arch Environ Contam Toxicol *39*, 413-419.

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Marechal-Drouard, L*., et al.* (2007). The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science *318*, 245-250.

Miller, S.M. (1999). Bacterial detoxification of Hg(II) and organomercurials. Essays Biochem *34*, 17-30.

Neumann, N.F., and Galvez, F. (2002). DNA microarrays and toxicogenomics: applications for ecotoxicology? Biotechnol Adv *20*, 391-419.

Neupert, J., Karcher, D., and Bock, R. (2009). Generation of Chlamydomonas strains that efficiently express nuclear transgenes. Plant J *57*, 1140-1150.

Pierron, F., Normandeau, E., Defo, M.A., Campbell, P.G., Bernatchez, L., and Couture, P. (2011). Effects of chronic metal exposure on wild fish populations revealed by high-throughput cDNA sequencing. Ecotoxicology *20*, 1388-1399.

Ramanathan, S., Ensor, M., and Daunert, S. (1997). Bacterial biosensors for monitoring toxic metals. Trends Biotechnol *15*, 500-506.

Ramanathan, S., Shi, W.P., Rosen, B.P., and Daunert, S (1998). Bacteria-based chemiluminescence sensing system using beta-galactosidase under the control of the ArsR regulatory protein of the ars operon. anal Chem Acta *369*, 7.

Rensing, C., Sun, Y., Mitra, B., and Rosen, B.P. (1998). Pb(II)-translocating P-type ATPases. J Biol Chem *273*, 32614-32617.

Rochaix, J.D., Goldschmidt-Clermont, M., and Merchant, S. (1998). The molecular biology of chloroplasts and mitochondria in Chlamydomonas (Dordrecht ; Boston, Kluwer Academic Publishers).

Russnak, R., Konczal, D., and McIntire, S.L. (2001). A family of yeast proteins mediating bidirectional vacuolar amino acid transport. J Biol Chem *276*, 23849-23857.

Salonen, J.T., Nyyssonen, K., Korpela, H., Tuomilehto, J., Seppanen, R., and Salonen, R. (1992). High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. Circulation *86*, 803-811.

Salvador, M.L., Suay, L., Anthonisen, I.L., and Klein, U. (2004). Changes in the 5'-untranslated region of the rbcL gene accelerate transcript degradation more than 50-fold in the chloroplast of Chlamydomonas reinhardtii. Curr Genet *45*, 176-182.

Schroda, M. (2006). RNA silencing in Chlamydomonas: mechanisms and tools. Curr Genet *49*, 69-84.

Scott, D.L., Ramanathan, S., Shi, W., Rosen, B.P., and Daunert, S. (1997). Genetically engineered bacteria: electrochemical sensing systems for antimonite and arsenite. Anal Chem *69*, 16-20.

Shao, N., and Bock, R. (2008). A codon-optimized luciferase from Gaussia princeps facilitates the in vivo monitoring of gene expression in the model alga Chlamydomonas reinhardtii. Curr Genet *53*, 381-388.

Shao, Z., and Zhao, H. (2009). DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res *37*, e16.

Si Shi-Hui, C.J.-H., He Feng-Jiao,Nie Li-Hua, Yao Shou-Zhuo (1996). Electrochemically modified piezoelectric crystal biosensor for detection of Staphylococcus aureus. Chinese Journal of Chemistry *14*, 5.

Simon, D. (2009). Determination of the genomic response of C. reinhardtii exposed to different metals and nanoparticles. In biology department (montreal, university of montreal).

Simon, D.F., Davis, T.A., Tercier-Waeber, M.L., England, R., and Wilkinson, K.J. (2011). In situ evaluation of cadmium biomarkers in green algae. Environ Pollut *159*, 2630-2636.

Simon, D.F., Descombes, P., Zerges, W., and Wilkinson, K.J. (2008). Global expression profiling of Chlamydomonas reinhardtii exposed to trace levels of free cadmium. Environ Toxicol Chem *27*, 1668-1675.

Slauch, J.M., and Silhavy, T.J. (1991). Genetic fusions as experimental tools. Methods Enzymol *204*, 213-248.

Snape, J.R., Maund, S.J., Pickford, D.B., and Hutchinson, T.H. (2004). Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquat Toxicol *67*, 143-154.

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

Stoiber, T.L., Shafer, M.M., and Armstrong, D.E. (2011). Induction of reactive oxygen species in Chlamydomonas reinhardtii in response to contrasting trace metal exposures. Environ Toxicol.

Trevors, J.T., Stratton, G.W., and Gadd, G.M. (1986). Cadmium transport, resistance, and toxicity in bacteria, algae, and fungi. Can J Microbiol *32*, 447-464.

Virta, M., Lampinen, J., Karp, M. (1995). A Luminescence-Based Mercury Biosensor Anal Chem *67*, 3.

Walker, J.M. (1994). The bicinchoninic acid (BCA) assay for protein quantitation. Methods Mol Biol *32*, 5-8.

Wu JX, H.Z., Wang CG et al (2008). Efficient expression of green fluorescent protein (GFP) mediated by a chimeric promoter in Chlamydomonas reinhardtii. Chin J Oceanol Limnol *26*, 6.

Yagi, K. (2007). Applications of whole-cell bacterial sensors in biotechnology and environmental science. Appl Microbiol Biotechnol *73*, 8.

Yang, M., Cobine, P.A., Molik, S., Naranuntarat, A., Lill, R., Winge, D.R., and Culotta, V.C. (2006). The effects of mitochondrial iron homeostasis on cofactor specificity of superoxide dismutase 2. EMBO J *25*, 1775-1783.

Zhou, Q., Zhang, J., Fu, J., Shi, J., and Jiang, G. (2008). Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal Chim Acta *606*, 135-150.