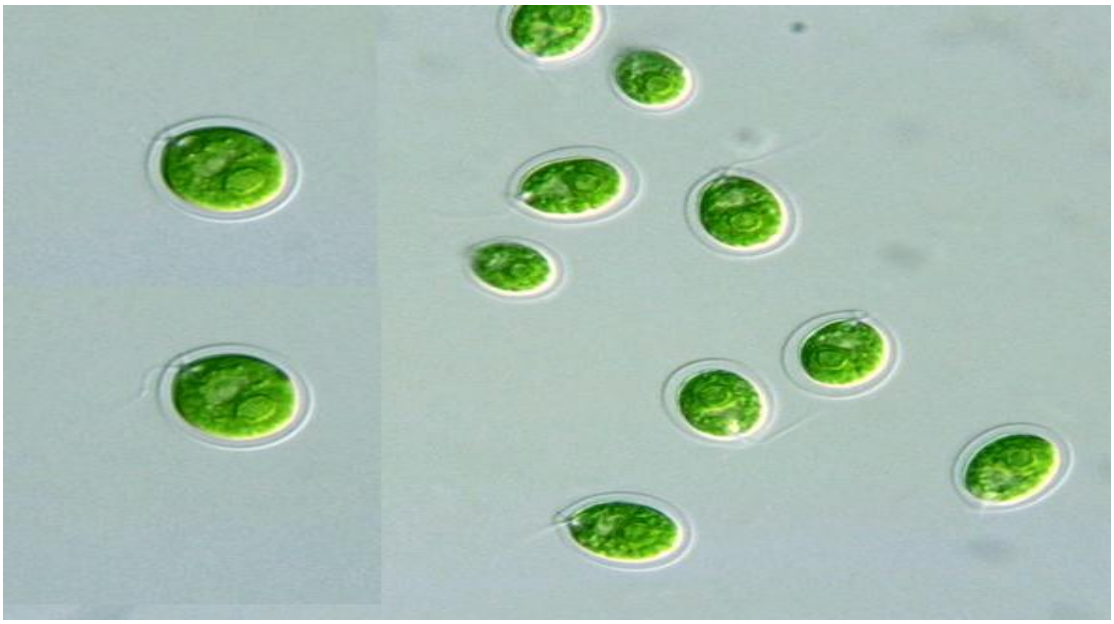
EFFECTS OF HEAVY METAL STRESS ON *CHLAMYDOMONAS REINHARDTII*: A COMPREHENSIVE STUDY



THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Geo-environmental Resources and Risks in the Department of Environmental and Natural Resources Engineering at the Technological Institute of Crete

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2016

Technological Educational Institute of Crete

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ABSTRACT OF THESIS

EFFECTS OF HEAVY METAL STRESS ON *CHLAMYDOMONAS REINHARDTII*: A COMPREHENSIVE STUDY

In marine and freshwater ecosystems, unicellular photosynthetic organisms are being considered as members of great importance for the early detection of potential hazards. Moreover, microalgae are in the base of the food chain and the exposure to potential pollutants such as heavy metal pollution is crucial for any ecosystem because they can transport the pollution to higher organisms in the food chain and can cause a major inhibition of photosynthesis. On these scores, biochemical and molecular biology biomarkers should be seriously take part in any environmental assessment program in order to give the best and most effective results. In this work, *Chlamydomonas reinhardtii* was chosen to be exposed in several sub – lethal levels of Cd^{2+}, Ni^{2+} and Pb^{2+} pollution and the effects of the metal exposure on cell growth rates, on the levels of certain metabolites and on specific enzyme activities were determined. In conclusion, at exposure to cations of Ni^{2+}, Cd^{2+},Pb^{2+} and tertiary mixtures of the abovementioned elements, showed higher cell division comparing to control for the single metal treatments of 14.58ppm and 7.29ppm Cd^{2+}, 6.26ppm, 12.51ppm Pb^{2+}. Exposure to 25.02ppm Pb^{2+} showed higher growth rate comparing to control at 168h, the same occurred at exposure to the mixture of 2.02ppm Ni^{2+}, 7.29ppm Cd^{2+} and 12.51ppm Pb^{2+}. Ni^{2+} cell content increased at its combination with Cd^{2+} and Pb^{2+} at both time points. At 120h, all Cd^{2+} treatments was showed to increase the cell Cd^{2+} content combined with treatments of Ni^{2+} and Pb^{2+}. Working on glutathione peroxidase (GPX) mRNA accumulation and using reverse transcription method, the gene was found expressed upon exposure to all conditions at 120h. Significant results obtained for the most conditions comparing to control at both time points. Enzyme activities were quantified involved in antioxidant cycles, e.g. Ascorbate – glutathione. Ascorbate peroxidase (APX) didn't offer any significant result comparing to control at both time points. Glutathione reductase (GR) quantification offered significant results only between the treatments, although there were no significant results comparing to control. Peroxidase (POX), Catalase (CAT), Dehydroascorbate reductase (DHAR), Glutathione S – Transferase (GST) and Superoxide Dismutase (SOD) assays gave similar results, as GR. Pyrroline 5 – carboxylate synthetase (P5CS), a key enzyme for proline biosynthesis, found to be inhibited under exposure to the lowest concentration of Pb^{2+} and to the mixture Ni 2.02ppm, Pb 12.51ppm, Cd 14.58ppm. Metabolites have been quantified, like non – enzymatic antioxidant activity, polyphenols content and a lipid peroxidation biomarker, malondialdehyde (MDA). Quantification of non – enzymatic antioxidant activity offer significant results between the treatments but also to control at 120h. Polyphenols content at 120h gave significant results between the treatments and also to the control. MDA quantification didn't offer statistical

results. Quantification of secondary metabolism was performed in order investigate the effect of pollutants to pigments of chlorophyll and carotenoids/xanthophylls. Chlorophyll *a* and *b* offer us interesting results and carotenoids/xanthophylls didn't give any statistical significant result. Flavonoids quantification gave significant results at 168h, especially for mixtures.

In conclusion, two biological replicates have been performed for each condition which proved by the high variations to be limited. Another one reason is that some concentrations may lead to high variations due to stress (e.g. Pb^{2+} 25.02ppm).

KEYWORDS: *Chlamydomonas reinhardtii,* heavy metal pollution, enzyme activity, metabolites

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September, 2017

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To my mother and beloved friends who encourage me all these years.

ACKNOWLEDGEMENTS

I would like to thank my main advisors, professors Dr Nikos Lydakis – Simantiris and Kris Vissenberg, for the great guidance, precious advices and huge support to carry out the whole process.

Great thanks go to prof. Dr. Ronny Blust for allowing me to grow *chlamydomonas* in his lab. Additionally, I would like to thank Sébastjen Schonaers for his great assistance during this project because he was never tired to answer my questions. I feel very grateful to Dr. Kayawe Valentine Mubiana for helping me to measure the metal concentrations using ICP – MS, to Femke De Croock and Karin Van den Bergh for introducing me into Chlamydomonas-culturing, to Prof. Dr. Els Prinsen for the determination of secondary metabolites, and to Dr. Hamada Abd Elgawad for the determination of metabolites and enzyme activity. Also, I would like to thank the other lab members of Systemic Physiological and Ecotoxicological Research (SPHERE) and Integrated Molecular Plant Physiology Research (IMPRES) for their huge assistance to achieve my goal.

I would like to thank my family for their support and love. Also, I would like to thank my roommates and colleagues who offered me great moments through these eight months of never ending work and helping me to decrease my stress providing to me more and more entertainment. I would like to thank all my professors and colleagues for making the last couple of years so enjoyable!

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Chapter 1: Introduction

1.1 The story behind

As the world population increases, developing countries follow alternative paths to take care of their increasing demands for sufficient living. Mining industry is still one of the most major sources which contribute to improve human living. The most important mining sectors are related to the extraction of coal, tin, tungsten, molybdenum, uranium and rare earth elements mines, as well as industrial mineral deposits (i.e. diamonds) and quarries. Despite of the obvious benefits of the mining industry for the financial growth of many developing countries, one of the biggest drawbacks is the heavy metal pollution which constantly increased all over the planet. The influence of heavy metal pollution on social living and the environment is a major problem and can highly affect agricultural productivity, impair with food security and quality, and dramatically impact on water resources along with the soil, human health and aquatic ecosystems through sequestration. Apart from this, there are financial impacts such like expensive cost of remediation projects and impacts on business sector about company's reputation. The mining activities have as major result depletion of the groundwater, for example in Bangladesh, India and Thailand because of the over – extraction and as a consequence of this, there is a major problem with arsenic contamination, mostly in India and Bangladesh. A very indicative example for the water pollution is China. According to the report on the State of Environment in China, only 40% of the water is suitable for drinking or fishing and 28% is not even suitable for industrial purposes (*Hu et al, 2014*). According to *Figure 1.1.,* many countries in the world are facing arsenic contamination due to extended mining activity. The highest number of people at risk is in Bangladesh, Nepal, western Bengal of India, and several regions of China, Argentina, northern Mexico, Hungary, Chile, Bolivia, Taiwan, Cambodia, Thailand, Vietnam, Pakistan and Burma (*Garelick and Jones, 2008*).

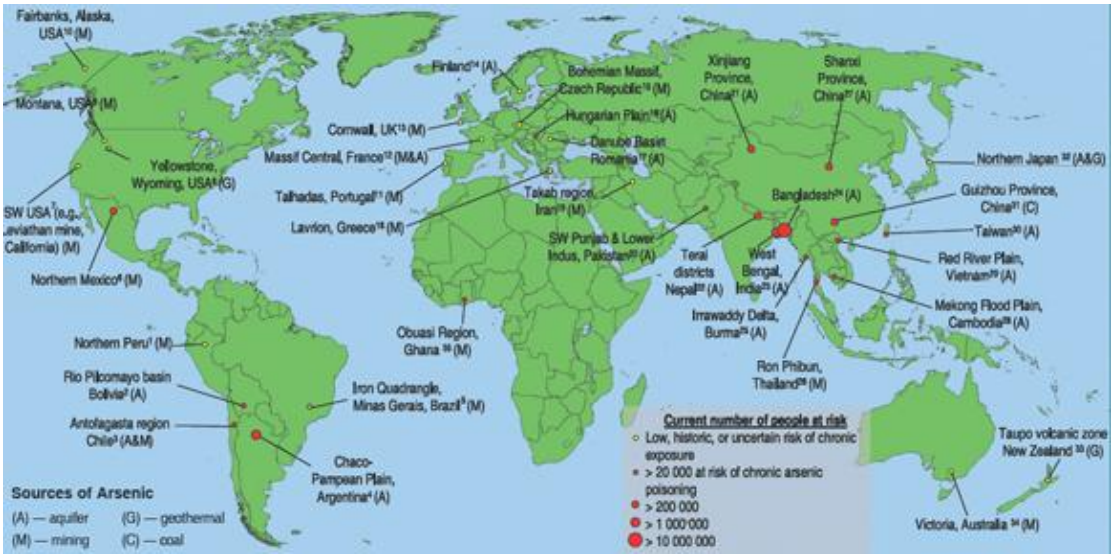

Figure 1.1.: Worldwide distribution of Arsenic contaminated regions (*Garelick and Jones, 2008*)

Table 1.1.: Global mine production and consumption 1970, 1990 and 2000 (*USGS, 2002a*)

Application	1970 (1000 tones)	1990 (1000 tones)	2000 (1000 tones)
Total mine production	3390	3370	3100
Total consumption	4502	5627	6494
Secondary production in percentage of total consumption	25%	40%	52%

Secondary production is defined as the difference between total consumption and mine production. According to *table 1.1,* secondary production is increased through the previous decades and total mine production is constantly decreased. That means, the primary production becomes more expensive and the secondary production relates to more effective and environmental friendly (recycling) way to have metal extraction. In highly industrialized countries, the rates of secondary production are higher than in other countries. For example, processing of lead scrap in North America is about 70%, in Western Europe about 60%, in Africa 50%, in Latin America under 50% and in Asia lees than 30% (*Thornton et al, 2001*). The main source of secondary lead is coming from used lead batteries.

1.2. Global heavy metal distribution

The definition of reserves according to USGS is the part of the resources which could be economically extracted or produced at the time of determination and reserves include only recoverable materials. According to *Table 1.2.1.*, the reserves follow the mine production of each country. Australia is the first one at the mine production, China along with United States of America follows closely. Lead rich materials are accompanied by other metals such like silver, zinc, copper and sometimes, gold. The two thirds of worldwide lead output are coming from mixed lead – zinc ores (*Ayres et al, 2002*).

Table 1.2.1. : Mine production and reserves by country, 2000 – 2001 (*USGS, 2002a*)

Country	Mine production 2000/1000 tones	Reserves 2001/1000 tones
Australia	699	15000
China	570	9000
USA	468	8700
Peru	271	2000
Mexico	156	1000
Canada	143	1600
Sweden	108	500
Morocco	80	500
South Africa	75	2000
Kazakhstan	40	2000
Other countries	490	22000
World (rounded)	3100	64000

1.2.1. North America

High values of arsenic have been found in U.S. groundwater, and high levels have been found in New England, the Great Lakes region, the western U.S. and New Mexico next to Middle Rio Grande basin (*Bexfield and Plummer 2003*). In Canada high arsenic levels observed and especially in regions of British Columbia, Manitoba, Alberta, New Branswick, Labrador, Nova Scotia, Quebec and Saskatchewan (*McGuigan et al, 2010*). Also, high copper and nickel levels have been found in some Canadian bottled drinking water (*Dabeka et al, 2002*). In Mexico and especially in the northern area, arsenic, mercury and lead were detected contamination in drinking water, and in other regions of Mexico as Coahuila and such as Chihuahua high concentrations of Arsenic are found mainly due to volcanic activities and mining processes (*Camacho et al, 2011*). It's notable to mention that in Kauai, Hawaii from diverse distant sources there is lead accumulation coming from anthropogenic sources in Asia and North America (*UNEP, 2010*).

1.2.2. South America

In Chile and only in some regions (i.e. Antofagasta) high levels of arsenic in drinking water have been found and especially next to the rivers which contain high inorganic arsenic levels (*Marshall et al, 2007*). In Venezuela and especially in the Coyuni river basin high levels of mercury have been observed due to nearby gold mining activities (*Garcia-Sanchez, 2008*). In Brazil, tin has been found in some water samples and drinking water samples have been found to contain high levels of iron, aluminum, manganese and arsenic (*Madrakian and Ghazizadeh, 2009*). In conclusion, 4.5 million people in Latin America seem to be exposed to high arsenic levels (*McClintock et al, 2012*).

1.2.3. Europe

In Greece and especially in eastern Thessaly high levels of arsenic and antimony contamination have been reported in drinking water samples (*Kelepetsis et al, 2006*). Manganese and iron have been observed in northern/eastern Greece (*Tsoumbaris et al, 2007* and *Doulgeris et al, 2007*). Lead has been observed in the drinking water of Austria (*Haider et al, 2002*) and Spain (*Extabe al, 2010*). Also, high arsenic levels have been found in regions of Serbia, Croatia (*Cavar et al, 2005*) and southern Tuscany in Italy (*Tamasi and Kini, 2004*). In Belgrade, high levels of radioactive uranium and strontium have been found in drinking water (*Rajkovic et al, 2008*). In Germany, and Greece elevated uranium has been observed using the method of taking samples from private wells. Also, nickel has been found in Denmark's water (*Nielsen, 2009*). Using the method of bottled water samples in Europe, 4.63% were shown to be over the acceptable limits for arsenic, barium, nickel or manganese and ten samples showed radioactive uranium concentrations to be over the acceptable limits (*Birke et al, 2010*). It's worth noting that in some bottled water in Germany high concentrations of tellurium have been found (*Andreae, 1984*).

1.2.4. Oceania

In Australia, and especially in northern Queensland, high levels of copper, lead and zinc have been reported (*Taylor and Hudson – Edwards, 2008*). The drinking water of Australia and New Zealand is coming from rainwater and the untreated water has been linked to heavy metal health risks.

1.2.5. Africa

Arsenic, mercury, manganese, iron and lead have been found in drinking water of Ghana (*Asante et al, 2007* and *Buamah et al, 2008*). Elevated cadmium and arsenic levels have been observed in water samples from South Africa and especially in Koekemoerspruit (*Dzoma et al, 2010*).

1.2.6. Asia

In Asia, and especially in northern and eastern countries such like Nepal, Thailand, Bangladesh, Taiwan, Vietnam, Cambodia and India high levels of arsenic have been observed in the groundwater and subsequently in the drinking water. In India, heavy metal pollution is linked to health problems such as skin lesions and reduced lung function. In India, and especially in Assam, excessive levels of lead have been observed in the drinking water (*Borah et al, 2010*). Regions next to the Kali River are exposed to high iron levels (*Chaudhary and Kumar, 2009*). Another country that has major problems with heavy metal pollution is Bangladesh in which high levels of uranium, manganese, arsenic, lead, nickel and chromium have been found (*Frisbie et al, 2009*). Drinking water of Pakistan has been found to contain high levels of arsenic in 58% of samples taken and in other regions of Pakistan (Skardu) shows excessive levels of zinc, iron, nickel, lead, cobalt, copper and chromium (*Lodhi et al, 2008* and *Nickson et al, 2005*). Vietnam has high levels of arsenic, barium, cadmium, nickel, tin, lead and uranium in the drinking water. Sri Lanka populations are at risk due to high levels of cadmium in drinking water (*Bandera et al, 2010*). In Indonesia (*Limborg et al, 2004*) and the Philippines (*Cortes – Maramba et al, 2006*) high levels of mercury have been observed in drinking water. Also, high levels of uranium concentrations have been found in central Asia countries, such as Kazakhstan and they are associated with kidney dysfunction problems (*Chiba and Fukuda, 2005*). In China, and especially in Shanghai, there are risks of copper, zinc and arsenic (*Xu et al, 2006*). A study has shown slow long – range transport of air pollution (including lead) from continental Asia (*UNEP, 2010*).

1.2.7. Antarctica

Stability of the water column and subsequently the trace metal distribution and speciation can be influenced by sea ice coverage and melt water percentage. Concentrations of heavy metals such as V, Cr, Mn, Cu, Zn, Co, Ag, Cd, Ba, Pb, Bi and U have been observed in snow samples from 1834 to 1990, collected at remote and low accumulation sites in Coats Land in Antarctica. Although, the concentrations were extremely low, down to 3×10^{-15} g g^{-1} for most metals. Lithogenic material is the major source of As, Cd, Cu, Mo, Pb, Sn and Sb in snow. Eolian deposition is the dominant source of As, Mo, Cu and Pb in Antarctic dry valley aquatic ecosystems comparing the distributions of these elements between snow, supra and pro-

glacial melt streams. Mercury is a globally dispersed toxic metal that affects even remote polar areas, atmospheric mercury depletion events are responsible of Hg transfer from the atmospheric reservoir to arctic snow packs (*Larose et al, 2011*). Arctic, where there are a few local sources for lead releases, as a remote region shows contribution of lead by regional and intercontinental atmospheric transport (*UNEP, 2010*).

1.3. Worldwide heavy metal pollution in numbers

The main emissions of metals to the atmosphere are nickel, lead and copper (*Table 1.3.1.*). Lead is often delivered from sulphide ores and most of the times combined with other elements like zinc, copper and silver (*UNEP, 2010*). Lead and zinc have the highest values of emission to the atmosphere from anthropogenic sources (*Table 1.3.2.*). About 2/3 of worldwide inputs are obtained from mixed lead – zinc ores (*UNEP, 2010*). Stationary combustion causes 59% of nickel emissions according to the publication of TNO in 1990 (*Berdowski et al, 1997*). Nickel sulfate and oxidic nickel are emitted by combustion plants. Other industrial sources may emit metallic nickel and in the case of two nickels refineries in Europe, nickel sub - sulfide. Heavy metal environmental pollution is very usual in territories which are surrounded by old mines and mining activities. The reduction of the pollution comes when the distance increases. These metals are leached out following sloppy areas and are carried by acid water downstream or run off to the sea (*Duruibe et al, 2007*). On the other hand, some metals are transferred by gas exchange at the sea surface, by dry (particles fall out) or wet (rain) deposition (*Valavanidis and Vlahogianni, 2016*). In addition, rivers make a huge contribution of metals in the marine environment. Lead, zinc, nickel and copper numbers indicate that anthropogenic sources are responsible for this pollution (*Tables 1.3.1. and 1.3.2.*). 2700 years ago, lead showed very low levels and the concentration increased when industrial activities rose rapidly and afterwards when lead was used as an antiknocking additive in gasoline fuels for vehicles. Natural sources for metal inputs are erosion, volcanic activity, wind - blown dust and forest fires. For example, aluminum transports via wind – blown dust of rocks and shales and mercury from volcano activities. It's very complex to distinguish between anthropogenic and natural sources of pollution and a very good example is mercury pollution. 150.000 tones of Hg released as a result of earth's crust degassing and 8.000 – 10.000 tones are coming from the industry and urban activities (*Goyer, 1991)*.

Table 1.3.1.: Worldwide emissions of metals to the atmosphere from natural resources (thousand tons per year) (*Clark et al, 1997*)

Metals	Thousand tones per year
Ni	26
Pb	19
Cu	19
As	7,8
Zn	4
Cd	1
Se	0,4

Table 1.3.2.: Worldwide emissions of metals to the atmosphere from anthropogenic sources (thousand tons per year) (*Clark et al, 1997*)

Metals	Thousand tones per year
Pb	450
Zn	320
Ni	47
Cu	56
As	24
Cd	7,5
Se	1,1

1.3.1. Cadmium

Cadmium is found to be applicable as stabilizers in PVC products, color pigments, several alloys and these days most commonly, in rechargeable batteries. The environment is being contaminated through the process of incineration of the household waste which contains garbage with heavy metal content. At the agricultural sector, the use of phosphate fertilizers and sewage sludge has as an impact the soil contamination and subsequently the cadmium uptake to crops and vegetables (*Table 1.3.1.1.*).

Table 1.3.1.1.: Sources of cadmium waste disposal in the E.U. about 2000 (*Scoulos et al, 2011*)

Sources	Tones/year	% Total
Cadmium processing	400 (approx)	16
Coal ash	113	4
Sewage sludge	70	3
Phosphate processing	60	2
Iron and steel processing	230	9
Cement production	280	11
Non ferrous metals processing	419	17
Total industrial sources	1572	62
Municipal waste or mixed direct input to landfills	800	32
MSWI ashes	150	6
Total municipal waste	950	38
Total to land	2.522	

According to *Table 1.3.1.2.*, the anthropogenic emissions of cadmium during 1983 was around 7600 tones and by the mid '90s a major decrease of 60.59% was observed. This decrease occurred due to improved fuel gas cleaning. Non – ferrous metal production is by far the major source of cadmium emission to the air. On the other hand, in Denmark, where

incineration is extensively used, cadmium emission by waste incineration accounts to 50% and from combustion of oil products accounts to 35% (*Drivsholm et al, 2000*). In United States, the combustion of coal and oil accounts to 76% of total cadmium emission to air and incineration only to 7% (*OECD, 1994*).

Table 1.3.1.2.: Global emissions of cadmium to air in mid 1990's (*Pacyna et Pacyna, 2001*)

Economic sector	Air emission (tones)	Percentage
Stationary fossil fuel combustion	691	23
Non – ferrous metal production	2.171	73
Iron and steel production	64	2
Cement production	17	0,6
Waste disposal (incineration)	40	1,3
Total	2983	
Total, 1983	7570	

Atmospheric deposition is one of the major sources of cadmium to soil accounted to 2500 – 15000 tones yearly and 7500 to 29500 tons per year are located in landfill as discarded products and production waste (*Table 1.3.1.3.*)(*Nriagu and Pacyna, 1988*). The yearly accumulation of cadmium at the top soil in Denmark, Sweden and The Netherlands is accounted on 0.3% for Denmark and 0.6% to 0.7% for The Netherlands and the major sources are the atmospheric deposition and the use of commercial phosphate fertilizers in farming activities (*OECD, 1994*). In countries, such as Austria, Ireland, Greece and the United Kingdom, the increase of cadmium pollution over 100 years is estimated between 4 – 43% due to the use of phosphate fertilizers. The use of fertilizers containing very low cadmium concentration (<7mg Cd/kg P_2O_5) are responsible for the change between -75% to 11% over 100 years for Finland and Sweden. At the farming sector of Belgium, the use of fertilizers with medium cadmium concentrations (~33mg cd/kg P_2O_5) is giving a change over 60 years of -75% to 120% (*Hutton et al, 2001*). Major sources of direct cadmium release to the water are atmospheric deposition (*Nriagu and Pacyna, 1988*), domestic wastewater, non - ferrous metal smelting/refining and manufacturing of chemicals and metals (*WHO, 2003*). According to OSPAR commission, the accumulation at river and lakes sediments is 5mg/kg, marine sediments 0.03 to 1.1 mg/kg, open seas 5 to 20ng/L, French and Norwegian coastal zones 80 – 250ng/L and European rivers 10 to 100ng/L (*OSPAR, 2002*).

Table 1.3.1.3.: Global cadmium releases to land, 1983 (*Nriagu et Pacyna, 1988*)

Source	1000 tones	% of discharge to land
Agricultural and food wastes	0 – 3	6
Animal wastes, manure	0,2 – 1,2	3
Logging and other wood wastes	0 – 2,2	4
Urban refuse	0,88 – 7,5	15
Municipal sewage sludge	0,02 – 0,34	0,7
Miscellaneous organic wastes including excreta	0 – 0,01	0,0
Solid wastes/metal manufacturing	0 – 0,08	0,1
Coal fly ash, bottom fly ash	1,5 – 13	26
Fertilizers	0,03 – 0,25	0,5
Peat (agricultural and fuel use)	0 – 0,11	0,2
Wastage of commercial products	0,78 – 1,6	4
Atmospheric fall out	2.2 – 8.4	19
Total to soil	5,6 – 38	
Mine tailings	2,7 – 4,1	12
Smelter slugs and wastes	1,6 – 3,3	9
Total discharge to land	10 – 45	

1.3.2. Lead

Lead as heavy metal element has very low exposure levels of toxicity. This heavy metal can affect plants, animals and microorganisms and it has been reported bioaccumulation in many organisms. Biological particles containing lead have residence times in surface waters for more than two years. The mobility of lead is very slow in soil and the only way to enter surface waters is by erosion of lead containing soil particles and the dumping of waste containing lead products (i.e. Batteries) (*UNEP, 2010*). Lead's oceanic time residence ranges from about 100 to 1000 years, which may is a potential for ocean transport. The contribution of lead to the marine environment from Europe and especially Belgium, The Netherlands, Denmark, France, Germany, Norway, Sweden and the United Kingdom using rivers as a pathway is currently larger than the airborne inputs (*UNEP, 2010*).

According to the *table 1.3.2.1.,* in 1983 the total emission of lead to air was almost three times higher than 1990. This decrease occurred due to the reduction to use of lead as a fuel additive. The total emissions of lead at the mid '90's were 120000 tones, of which 89000 were coming from petrol additives (*UNEP, 2010*).

Table 1.3.2.1.: Global emission of lead to air in mid – 1990's (*Pacyna et Pacyna, 2001*)

Economic sector	Air emission (tones)	Percentage
Stationary fossil/fuel combustion	11.960	10
Non – ferrous metal production	14.815	12
Iron and steel production	2.926	3
Cement production	268	0,2
Fuel additives	88.739	74
Waste disposal (incineration)	821	0,7
Total	119.259	100
Total emission to air (1983)	332.350	

According to the *Table 1.3.2.2.*, fuel additives, non – ferrous production and coal combustion were the main sources of lead emission to the air in 1998. In EU15, the major source of lead emission to the air was road transport because it used lead as a fuel additive. Nowadays, this percentage has been decreased and the main sources of lead emission to the air are non ferrous production and coal combustion.

Table 1.3.2.2.: Sector emissions of lead to air in the E.U. 15 in 1998 based on EMEP (*Tukker et al, 2001*)

Economic sector	Air emission (tones)	Percentage
Energy generation		
Combustion in energy and transformation industries	20	1
Combustion in manufacturing industry	441	17
Extraction and distribution fossil fuels and geothermal energy	0,0	0
Non – industrial combustion plants	20	1
Production processes	358	14
Road transport (petrol additives)	1560	61
Waste treatment (MSWIs)	124	5
Other (not included)		
Other mobile sources and machinery	34	1
Other sources and sinks	0,02	0
Solvent and other product use	1	0
Agriculture	0,1	1
Total	2.558	100

Apparently, according to the *Table 1.3.2.3.*, only atmospheric fall – out has decreased due to the use of unleaded fuels for the automobiles. No major differences among the distribution of other categories are noted. Farmlands in Denmark and The Netherlands show lead accumulation in top soil. The accumulation rate has been calculated for Denmark and The Netherlands, 0.08% and 0.06 – 0.2%, respectively. The major source of lead is atmospheric deposition and accumulation rates may be even higher for grasslands (*Bronnum and Hansen, 1998, Tukker et al, 2001*). Additionally, the use of lead shots in Greenland is the major source of lead and affects the human diets (*ASAP, 2002*).

Table 1.3.2.3.: Global lead releases to land in 1983 (*Nriagu at Pacyna, 1988*)

Source category	1000 tones/year	% of total to land
Agricultural and food wastes	1.5 – 27	1.1
Animal wastes, manure	3,2 – 20	0.9
Logging and other wastes	6.6 – 8.2	0.6
Urban refuse	18 – 62	3.1
Municipal sewage sludge	2.8 – 9.7	0.5
Miscellaneous organic wastes including excreta	0.02 – 1.6	0.1
Solid wastes, metal manufacturing	4.1 – 11	0.6
Coal fly ash, bottom fly ash	45 – 242	11
Fertilizer	0.42 – 2.3	0.1
Peat (agricultural and fuel use)	0.45 – 2.0	0.1
Wastage of commercial products	195 – 390	22
Atmospheric fall out	202 – 263	17
Total in soil	479 – 1113	
Mine tailings	130 – 390	19
Smelter slugs and wastes	195 – 390	22
Total to land	804 - 1820	

The speciation of lead in fresh and sea water is different. In fresh water, lead occurs is primarily as a divalent cation (Pb^{2+}) under acidic conditions and it forms $PbCO_3$ and $Pb(OH)_2$ under alkaline conditions. In seawater the speciation is a function of chloride concentration and the primary species are $PbCl_3^-$>$PbCO_3$>$PbCl_2$>$PbCl^+$>$Pb(OH)^-$ (*UNEP, 2010*). Environmental conditions as water hardness, pH and salinity affect the toxicity of inorganic lead salts (*UNEP, 2010*). Populations of invertebrates from polluted areas can show more tolerance to lead exposure than those of non – polluted areas. Lead toxicity can result in symptoms like spinal deformity and blackening of the tail region. The typical lead levels in aquatic environments, for seawater are <1μg/L and for freshwater <5μg/L (*OECD, 1994*), which is relatively low compared to lead levels causing effects. *Table 1.3.2.4.,* presents data on the accumulation tendency of lead in several species and the type of effects it may have.

Table 1.3.2.4..: Toxicity of lead in aquatic environments and bioaccumulation factors (*Brønnum et Hansen, 1998*)

Species	Effect	Concentration (µg/kg Ts)
Toxicity in fresh water		
Plankton algae	EC_{50}, growth rate etc	140 – 11000
Crustaceans	Chronic	10 – 200
Crustaceans	Acute	100 – 224000
Fish	Chronic	0.4 – 220
Fish	Acute	1000 – 540000
Toxicity in marine environment		
Plankton algae	EC_{50}, growth rate etc	20 – 950
Crustaceans	Chronic	20 – 40
Crustaceans	Acute	50 – 27000
Fish	Acute	300 - >10000
Bioaccumulation		
Algae	Bio – concentration factor	2000 – 4000
Invertebrates	Bio – concentration factor	400 – 12400

1.3.3. Nickel

Nickel is characterized as a hard silvery white metal and its compounds are practically insoluble in water include carbonate, sulfides (NiS and Ni_3S_2) and oxides (NiO and Ni_2O_3). Its compounds are formed in several oxidation states - its divalent ion seems to be the most important for both organic and inorganic substances, although the trivalent form may be generated by redox reactions in the cell. Soluble nickel salts include chloride, sulfate and nitrate. $Ni(CO)_4$ is a volatile and colorless liquid which has a boiling point of 43°C and can be decomposed at 50°C and above. Nickel can be found in sulfide or silicate – oxide ores, which in general contain no more than 3% nickel. In biological systems, nickel can form complexes with triphosphate adenosine, amino acids, peptides, proteins and deoxyribonucleic acid. As it seems from the data shown in *Table 1.3.3.1.*, windblown dust is the major source of nickel emissions into the atmosphere and the second is volcanogenic particles. According to *Table 1.3.3.2.,* the major distribution in nature is due to meteorites and for anthropogenic sources, the main reason is coal combustion.

Table 1.3.3.1.: Worldwide emission of nickel from natural sources in 10^3 t/a (*Crecelius et Sanders, 1980*)

Source	**Ni 10^3 t/a**
Windblown dust	20 (0.2 – 44)
Volcanogenic particles	3.8 (2.4 – 82)
Forest wildfires	0.6 (0.05 – 3.3)
Vegetation	1.6 (1.6 – 2.1)
Sea salt	0.04 (0.01 – 0.05)
Total	26

Table 1.3.3.2.: Nickel distribution worldwide (*WHO, 2000*)

Distribution in Nature	**%**
Earth crust	0,008%
Earth core	8,5%
Deep sea nodules	1,5%
Meteorites	5 – 50%
Natural Background levels	**µg/litre**
Open ocean water	0,228 – 0,693
Fresh water systems	<2
Anthropogenic sources	**mg/kg**
Agricultural soils	3 – 100
78 forest floors samples NE USA	8,5 – 15
Coal	300
Crude oils	<1 – 80

Nickel ore has two classes, the sulfide ores from which the most nickel comes out the silicate oxide. Canada and Russia account for 20 – 25% each of total annual production, which were 784.82 thousand tons in 1988.

The main anthropogenic sources for nickel emissions to the atmosphere are coming from procedures like burning of residual and fuel oils, nickel mining and refining and municipal waste incineration. In a remote area of Arctic Canada levels of 0,38 – 0,62 ng/m^3 have been recorded, in comparison to 124 ng/m^3 in the vicinity of a nickel smelter have been recorded. In Norway, 1 ng/m^3 was recorded in an unpolluted area, whereas this was about 5 ng/m^3 5 km away from a nickel smelter (average values 1990 – 91). Concentrations of 18–42 ng/m^3 were recorded in 8 United States cities (*Saltzman et al, 1985*). These values correspond to the average value of 37 ng/m^3 for 30 United States urban air national surveillance network stations for the period 1957 – 1968. There was a decrease of this average for 1965 – 1968, from 47 ng/m^3 to 26 ng/m^3. The arithmetic value of mean for 1970 – 1974 was 13ng/m^3 and in European cities ranges of 10 – 50 ng/m^3 and 9 – 60 ng/m^3 were reported (*Schmidt and Andren, 1990*). The observations of high values in heavily industrialized areas and they were around 110 – 180 ng/m^3 (*WHO, 2000*). According to *Table 1.3.3.3.*, the main intermediate us is steel production (metallurgy) and main end use is in transportation.

Table 1.3.3.3.: Intermediate and end uses of Nickel (*WHO, 2000*)

Intermediate uses	**%**
Steel production	42
Other alloys	36
Electroplating	18
End uses	**%**
Transportation	23
Chemical industry	15
Chemical equipment	12
Construction	10

1.4. Cadmium, Lead and Nickel origins and effects on human health

Heavy metals are defined as the metals which have a specific density of more than 5g/cm^3 and are poisonous even at low concentrations. Heavy metal pollution is caused by their release from both anthropogenic and natural sources, more significantly by mining and industrial activities and automoblile left over products (lead). Humans are exposed to cadmium by consumption of contaminated vegetables and crops and through cigarette smoking which increases the blood cadmium levels (B – Cd). The concentrations of blood Cd in smokers is on average 4 – 5 times higher than those in non – smokers. The most important source of cadmium is food, although this depends on the dietary habits of the individual which plays an important part for cadmium intake. It is well known that women's energy consumption is lower than that of men, so they have a lower daily cadmium intake. In general, B – Cd is an indicator of current exposure, but also partly of lifetime body burden. Cadmium concentration in urine shows a better image because it is mainly influenced by the body burden. U – Cd is proportional to the kidney concentration and smokers have higher urinary cadmium concentration than non – smokers (*Jarup, 2003*). The concurring health risks due to long term cadmium exposure are skeletal damage and cardiovascular mortality. Cadmium is classified as a human carcinogen (group I) and can cause lung, prostate and kidney cancer. However, the evidence for cadmium as a human carcinogen is rather weak.

Lead can be found in foodstuff originated from pots used for cooking and storage (*Jarup, 2003*). Lead emissions come from petrol, although nowadays the lead emissions have been decreased due to the introduction of unleaded petrol. Exposure to inorganic lead occurs in mines, smelters, welding of lead painted metal and battery plants. Also, low or moderate exposure may take place in the glass industry (*Jarup, 2003*). Airborne lead can be deposited on soil and water, so that it can subsequently reach humans via the food chain. More than 50% of inhaled inorganic lead may be absorbed in the lungs. More than 50% of lead intake in children occurs via the gastrointestinal tract whereas in adults this is around 10 – 15%. It is worth mentioning that the half life of lead in blood is about 1 month and in bones even around 20 – 30 years (*WHO, 1995*). About organic lead, its compounds can penetrate the body and cell membranes. Tetramethyl and tetraethyl lead penetrate the skin easily and have the ability to cross the blood brain barrier in adults that consequently can suffer from lead encephalopathy due to the acute poisoning by organic lead. Acute lead poisoning

evokes symptoms as headache, irritability, abdominal pain and various symptoms to the nervous system. Lead encephalopathy's symptoms are sleeplessness and restlessness. Behavioral disturbances and learning/concentration difficulties occurs in children. When individuals are exposed to lead for long time they show symptoms like memory deterioration, prolonged reaction time and reduced ability to understand (*Jarup, 2003*). That is the reason why IARC classified lead as a possible human carcinogen in 1987 (*Jarup, 2003*). However, since that period many results were obtained and they provided evidence that lead was a weak carcinogen. Lead absorption is related to lung cancer, stomach cancer and gliomas (*Steenland and Boffetta, 2000*).

Exposure to nickel may occur through inhalation, ingestion and skin contact. Most of the individuals take in nickel through food consumption and the average daily intake from food in the U.S.A. is estimated at 150 – 168 μg. In addition, the general population is exposed to nickel in nickel alloys and nickel plated materials such as coins, steel and jewelry and residual nickel can be found in soaps, fats and oils. Nickel in coinage, other manufactured products and household appliances maybe important for some health effects such as dermatitis (*WHO, 2000*). Nickel is a human carcinogen and nickel compounds, nickel sulfides and oxides can cause an elevated risk of death from lung and nasal cancer.

1.5. Project Goal

Biochemical and molecular biomarkers are crucial indicators for aquatic organisms exposed to chemical contaminants. Especially, biomarkers are estimated to be early warnings and signals of stresses and contaminants, subsequently offer detailed and comprehensive information about toxicity level in the environment. The examination of antioxidant enzymes, genes, metabolites and secondary metabolites of the unicellular photosynthetic organism *Chlamydomonas reinhardtii* may lead to monitor metals in the aquatic environment. The abovementioned biomarkers may lead to safe conclusions to improve our knowledge on how photosynthetic organisms deal with various concentrations of nickel, cadmium and lead, either as single exposures, either as mixtures. The study of differentially expressed genes could lead to different metabolic pathways. Although, cDNA microarray must be performed in order firstly to cover the whole genome of the organism and secondly to obtain a better screening about the mechanisms which cope with the responses of *Chlamydomonas reinhardtii*.

Chapter 2: Background

2.1. Microalgae

Microalgae are divided in prokaryotic and eukaryotic, terrestrial and aquatic and they have a wide range of environmental conditions in which the species can survive. Among the main benefits is the rapid rate of microalgal growth and the high photosynthetic efficiency, and for these reasons they are cultivated industrially. One of the main applications of microalgae is to work as a platform for the production of bioethanol because of advantages such as high capacity for CO_2 capture, large quantities of synthesized carbohydrates and great tolerance to differences in abiotic factors (temperature, pH, etc) (*Subhandra and Edwards, 2010*). Another application of microalgae is as food supplements and in pharmaceutical use. Some indicative therapeutic supplements are β – carotene, astaxanthin, polyunsaturated fatty acids (PUFA) and polysaccharides such as β – glucan (*Priyadarshani and Biswajit, 2012*). One of the major crucial advantages of microalgae is the metabolic engineering method which can provide very good results for increasing the lipid content and subsequently to achieve higher values of oil. For example, the overexpression of a DOF – type transcription factor (DNA – Binding with One Finger) in *Chlamydomonas reinhardtii* which was applied as an approach to increase the amount of lipids (*Ibanez – Salazar et al, 2013*). Another application at the molecular level using genetic engineering is to make a biopharm of the chloroplast of *Chlamydomonas reinhardtii*. In other words, one wants to produce health related products such as antibodies, antigens, human blood components, growth factors etc from plants. The most important advantages of genetic engineering in plants and algae are the following: the product is free of human pathogens and more quality consistent, larger quantities can be obtained in a reduced or confined area and the production cost is low (*Almaraz – Delgado et al, 2014*).

2.2. *Chlamydomonas reinhardtii* as a model organism

Chalmydomonas reinhardtii is a unicellular eukaryotic photosynthetic organism, oval shaped usually 10μm in diameter and containing two flagella for its motility. *Chlamydomonas* has served as a model organism to study the effects of environmental stresses, eukaryotic photosynthesis and to study the functions of eukaryotic flagella. For these reasons, the genome of *Chlamydomonas reinhardtii* has been completely sequenced by the researchers since 2007 (*Merchant et al, 2007*).

2.2.1. Growth model of *Chlamydomonas reinhardtii*

The growth model of algae culture has several phases and may be different depending on growth conditions. The first phase is the lag phase, in this phase the growing cells must adapt to the new environmental conditions. The length of the lag phase depends on the new conditions and the physiological condition of the inoculated cells. The next phase is the accelerating growth phase in which the growth increases because the cells are more adaptive to the new conditions. The next phase is the logarithmic phase of exponential growth where the cells haven't used all the nutrients and they don't have limitations to the

light due to the cells density. The next phase that follows is called the decreasing logarithmic growth where the growth rate is more linear and the culture is denser and with light limitations. The cells have also used most of the nutrients and begin to release toxic substances. The next phase is called the accelerated death phase in which the number of cells are decreased and this results in the release of growth inhibiting materials. During the last phase which is the log death phase, the death rate is exponential and the algae cells all die. During the growth of algae, the pH increases due to the release of hydroxide molecules from the photosynthesis process. During the growth, water reacts with carbon dioxide to result in carbonic acid, which can give hydrogen ions and bicarbonate. The algae take up the carbon in the bicarbonate form and release oxygen and OH^- molecules through photosynthesis, which increases the environmental pH.

2.3. Environmental stress responses in *Chlamydomonas*

There were several interesting results in publications about exposure of algae to heavy metal compounds (*Jamers et al., 2013; Jamers et al., 2006; Collard and Matagne., 1993; Nagel and Voigt, 1994*). The exposure of algae to several concentrations of heavy metal compounds can serve as a tool for the research for potential biomarkers such as the quantification of genes being expressed (*Hutchins et al., 2010; Jamers et al., 2013; Jamers et al., 2006*) and determination of enzyme activities and kinetics (*Aksmann et al, 2014*) at different time points during heavy metal exposure. It's notable that combinations of heavy metal exposure with different nutrient limitations (*Webster et al., 2011; Vega et al., 2005; Devriese et al., 2001; Mosulen et al., 2003*) and reduced light intensity have offered results(*Nowicka et al, 2016*). *Chlamydomonas reinhardtii* can offer several advantages such as easy manipulation and control of metal concentrations in the growth medium to create metal deficiency and metal overload situations. Additionally, due to its complete sequenced genome, its use can offer the identification and knowledge of several transition metal transporters, genes responsible for antioxidant activities and the preparation of mutants.

2.4. Risk assessment of metal mixtures

Most aquatic organisms are exposed to more than a single element. The responses of algae to metal mixtures need further consideration in order to find out potential interactions of their effects to the growing algae (*Flouty and Khalaf, 2015*). When applying different metals simultaneously, there is a complexity in the analysis of the results since there might be interactions of the metals with media constituents, with physiological processes and interactions at the area's toxicity (*Flouty and Khalaf, 2015*). The uptake of non essential metals appears to follow the same pathways as the essential metals, which are necessary to meet the metabolic requirement (*Flouty and Khalaf, 2015*).

2.5. Transition Metal Function

Transition metals are divided in essential and non – essential metals. Essential metals are important cofactors for many enzymes which are responsible during mitochondrial and plastid functions. Non – essential metals are in general characterized as toxic in low concentrations. Although, a non – essential element like cadmium behaves as a metal

cofactor of a carbonic anhydrase isoform produced under zinc deficiency in the diatom *Thlassiosira weissflogii*. This is evidence that occasionally non essential metals can function as prosthetic groups in biologically active metalloproteins. Copper is a notable example, which works as a cofactor for plastocyanin and cytochrome *c* oxidase for plastids and mitochondria, respectively. On the other hand, the non - essential metal ions lead to cell poisoning and affect the survival of the organism dangerously. For the regulation of this, it's necessary to develop a tightly controlled and sophisticated metal homeostasis network that offers the opportunity to control the balance among the metal uptake, chelation, distribution and storage processes. Using species with already sequenced genomes offers the opportunity of the identification of complete protein families that result in the construction of a metal homeostasis network. Cofactors that are involved in electron transport chains in chloroplasts serve as an essential component of the metal homeostasis network in photosynthetic cells. In conclusion, the investigation and subsequently the control of metal homeostasis in plants may lead to the achievement of aims such like pollution management, global primary productivity and development of solutions of human health issues.

2.5.1. Metal Transporters

2.5.1.1. Permeases

Permeases are divided in two functional groups. The first group is involved in the transportation of metal ions into the cytoplasm. These transporters consist of members which belong to NRAMP (Natural Resistance – Associated Macrophage Proteins), ZIP (Zrt -, Irt – like proteins), FTR (Fe Transporter) and CTR (Cu transporter) families (*Blaby – Haas and Merchant, 2012)*. Transporters of assimilation can be found in the plasma membrane. They are responsible for the increase of the intracellular concentration of metal when the equilibrium among chelating sites and metal ions is perturbed due to deficiency (*Blaby – Haas and Merchant, 2012)*. The second group is responsible for the decrease of the metal concentration in the cytoplasm. This group includes distributive transporters, which offer metal for organelle – localized – metal dependent proteins (*Blaby – Haas and Merchant, 2012*). This group consists of members from the CDF (Cation Diffusion Facilitator), P_{1B} – Type ATPases, FPN (Ferroportin) and CCC1 (Ca (II) – Sensitive Cross – complementary)/VIT1 (Vacuolar Iron Transporter 1) families. Their presence at the membranes of the secretory pathway can mediate the exocytosis of excess metal (*Blaby – Haas and Merchant, 2012*).

2.5.1.2. Cadmium associated metal transporters

Multidrug – resistance associated proteins are working as glutathione – S – conjugate pumps and belong to ABC (ATP – binding cassette) transporters. MRP2 is up regulated when *Chlamydomonas reinhardtii* is exposed to cadmium and shows a modified phytochelatin complex accumulation pattern (*Wang and Wu, 2006*). A faster rate of molecular weight PC – Cd complex formation occurs and shows an increased Cd sequestration by the PC – Cd complexes (*Wang and Wu, 2006*). In conclusion, MRP2 has a major contribution to Cd vacuolar storage. Other vacuolar transporters are SPHMT1 and eHMT – 1 and take part in the transportation of phytochelatin complexes from the cytoplasm into the vacuole and

shows contribution to cadmium tolerance. CDS1 gene is strongly affected by cadmium exposure and could directly take part in the transport of cadmium from the mitochondrial matrix and possibly works as a glutathione Cadmium conjugate pump and offers protection to the mitochondrial function from cadmium toxicity. In general, ABC transporters contribute in cadmium detoxification.

2.5.1.3. Nickel associated metal transporters

Nickel is an essential micronutrient in vascular plants and its deficiency affects the accumulation of toxic urea concentrations in several species. In *Chlamydomonas reinhardtii*, a putative high affinity nickel transporter called NIK1 has been observed as well as a putative nickel chaperone for urease or hydrogenease similar to the urease accessory protein ureG which was found in bacteria and *Arabidopsis thaliana*. Additionally, Ni^{2+} treatment shows a CuRE – dependent up regulation of CYC6, CPX1 and CRD1, similar to their induction under Cu deficiency (*Quinn et al, 2003*). This effect can be determined by studying the physiological or functional connection of the target genes and nickel metabolism, either the possible interference of Ni with Cu metabolism or the interaction with Cu signaling system.

2.5.1.4. Lead associated metal transporters

In *Chlamydomonas reinhardtii*, lead internalization is characterized by a very high rate and is already known that shows great sensitivity to dissolved lead. *Sanchez – Marin et al 2013,* has shown that Cu and Pb share a common transport pathway in *Chlamydomonas reinhardtii* at environmentally relevant metal concentrations. Nevertheless, the transport pathway of lead is still unknown so there is still a lot to be investigated.

2.6. Metal Toxicity and effects on the unicellular photosynthetic organism *Chlamydomonas reinhardtii*

The *Chlamydomonas* cell wall has a high affinity for metal cations and acts as a passive protection system against metal excess. *Chlamydomonas reinhardtii* has been exposed to several concentrations of many heavy metal elements such as arsenite, arsenate, copper, cadmium, nickel, lead, thalium, iron, mercury, selenite and zinc. Our interest is focused on cadmium, nickel and lead effects on this organism. At cadmium concentrations between 100 – 300 μM growth reduction (*Collard and Matagne, 1990*), reduction of photosynthesis by measuring the PSI activity (*Vega et al, 2006*) and reduction of chlorophyll content (*Howe and Merchant, 1992*) and inhibition of nitrate assimilation were observed (*Devriese et al, 2001* and *Mosulen et al, 2003*). The effects of cadmium exposure at lower levels (10 – 100μM) were an inhibition in photoactivation and subsequently of the mechanisms of photosynthesis due to the competition for binding to the calcium site of photosystem II during the assembly of the water – splitting complex (*Faller et al, 2005*). Concerning lead, *Chlamydomonas reinhardtii* has shown reduction of photosynthesis determined by measuring the oxygen evolution and ultrastructural changes, specifically in thylacoids, mitochondria and nucleus at Pb levels of 1 – 20μM. At the lowest concentration 2.5 – 10μM of lead exposure an inhibition of external carbonic anhydrase was observed (*Wang et al, 2005*). At 3 – 80μM of nickel exposure a stimulation of photosynthesis by measuring the

oxygen evolution was observed (*Danilov and Ekelund, 2001*). From these data it can be concluded that, photosynthesis constitutes the primary target of metal toxicity. When there is metal overload, the main response is the induction of oxidative stress protection mechanisms and the induction of protein chaperones, which also requires metabolic adaptations to support the synthesis of metal chelators, such as GSH and PC.

2.7. Reactive Oxygen Species (ROS)

The generation of Reactive Oxygen Species occurs during mitochondrial oxidative metabolism and in cellular response to xenobiotics, cytokines, and bacterial invasion (*Ray et al, 2012*). The production of ROS takes place in both unstressed and stressed cells and occurs at several subcellular locations. The endogenous generation is observed during certain developmental transitions and as a result of normal, photosynthetic and respiratory metabolism (*Grene, 2002*). ROS play a crucial role in plants and co - regulate processes such as growth, development, response to biotic and abiotic stresses, and programmed cell death (*Bailey – Serres and Mittler, 2006*). ROS are characterized as important environmental sensors and/or modulators of global patterns of gene expression in defense and development (*Grene, 2002*). They play a major role in endonuclease activation and consequently are responsible for DNA damage. Exposure to metal ions such as Fe or Cu (II), causes the rapid formation of hydroxyl radicals. Biologically important molecules like nucleic acids can be extensively damaged by the presence of hydroxyl radicals. Hydroxyl radicals can modify proteins and make them more susceptible to proteolytic threat. Specific endopeptidases such as one bound to the thylacoid membrane can break damaged proteins. A thylacoid – bound endopeptidase, the activity of which increased under photooxidative environmental conditions and treatment with an OH – generation system, was partially purified and characterized as a serinotype protease (*Casano et al, 1994*). The demonstration of a multicatalytic proteinase system in plant systems has been generated with the capacity to break down selected oxidative damaged proteins. Selective degradation of oxidatively damaged proteins allows proteolysis of systems to function directly in the removal of useless cellular debris and consequently to allow the prevention of the accumulation of potentially toxic fragments or large aggregates of cross – linked proteins (*Grune and Davies, 1997*). In eukaryotic organisms, the lumen of the rough endoplasmatic reticulum is the location of the formation of disulfide bonds (Cys – S – S – Cys), confined to secreted proteins and to the exoplasmatic domains of membrane proteins. These bonds stabilize the tertiary structure of these proteins and are therefore of great importance. The tripeptide glutathione is the major thiol – (SH-) molecule in eukaryotic cells and is responsible for the prevention of disulfide bonds formation at the cytosol and the catalysis of their formation at the rough endoplasmatic reticulum. Thiol redox regulation is partially mediated through the redox state of the glutathione pool (GSH/GSSG) and the regulation of the glutathione biosynthetic pathway. When oxidative stress occurred, the reduced glutathione pool is converted to oxidized glutathione (GSSG) and glutathione biosynthesis is stimulated. Biosynthesis of glutathione is affected by the step of gamma – glutamylcysteine synthetase, which is feedback regulated by GSH and is controlled by the level of available L – cysteine. The increase of glutathione biosynthetic capacity has shown to enhance resistance to oxidative stress. Glutathione acts as a redox sensor of environmental cues and is responsible for a part

of a multiple regulatory circuitry coordinating defense gene expression. GSH/GSSH redox state may act as a direct link between environmental cues and crucial molecular adaptive responses of plant cells (*Grene, 2002*). The cell is harmed by raising the level of oxidation through loss of cellular structure and function, subsequently the cells needs detoxification compounds like superoxide dismutase, glutathione S-transferase, Ascorbate oxidase, polyphenol oxidase, catalase, peroxidase and glutathione reductase and non – enzymatic compounds such like flavones, anthocyanin, carotenoids, reduced glutathione and ascorbic acid (*Muthukrishnan et al, 2014*). It is well known that hydrogen peroxide along with singlet oxygen, the superoxide anion and hydroxyl radicals induce oxidative stress in biological systems. All ROS can function as important signaling molecules that could alter gene expression and modulate the activity of specific defense proteins (*Carvalho et al, 2004*). In high concentrations ROS can lead to oxidization of proteins, lipids and nucleic acids and subsequently can result in organelle dysfunction, alterations in cell structure and mutagenesis (*Carvalho et al, 2004*).

2.8. Glutathione – Ascorbate cycle

The metabolism of plants can be disturbed by environmental stresses and subsequently can lead to negative effects on development and a reduction in productivity. Reactive oxygen species (ROS) like singlet oxygen, superoxide, hydrogen peroxide and hydrogen radical can be hazardous to cells. Ascorbate reduces intracellular and extracellular ROS with the formation of Monodehydroascorbate(MDHA) and Dehydroascorbate(DHA). The last one is reduced to Ascorbate by dehydroascorbate reductase (DHAR), through the oxidation of glutathione (GSH) and the subsequent reduction to glutathione disulfide (GSSG). Then, GSSG is reduced by Glutathione reductase (GR) to GSH. Following this processes Ascorbate and Glutathione are continuously recycled (*Iriti et al, 2007*).

2.8.1. Glutathione (GSH)

Glutathione occurs as a tripeptide of 307 Daltons consists of a glutamate, a cysteine and a glycine (γ – L – glutamyl – L – cysteinyl – glycine) and plays an important role in the coordination of antioxidant defense processes. The synthesis of this molecule occurs in the cytosol and chloroplasts and is synthesized by glutamate, cysteine and glycine in reactions catalyzed by the enzymes γ-glutamylcysteine synthetase and glutathione synthetase (*Iriti et al, 2007*). This molecule can be found in two forms, as GSH (reduced) or GSSG (oxidized). NADPH is being used as a reductant by glutathione reductase to regenerate GSH from GSSG. Glutathione is considered to be responsible as a redox buffer for the maintenance of the intracellular environment in a reduced state. Glutathione has many functions in plants including ROS, heavy metal and xenobiotic detoxification but also G1 – S phase transition during cell cycle, cell differentiation, flowering, accumulation of anthocyanin, programmed cell death and resistance against pathogens. Glutathione is the precursor of phytochelatins which are involved in the detoxification of heavy metals. Glutathione can be used as a substrate of an important group called glutathione transferases (GST) which are involved in the detoxification of xenobiotics (*Iriti et al, 2007*). Another worth notable function of glutathione is its involvement to glutathionylation which is a reversible post – translational

modification, which contains the formation of a mixed disulfide between the free thiol of a protein and a molecule of glutathione.

2.8.2. Ascorbate (ASC)

Ascorbate is a highly abundant water soluble antioxidant and serves as an enigmatic component of the plant defense system (*Anjum et al, 2014*). Ascorbate is an important component which is part of the ascorbate – glutathione cycle and it carries the responsibility to perform multiple vital functions in plants including control of growth and development by participating in the metabolism of reactive oxygen species (ROS) and its products (*Anjum et al, 2014*). One of the most important enzymes in the Ascorbate – Glutathione cycle is Ascorbate Peroxidase(APX), an important player in the elimination of hydrogen peroxide, a typical product during oxidative stress in the cell (*Esmailzadeh et al, 2017*). Ascorbate peroxidase is responsible for breaking down hydrogen peroxide during which ascorbate serves as an electron donor (*Esmailzadeh et al, 2017*). As a result oxidized ascorbate and monohydrate ascorbate are produced. This enzyme is located in chloroplast, peroxisome, cytosol and mitochondria (*Esmailzadeh et al, 2017*).

$$2Ascorbate + H_2O_2 \rightarrow APX2monohydroascorbate+2H_2O$$

2.8.3. Superoxide Dismutase (SOD)

Superoxide dismutase is one of the enzymes that is defined as the cell's first line of defense against reactive oxygen species and catalyzes the disproportionation of O_2^- to O_2 and H_2O_2 (*Carvalho et al, 2004*).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Three isoforms of Superoxide Dismutases are using different metal cofactors are found in plants at distinct intracellular localizations. Cu/Zn SOD locates in the cytosol, plastid and peroxisome. MnSOD is found in mitochondria and FeSOD is in the plastid (*Kliebenstein et al, 1998*). In *Chlamydomonas,* one FeSOD and two MnSOD have been identified. MnSOD activity is located not only in mitochondria, but also in the chloroplast (*Sakurai et al, 1993;Kitayama et al, 1999;Allen et al,2007*).

2.8.4. Glutathione Reductase (GR)

One of the main enzymes involved in the glutathione cycle is glutathione reductase which is an NADPH – dependent oxidoreductase. Glutathione reductase is responsible for the reduction of glutathione disulfide (GSSG) to the sulfhydryl form (GSH) which plays a critical part for the maintenance of the cell's reducing environment. Also, glutathione can serve as a substrate for glutathione peroxidase (*Muthukrishnan et al, 2014*). Glutathione reductase functions as a dimeric disulfide oxidoreductase and uses a FAD prosthetic group and a NADPH to achieve a reduction of one molar equivalent to GSSG to two molar equivalents of GSH. In *Chlamydomonas*, two genes encode glutathione reductases, GSH1 and GSH2. GSH2 has a close relationship to an *Arabidopsis* glutathione reductase targeted to both chloroplasts and mitochondria. In *Arabidopsis*, glutathione reductases are localized in the cytosol, mitochondria and chloroplasts.

2.8.5. Glutathione disulfide (GSSG)

In the glutathione cycle, GSSG results to the sulphydryl form (GSH). In a few words, the electrons are subsequently transferred to glutathione disulfide yielding two molecules of GSH. Alternatively, GSSG can also oxidize thiols under certain conditions depending on thermodynamic and kinetic parameters (*Deponte, 2013*). GSSG is also involved in glutathione redox potential which depends on the ratio GSH/GSSG, the temperature, the pH and on the actual concentration of glutathione (*Deponte, 2013*). The concentration of GSH is several orders of magnitude higher than this of GSSG, although this depends on the subcellular compartment, the cell type and the organism. Also, the cell cycle and cell condition (stressed or unstressed) is responsible for the ratio GSH/GSSG.

2.8.6. Catalase (CAT)

Catalase is a tetrameric protein and constitutes of four heme groups, which is responsible for the catalysis of the dismutation of hydrogen peroxide in water and oxygen (*Muthukrishnan et al, 2014*). Catalase occurs as a molecule of 60 or 75 kDa and can bring a rapid destruction of H_2O_2 in two steps. A molecule of H_2O_2 binds and is broken apart. One oxygen atom is extracted and subsequently attached to the iron atom, and the rest is released as H_2O. Continuously, a second H_2O_2 molecule binds and it is also broken apart and the parts are combined with the iron bound oxygen atom, releasing H_2O and molecular oxygen. The oxygen atom is bound to the iron, ready for the second H_2O_2 molecule to bind. Two amino acids assist for this reaction, asparagines and histidine. Superoxide dismutase is responsible for the conversion of superoxide radicals into H_2O_2 where catalase is responsible for the conversion of H_2O_2 into O_2 and H_2O. Catalase is found in compartments such as peroxisomes, cytosol, glyoxysome and mitochondria (*Esmaeilzadeh et al, 2017*).

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

2.8.7. Glutathione S – Transferases (GST)

An important group of cytosolic multifunctional enzymes which are involved in the detoxification of endogenous compounds and foreign chemicals are Glutathione S Transferases. GSTs are dimeric, mainly cytosolic, enzymes that have extensive ligand binding proteins in addition to their catalytic role in detoxification. Glutathione S-Transferases take part at the catalysis of the conjugation of electrophilic substrates to glutathione (GSH), at the inhibition of Jun N – terminal kinase which binds and phosphorylate c – Jun on Serine 63 and 73 within its transcriptional activation domain, thus protecting cells against H_2O_2 and they have the ability non – catalytically bind a wide spectrum of endogenous and exogenous ligands. Two phases involve the conversion of a lipophilic, non – polar xenobiotic into a more soluble and less toxic metabolite which can be then easier and more efficient eliminated from the cell. The catalysis of Phase I is done by the cytochrome P_{450} system, which consists of microsomal with oxidation as their main function. At phase II, the conjugation of activated xenobiotics to endogenous water - soluble substrate is catalyzed by enzymes such as reduced glutathione, UDP – glucuronic acid or glycine. It has to be mentioned that UDP glucuronic acid is involved in the creation of polysaccharides and is an intermediate in the

biosynthesis of ascorbic acid. Quantitatively, in many species, the conjugation to GSH, which is catalyzed by the GST's is the major reaction at Phase II.

$$R + X + Glutathione \rightarrow H - X + R - S - glutathione$$

R: Possible an aliphatic, aromatic or heterocyclic group

X: Possible a sulfate, nitrile or halide group

2.8.8. Peroxidases (POX)

Peroxidases are one of the main contributors for the detoxification of the reactive oxygen species. Peroxidases are responsible for the elimination of hydrogen peroxide produced by a variation of stressors such as heavy metals, drought, salinity etc (*Esmailzadeh et al, 2017*). Peroxidases offer protection to cells against harmful concentration of hydrogen peroxide. Increase of peroxidase activity has a crucial role in the cells defense mechanisms against toxicity (*Esmailzadeh et al, 2017*).

$$Donor + H_2O_2 \rightarrow Oxidized\ donor + 2H_2O$$

2.8.9. Other enzymes of the Ascorbate – Glutathione cycle

There are two more enzymes that are involved in the glutathione – ascorbate cycle. There are the monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR). At first, H_20_2 is reduced to H_20 using ascorbate as an electron donor. The regeneration of monohydroascorbate is catalyzed by monohydroascorbate reductase (MDAR). MDAR is radical if will not reduced rapidly, so dehydroascorbate reductase (DHAR) is responsible to reduce dehydroascorbate to ascorbate at the expense of GSH, resulting to oxidized glutathione (GSSG) (*Shimadka et al, 2003*).

2.9. Proline Biosynthesis

There are a lot of documentations on the accumulation of proline due to increased synthesis and decreased degradation under several stress conditions such as salt stress, drought and metal stress. High rates of cellular proline accumulation (up to 80% of the amino acid pool under stress and 5% under normal conditions) have been reported in many publications when the plants were exposed to salt and drought stress. Proline has a cyclic ring that is produced by formation of a covalent bond between its R group and the amino group on C_a. Sometimes proline and glycine are found at points on a protein's surface where the polypeptide chain loops back into the protein.

2.9.1. Proline biosynthesis via glutamate

Proline is synthesized via the phosphorylation of glutamate which is then conversed to γ – glutamylphosphate and subsequently to glutamic –γ – semialdehyde (GSA) catalyzed by the enzymes γ – glutamyl kinase and glutamic – γ – semialdehyde dehydrogonase, respectively. GSA gets converted to pyrroline 5 – carboxylate (P5C) by spontaneous cyclization. On the other hand, glutamate is directly catalyzed to GSA by Pyrroline 5 – carboxylate synthetase

(P5CS) in plants and other eukaryotes. Afterwards, P5C is reduced to proline by P5C reductase (P5CR) in both prokaryotes and eukaryotes (*Kavi Kishor et al*, *2005*).

2.9.2. Proline biosynthesis via arginine/ornithine

The conversion of arginine to ornithine is catalyzed by the enzyme, arginase. In bacteria, the degradation of ornithine to α – keto – aminovalerate is catalyzed by the enzyme ornithinea – α – aminotransferase (α – OAT), which then spontaneously gets cyclized to pyrroline 2 – carboxylate (P2C). At the end, P2C is catalyzed to proline by P2C reductase. In contrast, in plants GSA is derived directly from ornithine by the enzyme ornithine δ – aminotransferase (*Kavi Kishor et al*, *2005*).

2.10. Metabolites involved in the antioxidant system

2.10.1. Malondialdehyde (MDA)

In general, free radicals affect the lipid peroxidation process and cause overproduction of MDA. Malondialdehyde is one of the final products of polyunsaturated fatty acids peroxidation in the cells (*Gawel et al, 2004*). Commonly, MDA is one of the most important markers for oxidative stress, although, there are several limitations such as low stability in biological samples because of its high tendency to have reactions with proteins, amino acids and rapid enzymatic degradation. Also, the non - specificity of TBA (Thiobarbituric acid) reactivity on MDA and production of MDA from reactions other than lipid peroxidation, effects of procedural modifications on MDA – TBA adduct development, poor reproducibility of analytical results and low recovery test results (*Khoubnasabjafari et al, 2015*).

2.10.2. Polyphenols

Phenylpropanoids are phenylalanine derivatives and this group contains a wide range of plant secondary metabolites with a wide spectrum of antioxidant activities (*Iriti et al,* 2007). The most important water soluble antioxidants found in algae are polyphenols, phyco – billiproteins and vitamins (vitamin c) (*Shalaby, 2011*). In particular, phenolic compounds are one of the most powerful classes of natural antioxidants. Their molecules consist of one or more aromatic rings with one or more hydroxyl groups. There are several classes such as phenolic acids (hydrobenzoic acids, hydroxycinnamic acids), flavonoids (flavones, flavonols, flavanones, flavanonols, flavanols and anthocyanins), isoflavonoids (isoflavones and coumestans), stilbenes, lignans and phenolic polymers (proanthocyanidins – condensed tannins and hydrolysable tannins) (*Machu et al, 2015*). The primary function of polyphenols is the protection against unltraviolet radiation and pathogens (*Machu et al, 2015*). Other roles include pigmentation, reproduction and development/growth of plants (*Machu et al, 2015*) and they can be stored in the vacuole or become secreted into the apoplastic space (*Iriti et al, 2007*).

2.10.3. Non – enzymatic antioxidant activity

Ascorbic acid (Vitamin *C*) and *α*- tocopherol (Vitamin *E*), along with the GSH and carotenoids are the main low – molecular compounds of a non – enzymatic antioxidant system. Ascorbic

acid is water soluble and functions as an intracellular and extracellular agent providing an aqueous phase antioxidant capacity by scavenging oxygen free radicals. Its responsibility is the conversion of vitamin *E* free radicals back to vitamin *E* (*Bunker, 1992* and *Mezzetti et al, 1996*). *Alpha* - tocopherol is lipid soluble and resides in phospholipid membranes in cells. It carries the responsibility to be the principal defense against oxidant induced membrane harm. Vitamin E donates electron to peroxyl radical, which is produced by lipid peroxidation. *A* – tocopherol is the most important membrane – bound antioxidant compound in cell (*Shannon et al, 2007*).

2.10.4. Chlorophyll *a* and *b*, Carotenoids

The most important and predominant chlorophylls are *a* and *b*, and these consist of two components, a tetrapyrrole pigment and the isoprenoid phytol, which get covalently linked at a late stage of chlorophyll biosynthesis (*Stern, 2009*). Tetrapyrolles carbon atoms are derived from glutamate in a multiple biosynthetic pathway that occurs completely within the chloroplast (*Stern, 2009*). The nuclear genome encodes the most of the enzymes which participate in chlorophyll biosynthesis (*Stern, 2009*). Recently it was demonstrated that heavy metals stress can result in a major chloroplasts alterations, color alteration, cellular deformation and death (*Basel, 2015*). To be more specific, using microscopy an inhibition in different physiological parameters was shown such as reduction in algal specific growth rate (SGR%), pigmentation including chlorophyll a and b, carotenoids and total chlorophyll(*Basel, 2015*). On the other hand, one more component that is essential to plants and that can be found even in fungi and bacteria are carotenoids which have gained increased interest due to their photoprotective and antioxidant properties. The carotenoids belong to the isoprenoids and their basic structure consists of eight isoprene units giving a C_{40} backbone (*Stern, 2009*). Carotenoids consist of an extensive double bond system and are divided in two types, carotenes which are pure hydrocarbons and xanthophylls which are derivatives and contain one or more oxygen functions (*Stern, 2009*). These compounds have lipophilic properties and offer protection to thylacoidal membranes from lipid peroxidation and the integrity of photosynthetic processes (*Iriti et al, 2007*). The highest reduction was found in Cd treatment (22%), while the lowest one was reported in Zn treatment (20%) (*Basel, 2015*).

2.11. Glutathione Peroxidase (GPX)

Thiol/selenol peroxidases are ubiquitous non-heme peroxidases. The subfamilies are Peroxiredoxins (PRXs) and glutathione peroxidases (GPXs). The discovery of glutathione peroxidase has occured in human as an erythrocyte enzyme protecting hemoglobin from oxidative damage. The enzyme of Glutathione Peroxidase is responsible for the catalysis of hydroxyperoxides reduction by glutathione. The reaction is the following:

$$2\ GSH + H_2O_2 = GSSG + 2H_2O$$

Its main function is the protection against the damaging impact which is coming from endogenously formed hydroxyperoxides. The isoforms of glutathione peroxidase consists of three amino acids characterized by stricted conservation which involved in the catalytic mechanism for mammalian selenoGPXs. The catalysis of glutathione peroxidase activity consists of a primary oxidation of the catalytic Cys or Se – Cys residue to sulfenic or selenic

acid, respectively. In selenoGPXs, nucleophilic attack of the selenic acid by GSH results to a formation of a selenylsulfide which reacts with a second molecule of GSH to lead to a regeneration of a reduced selenol at the Se – Cys residue and liberate oxidized glutathione (GSSG). pKa of Se – Cys and cysteine have values of 5.2 and 8.3, respectively. This confers to a high reactivity of selenoproteins and subsequently offering an advantage of employing the reactivity of Se – Cyc during the catalytic cycle, such like GPXs. The selenogroup is mainly in its anion selenolate form at physiological pH while the thiol of a Cys residue is only partially ionized, showing Se – Cys more reactive than Cys. On the other hand, site directed replacement of Se – Cys by Cys leads to a decrease of the activity of selenoGPXs (*Dayer et al, 2008*). The expression of glutathione peroxidase genes is located in most tissues although some only accumulate low mRNA levels. Experiments with *Arabidopsis thaliana* using hormones such as salicyclic acid, jasmonic acid, abscissic acid and auxin have shown an increase in GPX mRNA levels characterized by different patterns for each individual gene, resulting to the involvement of possible and multiple signaling pathways. There have been several reports mentioning diverse stress conditions can be responsible for the regulation of Glutathione peroxidase expression. *Chlamydomonas reinhardtii* exposed to sulfur starvation showed an induction of GPX expression (*Zhang et al, 2004*) and manganese deficiency has shown an upregulation of GPX3 and GPX4 transcript levels probably coming from the resulting oxidative stress (*Allen et al, 2007*). In addition, glutathione peroxidases are more efficient on a molar basis than other enzymes (*Muthukrishnan et al, 2014*).

2.12. Transcript analysis

Transcript analysis has been extensively used in many publications related to *Chlamydomonas reinhardtii*. There have been many approaches relating to genes involved in photosynthesis, oxidative stress, lipid metabolism, evaluating the phosphorus deprivation, survival during sulfur starvation, showing the gene expression patterns during the exposure to the explosive 2, 4, 6 – trinitrotoluene (TNT), studying the anoxic gene expression after anaerobic acclimation and to search the plastid and mitochondrial transcriptomes for changes in RNA profile as a response to certain biotic and abiotic stimuli. For the above research approaches microarray analysis was the main method to assess differential gene expression. Another method is RNA Seq that has been used for transcriptome analysis as well. *Gonzalez – Ballester et al 2010,* show that quantitative real time PCR evaluation of the results obtained by this technique showed that RNA – Seq is more efficient because reports a larger dynamic range of expression levels that do microarray hybridizations. It makes genome wide transcript analyses both sensitive and quantitative. These techniques are efficient when the purpose is to scan the genome of the model organism and finding the differential gene expression. On the other hand, under certain circumstances there is an alternative way to show if gene expression occurs or not. As shown in *figure 2.9.1.,* when the genes are known to us, subsequently the primer design is efficient and following the method of reverse transcription and RT – PCR we reach out to the desired result.

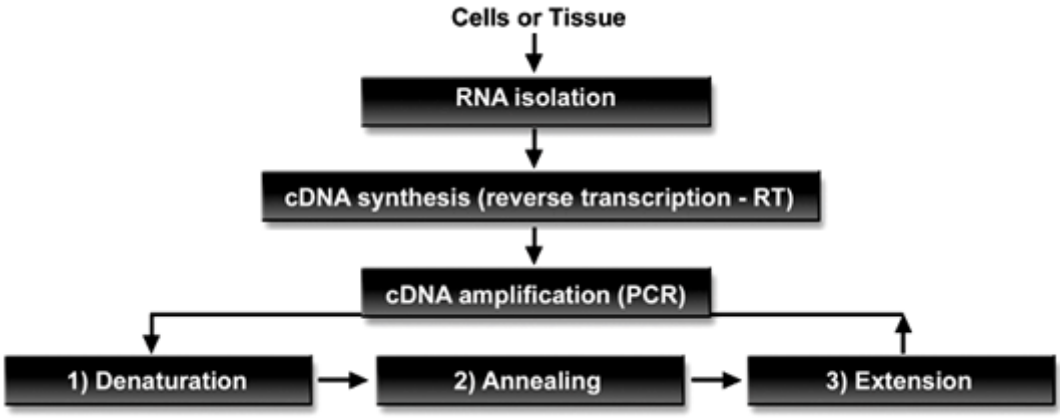

Figure 2.9.1.: Reverse Transcription method

2.13. Internal metal concentration and homeostasis

The uptake and the concentration of metal in the cell during heavy metal exposure is a crucial parameter to check. Many studies, *Wang et al (2005),* made excellent studies on the biokinetics of metals in algae and more specific in natural coastal phytoplankton assemblages and in the coastal diatom *Thalassiosira pseudonana. Jamers et al (2006)* performed a very good study on the uptake of Cu during copper exposure in *Chlamydomonas reinhardtii* and in 2013 they performed another study on the uptake of Cd during cadmium exposure. A review on the majority of chemical and biological processes in uptake of trace metals in aquatic organisms has been written by *Worms et al (2006)*. Another interesting parameter is the competition of metals in aquatic organisms exposed to heavy metal mixtures. *Topperwien et al*, (2007) show the competition of cadmium with other metals in *Scendesmus vacuolatus* under freshwater conditions. *Jarvis et al (2015)* show the accumulation and effects of metal mixtures in two seaweed species. To be more specific, the green seaweed *Ulva lactuca* and the red seaweed *Agardhiella subulata* were exposed to five metals (Cu, Ni, Pb, Cd and Zn) simultaneously and *U. lactuca* was also exposed to each metal individually for 48h. Inductively Coupled Plasma Mass Spectrometry (ICP – MS) is a wide used analytical technique used for elemental determinations. ICP – MS offers several advantages such as the fact that detection limits are 10 – 100 times lower comparing to ICP – AES, it provides elemental isotopic ratio information and a large dynamic working range. The use of radioisotopic tracers is one of the methods to determine the uptake and subsequently the concentration of metal in the cell of an organism. In contrast, there are two major drawbacks for the study of biokinetics using radioisotopic tracers. The first is that suitable radiotracers are lacking for some elements and the second is that the specific activity of the isotopes inside the organism is not often known. So, the use of stable isotopes comes up to provide information on all isotopes on the same metal present in a given system and for the most metals a number of chemically identical but isotopically forms exist, allowing the labeling and the distinguishing of uptake routes or different compartments in an organism.

Chapter 3: Materials and Methods

3.1. *Chlamydomonas reinhardtii* strain and growth cultures

Chlamydomonas reinhardtii strain 11 – 32a was used. It was provided by the culture collection of algae located at the University of Gottingen, Germany. The maintenance of the cultures was in Tris – Acetate – Phosphate (TAP) (Supplement 1.1.) liquid medium (*Harris, 1989*) using growth chamber (Innova 44, Brunswick, USA), at 25°C±1°C under continuous illumination with light provided by a photosynthetic light bank. For the cultures exposed to lead and cadmium concentrations, glycerophosphate was added instead of inorganic phosphate to avoid the precipitation of the insoluble inorganic salts which are formed by these two metals in the nutrient medium. The polluted medium was prepared in 2L bottles provided by VWR (Leuven, Belgium) and the total volume was 1.700mL. For the maintenance of the culture, two Erlenmeyer flasks of 250mL were inoculated with one 1mL every five days under the same conditions of the exposured cultures. The heavy metal concentrations in which each culture was exposed are presented in *Table 3.1.* and the heavy metal mixtures or combinations are presented in *Table 3.2.*. The chosen time points for cell harvesting were 120h and 168h of exposure. These time points were chosen where the growth of cells was at the beginning (120 hours) and at the end of the logarithmic phase (168h). The experiment had two biological replicates of each condition instead of three because of the time limitations.

Table 3.1.: Metal concentrations in parts per million (ppm) of the polluted *Chlamydomonas reinhardtii* cultures

Element	1st Concentration	2nd Concentration	3rd Concentration
Ni^{2+}	2.02	6.06	8.01
Cd^{2+}	3.64	7.29	14.58
Pb^{2+}	6.26	12.51	25.02

Table 3.2.: Metal concentrations in parts per million (ppm) of the polluted metal mixed conditions of *Chlamydomonas reinhardtii* cultures.

Metal Mixture	Ni^{2+}	Cd^{2+}	Pb^{2+}
First	2.02	3.64	6.26
Second	2.02	7.29	12.51
Third	2.02	14.58	12.51
Fourth	6.06	14.58	12.51

3.2. Determination of algal growth rates

The construction of growth curves was the preliminary experiment that was executed before the main research was done and that was necessary to choose the right time points of sampling. Three biological replicates of each condition in the volume of 100mL in 250mL

Erlenmeyer flasks were cultured under the same conditions as well as the main experiment. Growth rates of each condition were determined by counting cell numbers at the 0, 24, 48, 72, 96, 120, 144, 168h, 192, 216, 240 and 264 hours time points. A volume of algal culture was diluted in balanced electrolyte solution (Coulter Isoton II Diluent, Beckman Coulter, U Brea, Ca, USA) and cells were counted using a Multisizer 3 coulter counter (Beckman coulter, USA).

3.3. Metal Concentrations

3.3.1. Sample preparation

At time points of 120h and 168h after the initiation of the experiment, heavy metal concentrations were measured. From 1700mL of the cell culture, 250mL was taken and the cells were counted using Coulter counter then the culture was centrifuged for 15 minutes at 3000xg and temperature of 4°C. Supernatant was collected in a 10mL sterilized polypropylene falcon and the pellet was re-suspended in washing buffer solution and stored in -80°C freezer.

3.3.2. Sample preparation for ICP – MS

The element which has been used for the determination of heavy metal content was Element 2 HR – ICP – MS (Thermoscientific, USA). Initially for the sample digestion, 2 mL of high purity HNO_3 was added and the samples were let at least for 12 hours at room temperature to be digested. Afterwards, the tubes were placed in the hot block. The temperature of hot block was approximately 115°C and the samples were removed after 30 minutes. After five minutes 10mL of H_2O_2 were added to the samples and a digestion was begun for 30 minutes. Then, the samples were removed from the hot block and MQ was added to a final volume of 40mL. Finally, the tubes were closed tightly and stored at room temperature.

3.4. Determination of secondary Metabolites

3.4.1. Flavonoids determination

3.4.1.1. Extraction

Initially, the samples were thawed on ice very carefully and the biomass was transferred to new eppendorf tubes. Secondly, the biomass was determined and then a centrifugation at 4000xg, for 5 minutes at 4°C was performed. The supernatant was decanted and immediately 500µL of ultra pure water was put to each tube. Then, a second centrifugation was performed under the same conditions for 3 minutes. Subsequently, the samples were sonicated under the specific conditions of 8 seconds ON and 9 seconds OFF using an ice bath for 10 cycles. After the sonication of each sample, liquid nitrogen was used to prevent the samples from heating. After the sonication of each sample an equal volume of flavonoid extraction buffer was used to collect all the biomass from the sonication probe and then the probe was rinsed with EtOH to prevent contamination of the samples.

3.4.1.2. Analysis

A centrifugation at 30074xg, for 20 minutes, at 4°C was performed and the supernatant was transferred to a new tube which contained grace. This was immediately followed by a centrifugation, for 10 minutes at 4°C. A dilution 1/10 was performed and 80µL were put into a UV Star Platte of 96 wells and the measurements were performed in scanning mode of 250 – 350nm spatial resolution. The calibration curve was constructed with Quercetin – riboside and the data are expressed in µmol.

3.4.2. Analysis of chlorophyll and carotenoids

The analysis of chlorophyll and carotenoids/xanthophylls was performed using a protocol that was based on the studies of *Harmut et al (1983)* and *Warren (2008)*.

3.4.2.1. Extraction

The same procedure is followed till sonication, which has been described at the previous subchapter (*3.4.1.1.*). The sonication conditions were exactly the same and the cells were broken in 500µL extraction solvent 96% of EtOH and the probe was rinsed with equal volume of solvent to collect all the biomass. Then, a centrifugation of 14000xg, for 10 minutes, at 4°C was performed and the supernatant was collected. The pellet was re – extracted with an additional 500µL 96% EtOH and a second centrifugation was performed under the same conditions. In the end, supernatant was pooled with the previous supernatant fraction.

3.4.2.2. Analysis

The analysis was performed using Greiner 96 well plates. Each sample was pipetted in duplicate, once 100µL, and for duplicate 200µL. Then, OD was measured with platereader in scanning mode 450 – 700nm. It's notable that OD values should be above the background and below 0.9 absorption units in case they are higher, proper dilution should be made.

3.4.2.3. Calculations

Based on the study of *Warren, 2008* the conversion factor for path length correction is:

- 100uL Sample: $A_{1cm}=A_{100uL}/0.24$
- 200uL Sample: $A_{1cm}=A_{200uL}/0.53$

The absorption values were corrected and then the corrected A – values could be used for calculations using the formulas of *Hartmut et al, 1983*. These formulas are specific for Ethanol as extraction solvent.

- Concentration Chla=C_a=13.95 $A_{665\ 1cm}$ – 6.88 $A_{649\ 1cm}$
- Concentration Chlb=C_b=24.96 $A_{649\ 1cm}$ – 7.32 $A_{665\ 1cm}$
- Concentration Carotenoids and Xanthophyl=C_{x+c}=(1000 $A_{470\ 1cm}$ – 2.05 C_a – 1148 C_b)/245

3.5. Metabolites

3.5.1. Introduction

Malondialdehyde (MDA) is used as biomarker for oxidative stress, total non – enzymatic antioxidant activity is measured to find if there is an activity of vitamins (Tocopherol, Ascorbic acid etc) and polyphenols concentration is measured to determine their antioxidant activity. For sample preparation the procedure described in paragraph *3.4.1.1.*, was followed up to the sonication step. The samples were broken in 80% EtOH which is the solvent extraction. This sample can be used for Ferric Reducing Antioxidant Power (FRAP), polyphenols and Malonudialdehyde(MDA) content.

3.5.2. Analysis of FRAP assay for total non – enzymatic antioxidant activity

This assay is based on the measurement of the reduction of ferric to ferrous ions by biological material. Alcoholic extract of homogenized plant samples is reacted with a reagent mix that produces a blue color when Fe is reduced. The standard curve, *Figure 3.5.2.1.,* was prepared with TROLOX (6 – Hydroxy – 2,5,7,8, Tetramethylchromane – 2 – carboxylic acid). The standard curve was prepared as follows in the *Table 3.5.2.1.*:

Table 3.5.2.1.: Standard curve for FRAP assay for total non – enzymatic antioxidant activity

Concentration of TROLOX	250µM TROLOX (in µL)	80% Alcohol (µL)
0	0	100
62.5	25	75
125	50	50
187.5	75	25
250	100	0

Then, were added 100µL of reaction mixture to 100µL of sample in a micro plate. The plate was kept on ice for 20 minutes and then it was read at 600nm.

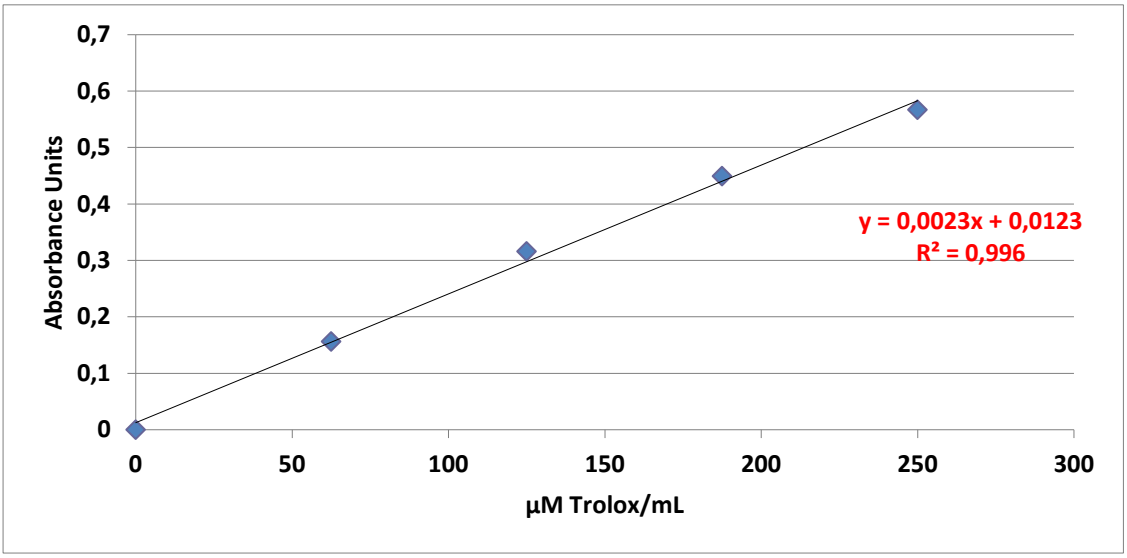

Figure 3.5.2.1.: Standard curve using dilutions of 6 – Hydroxy – 2,5,7,8, Tetramethylchromane – 2 – carboxylic acid (TROLOX) measured at 600 nm

3.5.3. Analysis for Polyphenols content

First, 20µL of extract was added in 50µL of Folin Cioculteu Phenol reagent and was allowed to react for 5 minutes. Then, 40µL of 10% saturated Na_2CO_3 solution was added and allowed to stand for 60 minutes in the dark. The absorbance of the reaction mixture was read at scanning mode of 725nm using micro plate ELISA reader. The total Polyphenol content (TPC) of each extract was expressed as mg gallic acid equivalents. At the end, a gallic acid standard curve was prepared, *Figure 3.5.3.1.,* for the calculation of phenolic content. The standard curve was prepared as shown in *Table 3.5.3.1.*:

Table 3.5.3.1.: Standard curve for Polyphenols content

Concentration (µg/mL)	Stock of 100µg/mL Gallic acid (µL)	80% EtOH (µL)
0	0	1000
5	50	950
10	100	900
20	200	800
30	300	700
40	400	600
50	500	500
60	600	400
70	700	300
80	800	200
90	900	100
100	1000	0

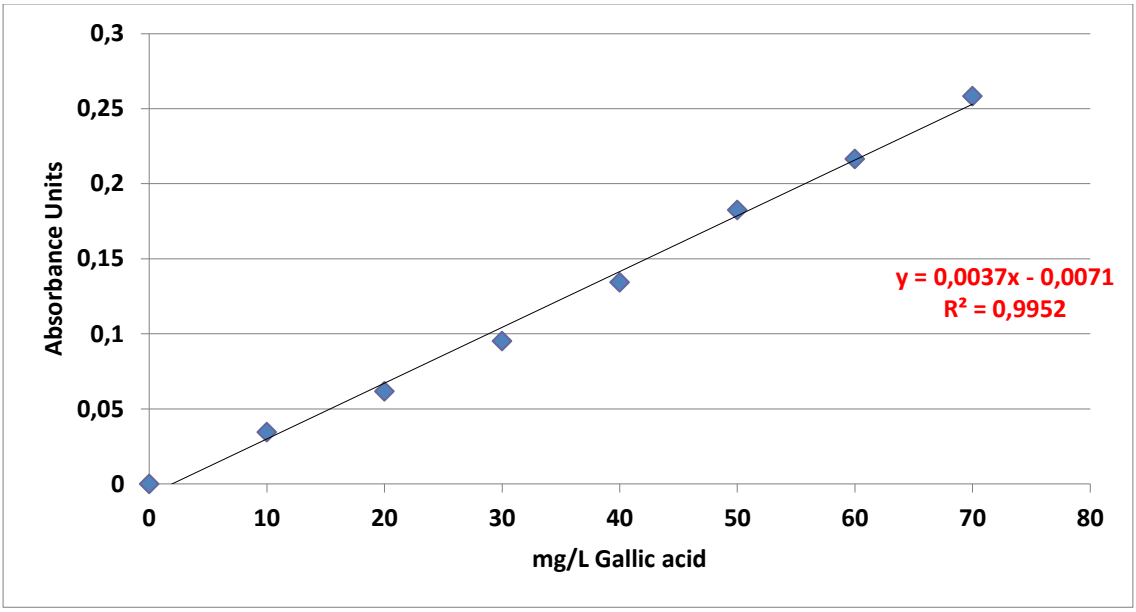

Figure 3.5.3.1.: Standard curve using dilutions of Gallic acid (GA) measured at 725 nm

3.5.4. Malondialdehyde content analysis

A centrifugation was performed at 1200g for 15 minutes with homogenized frozen cells in 80% (w/v) EtOH. Then, 0.5mL of the supernatant was added in 1mL 0.5% (w/v) TBA (in 20% TCA). Immediately, both mixtures were incubated in boiling water for 30 minutes. To continue, reaction was stopped by placing the reaction tube in an ice bath and was vortexed tube thrice. In the end, 200uL of aliquots from each tube were transferred in 96 well plate. The read of the absorbance of the supernatant was at scanning mode of 450, 532 and 600nm in a micro plate reader.

3.6. Enzyme activity

The protocols are cited in supplements and are based on *Murshed et al, 2008* study. The extraction procedure of Ascorbate – Glutathione recycling enzymes which are extracted together into 750 – 1000µL of 50mM MES/KOH buffer (pH: 6.00) or KPO_4 buffer (pH: 7.00). 250mM AsA is freshly prepared and added to the extraction medium. Improving the extraction by vortexing and transfer it back on ice. A centrifugation if performed to homogenate at maximum speed for 30 minutes at 4°C, if the supernatant is not totally separated from the pellet, the centrifugation should be repeated for 10 minutes at least under the same conditions. The supernatant was analyzed immediately for enzyme activities. Additionally, an aliquot is taken before the measurement for protein determination (*Table 3.6.1.* and *Figure 3.6.1.*). At *Table 3.6.1.*, the column with correction is defined as the average of measured BSA concentration (mg/mL) subtracts the BSA concentration to obtain the corrected value. In the end, all enzyme assays are performed in a final volume of 200µL per well and at 25°C. The enzymes that have been measured were Ascorbate Peroxidase(APX), Superoxide Dismutase(SOD), Glutathione Reductase(GR), Glutathione –S – Transferase(GST), Proline Enzyme activity (P5CS), Peroxidase (POX), Catalase (CAT), monodehydroascorbate reductase (MDHAR) and Dehydroascorbate Reductase (DHAR).

Table 3.6.1.: Standard calibration curve for BSA

BSA concentration	BSA Concentration (mg/mL)				mean	correction
0(H2O)	0,063	0,045	0,056	0,055	0,05475	0,000
125	0,13	0,123	0,122	0,117	0,123	0,068
250	0,186	0,18	0,173	0,169	0,177	0,122
500	0,28	0,269	0,268	0,241	0,2645	0,210
1000	0,423	0,432	0,392	0,359	0,4015	0,347
1500	0,498	0,527	0,452	0,437	0,4785	0,424

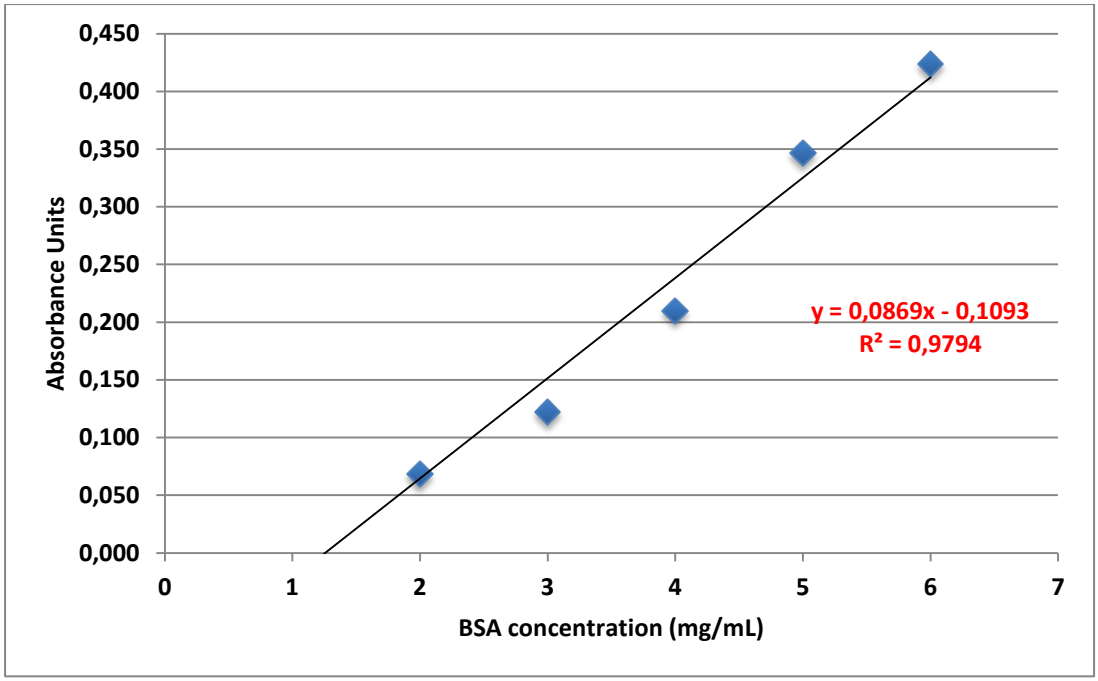

Figure 3.6.1.: Protein standard curve using dilutions of bovine serum albumin (BSA) measured at 660 nm

3.7. Transcript analysis

From the related literature eight genes have been chosen to study the differential gene expression in cells exposed to several heavy metal concentrations. These genes were Glutathione S Transferase, Glutathione Peroxidase, Catalase, Ascorbate Peroxidase, Superoxide Dismutase, P5CS, HSP 70B and Thioredoxin h cytosolic and primers were designed, respectively, using AmplifX software (Informer Technologies, Inc). The primers are cited in the Table *3.7.1.* and PCR efficiencies were checked using a dilution series of cDNA. Reverse transcription was performed which began with RNA isolation (RNeasy Plant Mini Kit, Quiagen, 2011) and the RNA was converted to cDNA using Superscript II RNase H – Reverse Transcriptase (Gibco BRL, Invitrogen, Merelbeke, Belgium), adding OLIGO $(dt)_{15}$ primer, dNTP Mix (10mM each), 5x First Strand Buffer, 0.1M DTT and Superscript II Reverse transcriptase enzyme. The full protocol is cited in supplement. Real time PCR (Step One Real time PCR Systems, Thermoscientific) was performed using TAKYON Sybr green (Eurogentec) as detection dye. Total amount of RNA present in each reaction was normalized by choosing CBLP and Actin as housekeeping genes.

Table 3.7.1.: Pair of Primers list

Gene	Forward	Reverse	AFS** Nucl.	GC%*	Annealing Temperature C °
Glutathione S Transferase (GST1)	5' TTCAGTTCGAGGACGT GCGTA 3'	5' TGTTCCTGGTTC AGGACGTCT 3'	219	64	58
Glutathione Peroxidase (GPX5)	5' TGGCTAGCAAGTGCG GCTTTA 3'	5' AAGTCGGACGT GAACGGCAA 3'	208	59	57
Ascorbate Peroxidase (APX)	5' CGACAAGAACATCGC TGAGT 3'	5'TGTTCCAAATG GCATCAGCC 3'	178	58	56
Superoxide Dismutase (SOD)	5' GGTCACGTCAACCAT GACATT 3'	5' CTACCTGCAGTA CAAGAACG 3'	307	64	56
Thioredoxin h (TRXh)	5' AGGAGCACAAGCCGA TTGTT 3'	5' TACAAGGATGG CGTGAAGGC 3'	208	58	56
Glutamic Gamma dehydrogonase semialdehyde (P5CS)	5' AGAAGGTGCTCATCC ACAAG 3'	5' ACACTGGAGCTG GTTGACAA 3'	190	68	57
Heat Shock Protein 70B (HSP 70B)	5' AAGGTCGTGGGTATC GATCT 3'	5' CAAGTGCCTTAC CGGGTGATT 3'	270	63	57
Actin (ACTIN)	5-ATGGGCCAGAAGGAC TCGTA-3	5-GTCGTCCCAGTT GGTCACAA-3	*Xu et al, 2015*		
***Chlamydomonas* bet a subunit-like polypeptide (CBLP)**	5' CTTCTCGCCCATGACC AC 3'	5' CCCACCAGGTTG TTCTTCAG 3'	*Jamers et al, 2013*		
Catalase (CAT)	5' TCGAGGTGACGCACGA CAT 3'	5' CAACATGCCGGT GTTCTTCA 3'	210	66	57

*Percentage of Guanine/Cytosine content

**Allele frequency spectrum

Chapter 4: Results

4.1. Algal growth

The first part of the total experimental set up of this project was the construction of the growth curves of *Chlamydomonas reinhardtii*, exposed to different pollution levels of Ni^{2+}, Cd^{2+}, and Pb^{2+}, either as single pollutants, or as mixtures. In general, the levels of the heavy metals in the growing media were not lethal for the cells, with the exception of Ni^{2+} 8.01 ppm and the mixture Ni 6.06 ppm, Cd 3.64 ppm, Pb 6.26 ppm (*Figure 4.1.1.*). With the exception of the condition Ni^{2+} 6.06ppm and the mixture Ni 2.02ppm, Cd 14.58 ppm, Pb 12.51 ppm, where the growth rates were slower, all pollution levels didn't show any significant effect either in the growth rate, or in the total cell production at the steady – state phase of the mixtures (*Figures 4.1.1., 4.1.2., 4.1.3. and 4.1.4.)* as compared to the control, unpolluted cultures. For cell harvesting, two time points were selected: One at 120h after inoculation, which corresponded to the beginning of the logarithmic phase, and another one at 168h after inoculation, around the end of the logarithmic phase.

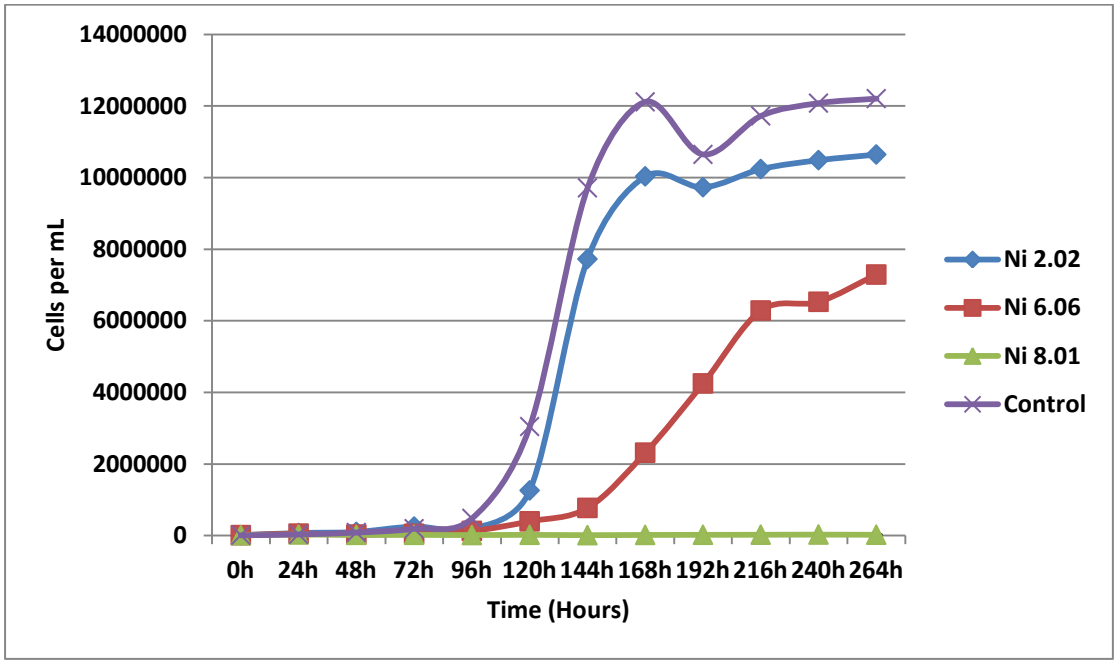

Figure 4.1.1.: Growth rates of *Chlamydomonas reinhardtii* exposed to three concentrations of Ni^{2+} (n=3)

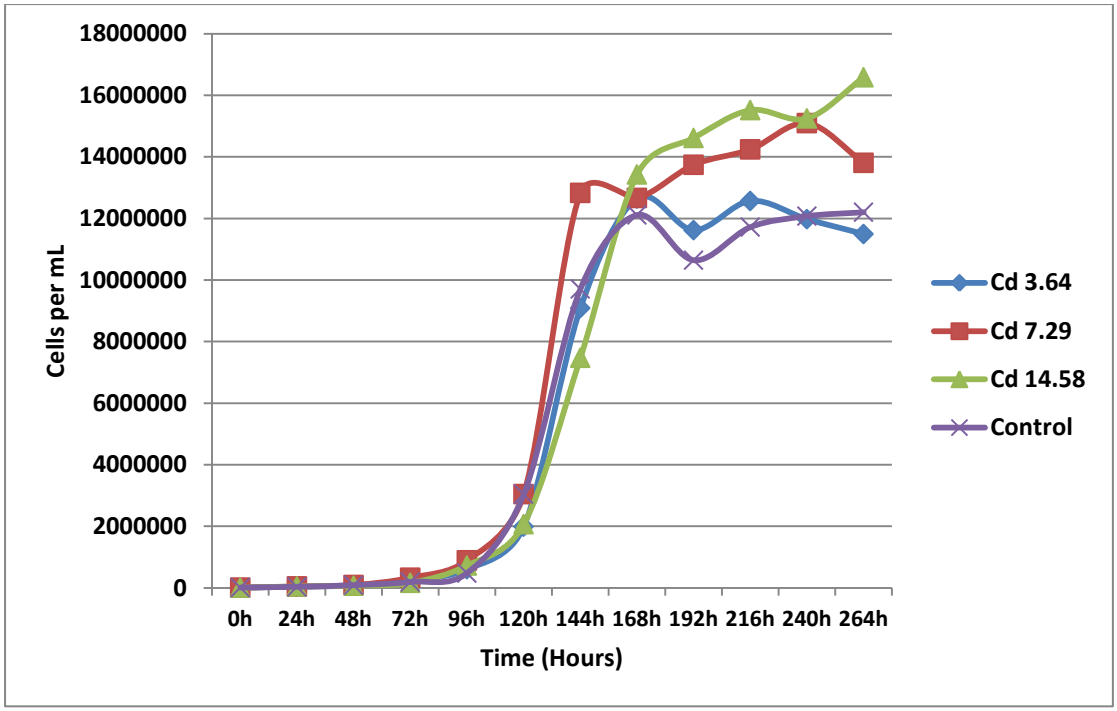

Figure 4.1.2.: Growth rates of *Chlamydomonas reinhardtii* exposed to three concentrations of Cd^{2+}. (n=3)

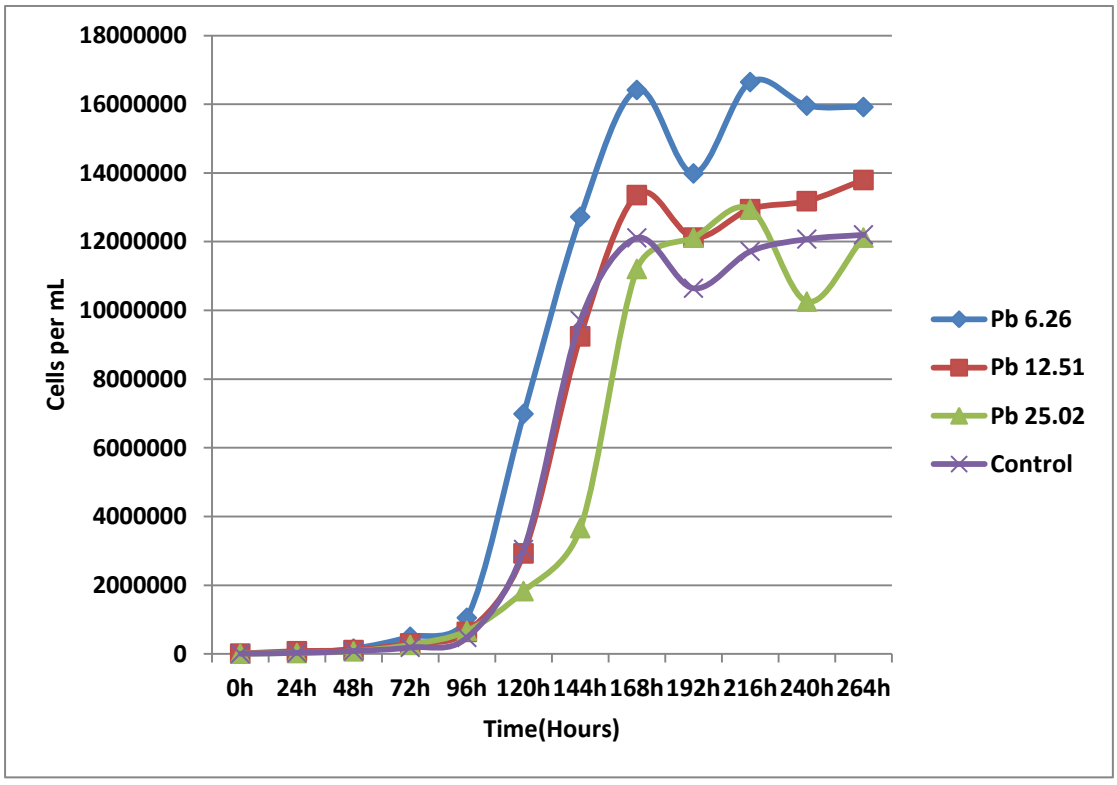

Figure 4.1.3.: Growth rates of *Chlamydomonas reinhardtii* exposed to three concentrations of Pb^{2+} (n=3)

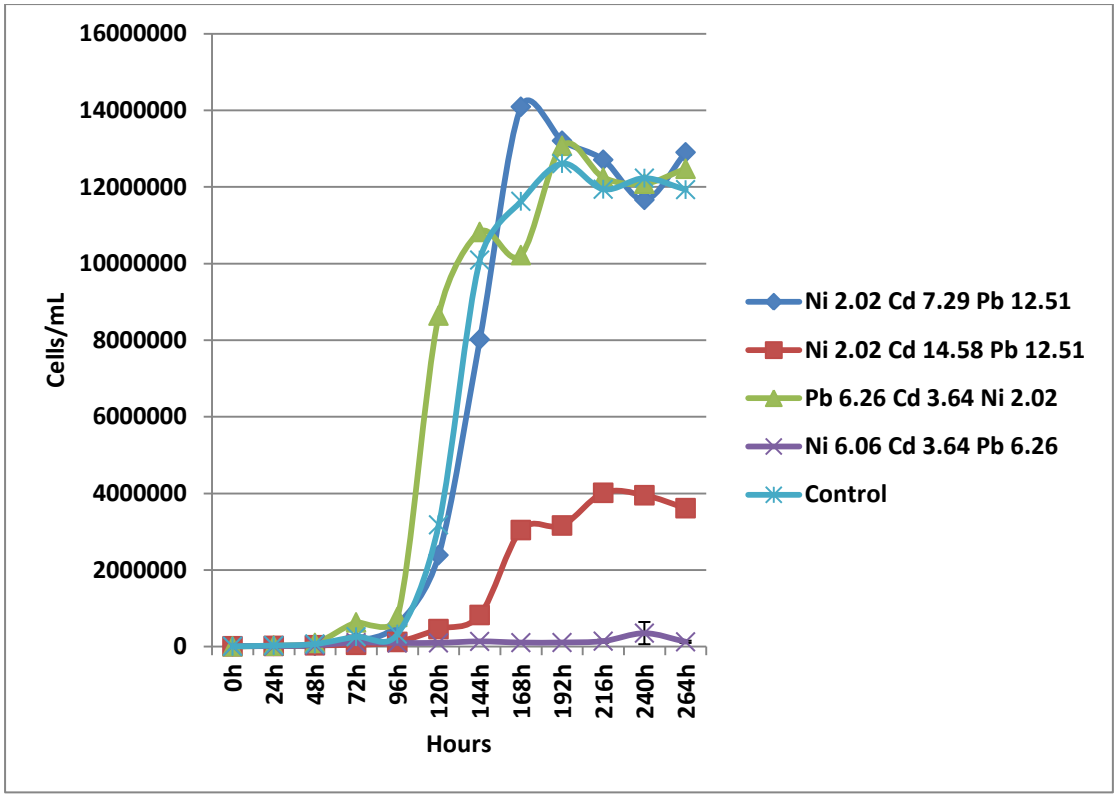

Figure 4.1.4.: Growth rates of *Chlamydomonas reinhardtii* exposed to four tertiary mixtures of metal concentrations (n=3)

4.2. Metal Concentrations

The condition of 2.02 ppm Ni^{2+} remains in very low levels comparing with its combination in all the tertiary mixtures (*Figure 4.2.1.*). All the concentrations of Cd^{2+} at single metal exposures have very low levels compared to tertiary mixtures (*Figure 4.2.2.*). *Figure 4.2.3* shows that at the lowest concentration of Pb^{2+} there is a small increase, on other hand the concentration of 12.51 ppm Pb^{2+} remains almost the same as the level of its single exposure at its combination with 2.02 ppm Ni^{2+} and 7.29 ppm Cd^{2+}. Although, at the exposure to the mixture of Ni^{2+} 2.02 ppm, Cd^{2+} 14.58 ppm and Pb^{2+} 12.51 ppm there is an increase at the internal cell concentration of lead. Ni^{2+} and Cd^{2+} internal cell content show an increase at the conditions of all tertiary mixtures. At 168h the most of the exposed cultures show a decrease at the internal metal concentration or a very small increase for example about the mixture Ni^{2+} 2.02 ppm, Pb^{2+} 12.51 ppm and Cd^{2+} 3.64 ppm or 7.29 ppm.

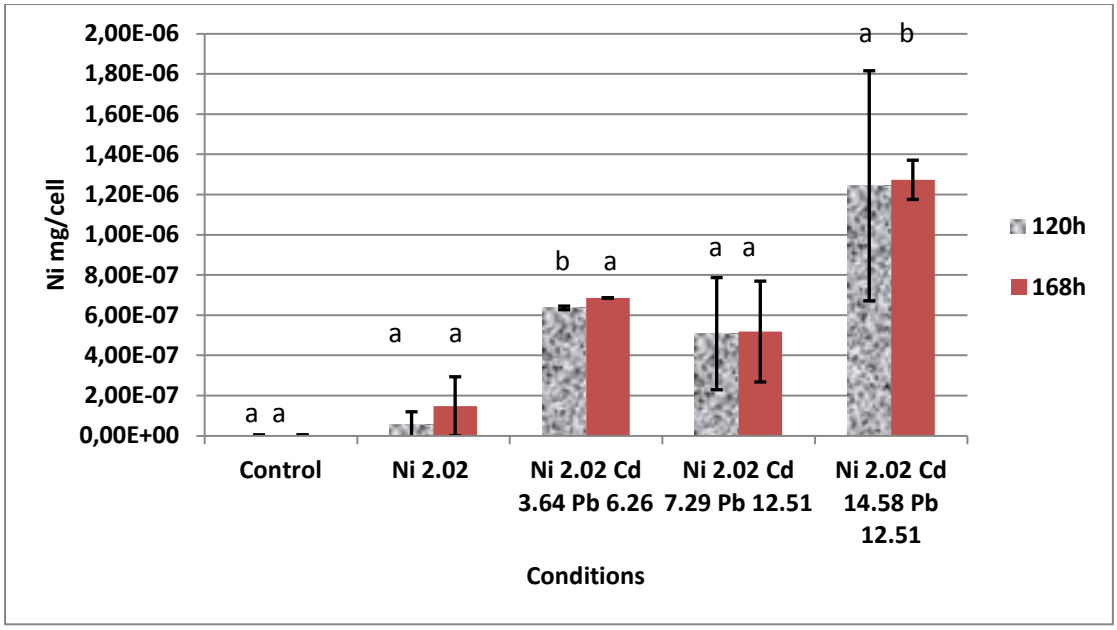

Figure 4.2.1.: Interior cell Ni^{2+} content (mg/cell) of *Chlamydomonas reinhardtii* exposed to single and tertiary metal mixtures at 120h and 168h (n=2). Different letters indicate significant differences between the treatments and control. (*a=0.05*). About the mixture of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm at 168h (n=1)

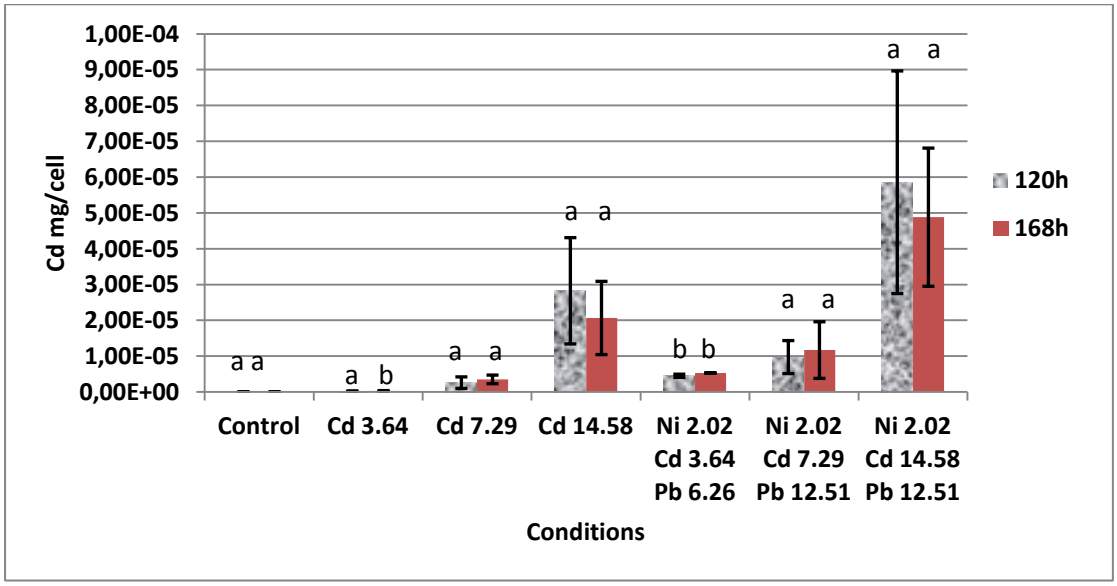

Figure 4.2.2.: Interior cell Cd^{2+} content (mg/cell) of *Chlamydomonas reinhardtii* exposed to single and tertiary metal mixtures at 120h and 168h (n=2) Different letters indicate significant differences between the treatments and control. (*a=0.05*). About the mixture of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm at 168h (n=1)

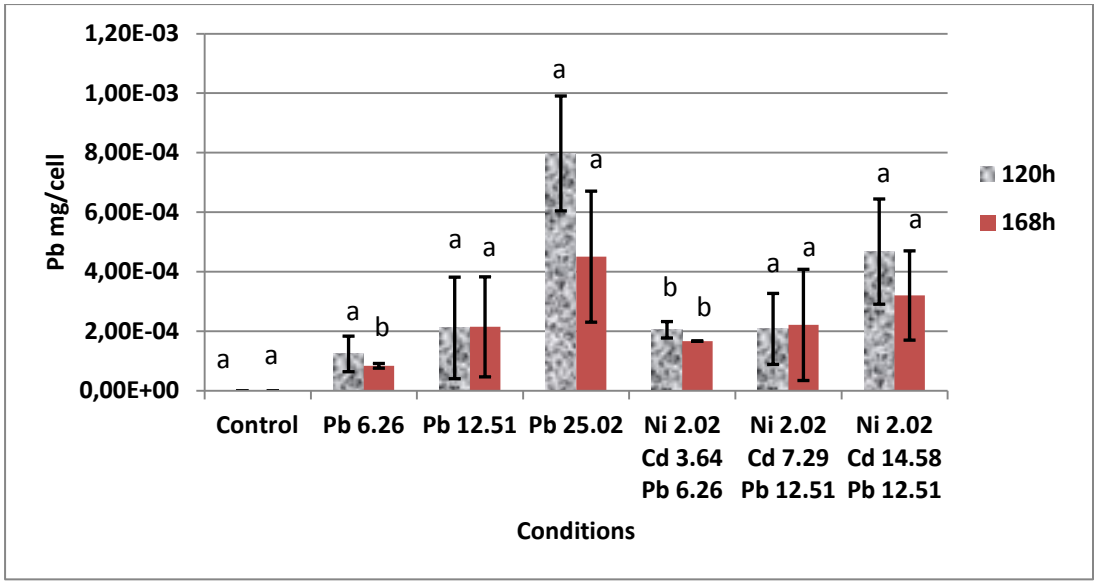

Figure 4.2.3.: Interior cell Pb^{2+} content (mg/cell) of *Chlamydomonas reinhardtii* exposed to single and tertiary metal mixtures at 120h and 168h (n=2). Different letters indicate significant differences between the treatments and control. (*a=0.05*). About the mixture of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm at 168h (n=1).

4.3. Gene Expression

According to *Table 4.3.1.*, at 120h there is an over expression of glutathione peroxidase for all the exposed cultures (*Figure 4.3.1.*). On the other hand, at 168h of exposure there is a down regulation of the gene for the cultures exposed to 7.29 ppm and 14.58 ppm of Cd^{2+} (*Figure 4.3.2.*). The same behavior belongs to the cultures exposed to 12.51 ppm of Pb^{2+} (*Figure 4.3.1.*) and the mixture of Ni^{2+} 2.02 ppm Cd^{2+} 14.58 ppm Pb^{2+} 12.51 ppm (*Figure 4.3.1.*). The gene is still over expressed at the rest of the conditions. Although, at 168h of exposure for the condition of 25.02 ppm of Pb^{2+} there occurs an extreme value compares to the rest, almost 50 times fold compares to the rest conditions and this might be an artifact (*Figure 4.3.2.*). At the exposure at 168h there is a decrease of fold change for all the exposed cultures, although the condition 2.02 ppm of Ni^{2+} is the only increased (*Figure 4.3.2.*). In general, the expression of glutathione peroxidase at 168h time point exhibits a decrease to the expression of corresponding conditions at 120h time point (*Figures 4.3.1., 4.3.2.*). Exception is the Ni^{2+} 2.02 ppm condition. Due to time limitations the rest of the genes that have been abovementioned in *Chapter 3* haven't been analyzed.

At 120h (*Figure 4.3.1.*, *Table 4.3.1.*), there were significant differences between the control and the conditions of Cd^{2+} 3.64ppm (0.000187<0.05), Cd^{2+} 7.29ppm (0.035871<0.05), Cd^{2+} 14.58ppm (0.000159<0.05), Ni^{2+} 6.06ppm ($2.22197*10^{-5}$<0.05) and the mixture of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm (0.03256<0.05). Other significant differences were between the conditions of Cd^{2+} 3.64ppm and Ni^{2+}2.02ppm (0.018556<0.05), Pb^{2+} 12.51ppm (0.0091<0.05) and the mixture of Cd 3.64ppm, Pb 6.26ppm, Ni 2.02ppm (0.0129<0.05). The highest concentration of Ni^{2+} found significant different with the conditions of Ni^{2+} 2.02ppm

(0.0028<0.05), Pb^{2+} 12.51ppm (0.001671<0.05), Cd^{2+} 14.58ppm (0.005898336<0.05) and the mixture of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm (0.00088251<0.05). The condition of Cd^{2+} 14.58ppm found significant different with Ni^{2+} 2.02ppm (0.03167<0.05), Pb^{2+} 12.51ppm (0.0078<0.05) and the mixture of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm (0.025562<0.05). At 168h (*Figure 4.3.2., Table 4.3.1.*), the only conditions that were significant to control were Ni^{2+} 2.02ppm (0.009357<0.05) and Pb^{2+} 12.51ppm (0.0083<0.05). The lowest concentration of Ni^{2+} was found to be significant different to Cd^{2+}3.64ppm (0.0254<0.05), Cd^{2+} 7.29ppm (0.002783<0.05), Cd^{2+} 14.58ppm (0.0178<0.05) and Pb^{2+}12.51ppm (0.000336<0.05). The lowest concentration of Cd^{2+}3.64ppm was found significant different with the condition of Pb^{2+} 12.51ppm (0.008354<0.05). The condition of Pb^{2+}12.51ppm was found significant different to Pb^{2+} 6.26ppm (0.008863<0.05) and to mixtures of Ni 2.02ppm, Cd 7.29ppm, Pb 12.51ppm (0.008241<0.05) and Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm (0.004569<0.05). The highest concentration was found significant different to the mixtures of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm (0.0455<0.05) and Ni 2.02ppm, Cd 7.29ppm, Pb 12.51ppm (0.036875<0.05).

Table 4.3.1.: Fold change (relative to control) of Glutathione Peroxidase gene expression

	Fold change(relative to control)	
Condition	**Timepoint 120h**	**Timepoint 168h**
Control	1	1
Ni^{2+} 2.02 ppm	1,305239618	1,835738348
Ni^{2+} 6.06 ppm	3,901673314	2,132696104
Cd^{2+} 3.64 ppm	3,01131838	1,131146008
Cd^{2+} 7.29 ppm	2,506715578	0,594073697
Cd^{2+} 14.58 ppm	2,743053625	0,442729775
Pb^{2+} 6.26 ppm	5,730259989	1,178259527
Pb^{2+} 12.51ppm	1,452631833	0,452296649
Pb^{2+} 25.02 ppm	4,966838298	52,42028893
Ni^{2+} 2.02 ppm Cd^{2+} 3.64 ppm Pb^{2+} 6.26 ppm	1,803500817	1,558739559
Ni^{2+} 2.02 ppm Cd^{2+} 7.29 ppm Pb^{2+} 12.51 ppm	2,920688583	1,266100998
Ni^{2+} 2.02 ppm Cd^{2+} 14.58 ppm Pb^{2+} 12.51 ppm	11,86529284	0,795994274

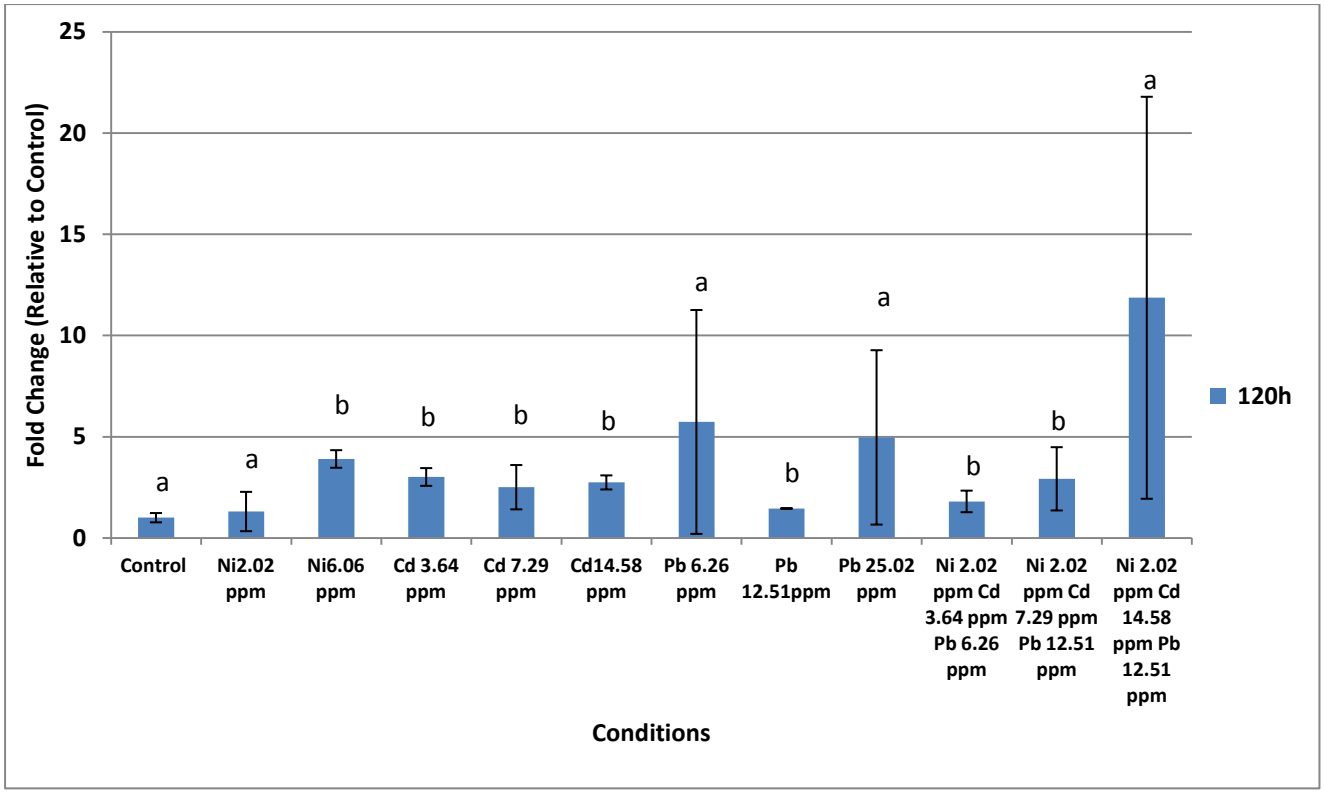

Figure 4.3.1.: Gene expression of Glutathione peroxidase at 120h of exposure to single and tertiary metal mixtures

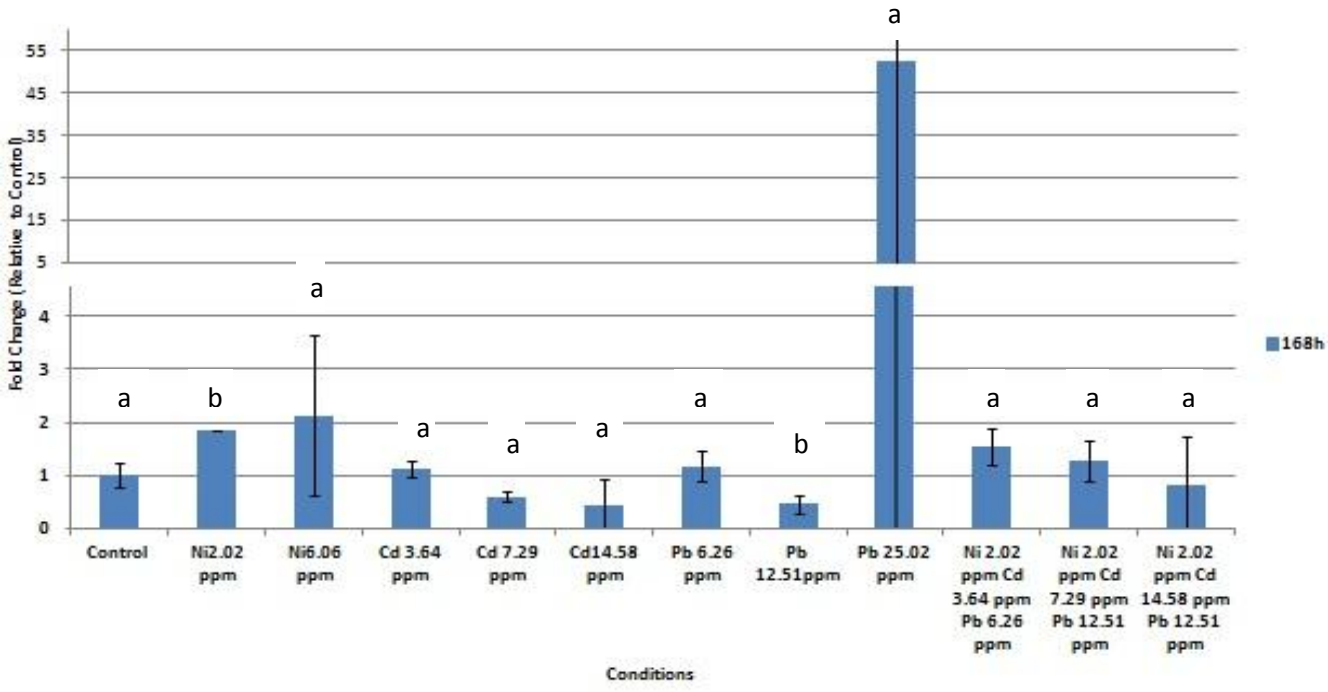

Figure 4.3.2.: Gene expression of Glutathione peroxidase at 168h of exposure to single and tertiary metal mixtures

4.4. Enzyme Activity

4.4.1. Ascorbate peroxidase

When the cells are exposed for 168h to Cd^{2+} a decrease of enzyme activity occurs comparing to 120h of exposure to 3.64 ppm (0.913±0.94) and 7.29 ppm (0.370±0.22) of Cd^{2+} when an increase occurred comparing to control condition (0.30±0.20) (*figure 4.4.1.1., Tables 4.4.1.1., 4.4.1.2.*). At 120h of exposure to Pb^{2+} there is only an increase of enzyme activity for the culture exposed to 25.02 ppm (0.314±0.09) (*figure 4.4.1.1.*). At 168h of exposure there is an increase only for the condition 6.26 ppm of Pb^{2+} (0.866±0.90) (*figure 4.4.1.1., Table 4.4.1.2.*). When the cells are exposed to tertiary mixtures of Ni^{2+}, Cd^{2+} and Pb^{2+} an increase occurs at 120 of exposure for all conditions and a decrease for all at 168h (*figure 4.4.1.1. Tables 4.4.1.1., 4.4.1.2.*). No significant results obtained from this quantification (*Tables A1.20., A1.21.*), although there is a high correlation with nickel, cadmium and lead content at the mixtures at the time point of 120h (*Tables A2.7., A2.9., A2.11.*).

Table 4.4.1.1.: Effect of single and tertiary metal mixtures on the enzyme activity of ascorbate peroxidase at 120h of exposure

Conditions	**µmol AsA/mg(protein).min**	**Standard Deviation**
Control 120h	0,307822638	0,206596512
Ni 2.02 ppm 120h	0,141096433	0,107283948
Ni 6.06 ppm 120h	0,243232644	0
Cd 3.64 ppm 120h	0,913278215	0,944886603
Cd 7.29 ppm 120h	0,370150789	0,223650405
Cd 14.58 ppm 120h	0,279545527	0,047166508
Pb 6.26 ppm 120h	0,180113811	0,035302085
Pb 12.51 ppm 120h	0,172540253	0,180679157
Pb 25.02 ppm 120h	0,31418923	0,095266965
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	0,476513488	0,122753433
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	0,512649268	0,167819318
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	0,915538848	0,7713755

Table 4.4.1.2.: Effect of single and tertiary metal mixtures on the enzyme activity of ascorbate peroxidase at 168h of exposure

Conditions	µmol AsA/mg(protein).min	Standard Deviation
Control 168h	0,612280426	0,616230705
Ni 2.02 ppm 168h	0,358128603	0,056716052
Ni 6.06 ppm 168h	0,098714725	0,056716052
Cd 3.64 ppm 168h	0,459332374	0,030966158
Cd 7.29 ppm 168h	0,328337292	0,100300607
Cd 14.58 ppm 168h	0,16746064	0,22523909
Pb 6.26 ppm 168h	0,866955575	0,907862865
Pb 12.51 ppm 168h	0,383851331	0,239297234
Pb 25.02 ppm 168h	0,076579807	0,22702435
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	0,39513311	0,101600473
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	0,453460693	0,237534065
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	0,35823292	0,118060171

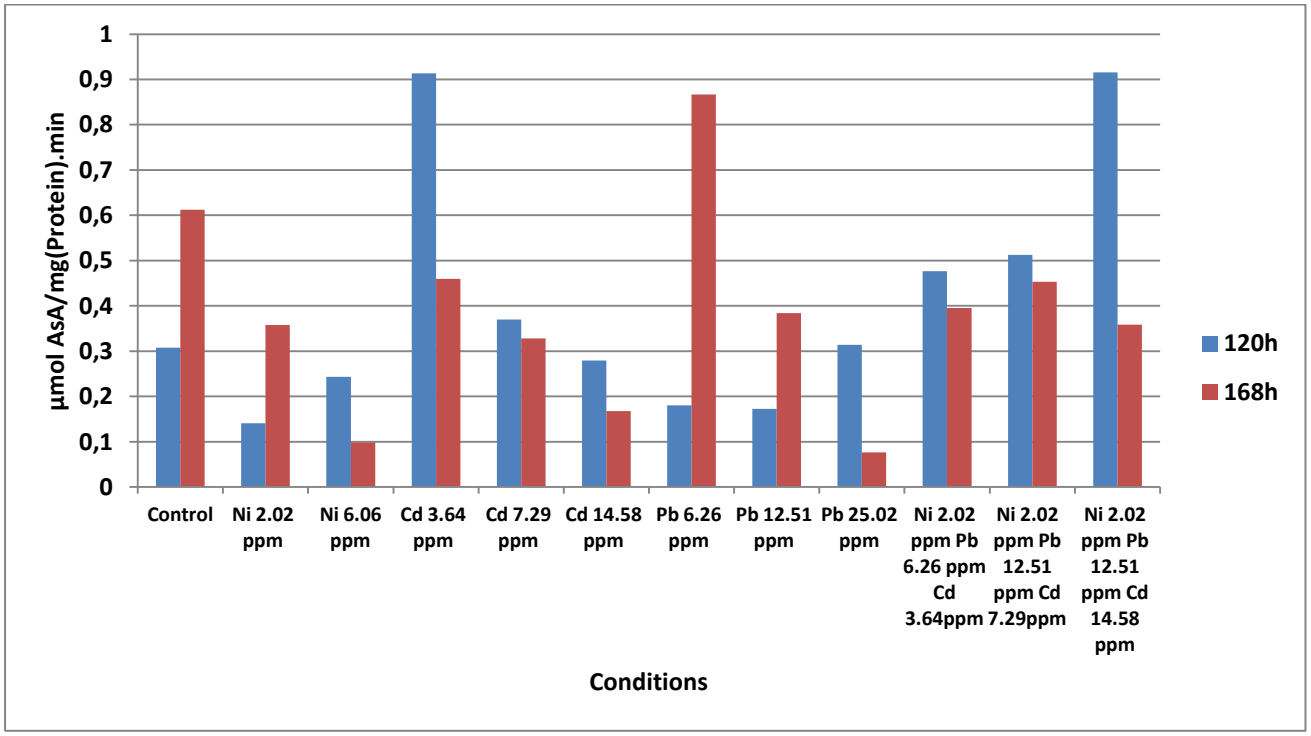

Figure 4.4.1.2.: Enzyme Activity of Ascorbate peroxidase at 120h and 168h of exposure to single and tertiary metal mixtures. About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.4.2. Enzyme Activity of Glutathione Reductase

At 120h and 168h of exposure to Ni^{2+} concentrations, glutathione reductase enzyme activity was lower than control (*Figure 4.4.2.1., Tables 4.4.2.1., 4.4.2.2.*). At Cd^{2+} treatments, 7.29 ppm Cd^{2+} was higher than control at 120h, from the results in *Figure 4.4.2.1.* it seems that glutathione reductase activity is reduced after the exposure to Cd^{2+} for 168h. This reduction seems to be larger with higher concentrations of Cd^{2+}. Pb^{2+} exposure did not result to a clear change in enzymes activity, with the only probable exception of 25.02 ppm at 168h where a reduction was recorded (*Figure 4.4.2.1.,Table 4.4.2.2.*). Experiments with mixtures of pollutants did not result to clear effects on enzymes activity. There seems to be a possibility of increase of activity for the first condition at 120h time point and a decrease of activity for the third condition at 168h time point, as they are presented in *Figure 4.4.2.1..* Glutathione reductase seems to have high correlation along with the increased metal content of lead at the single treatments (*Table A2.5.*), although this has to be proved with significance.

Table 4.4.2.1.: Effect of single and tertiary metal mixtures on the enzyme activity of Glutathione reductase at 120h of exposure

Conditions	**μmol NADPH/mg(protein).min**	**Standard deviation**
Control 120h	0,201615336	0,131688403
Ni 2.02 ppm 120h	0,108473542	0,014970501
Ni 6.06 ppm 120h	0,07018833	0
Cd 3.64 ppm 120h	0,200263511	0,037782235
Cd 7.29 ppm 120h	0,237954238	0,127931139
Cd 14.58 ppm 120h	0,133012164	0,017126444
Pb 6.26 ppm 120h	0,173252696	0,090078172
Pb 12.51 ppm 120h	0,166287253	0,171237525
Pb 25.02 ppm 120h	0,27180598	0,041181677
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	0,338642933	0,113431473
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	0,344644443	0,072355315
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	0,187538194	0,011367214

Table 4.4.2.2.: Effect of single and tertiary metal mixtures on the enzyme activity of Glutathione reductase at 168h of exposure

Conditions	**µmol NADPH/mg(protein).min**	**Standard deviation**
Control 168h	0,419038672	0,39683544
Ni 2.02 ppm 168h	0,207336923	0,124202412
Ni 6.06 ppm 168h	0,136274991	0
Cd 3.64 ppm 168h	0,260172523	0,175882945
Cd 7.29 ppm 168h	0,198023908	0,011123662
Cd 14.58 ppm 168h	0,130539382	0,014235239
Pb 6.26 ppm 168h	0,501860941	0,214205517
Pb 12.51 ppm 168h	0,330757531	0,166271804
Pb 25.02 ppm 168h	0,160932794	0,012908954
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	0,229866927	0,145946115
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	0,288915621	0,21606251
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	0,130349874	0,039886696

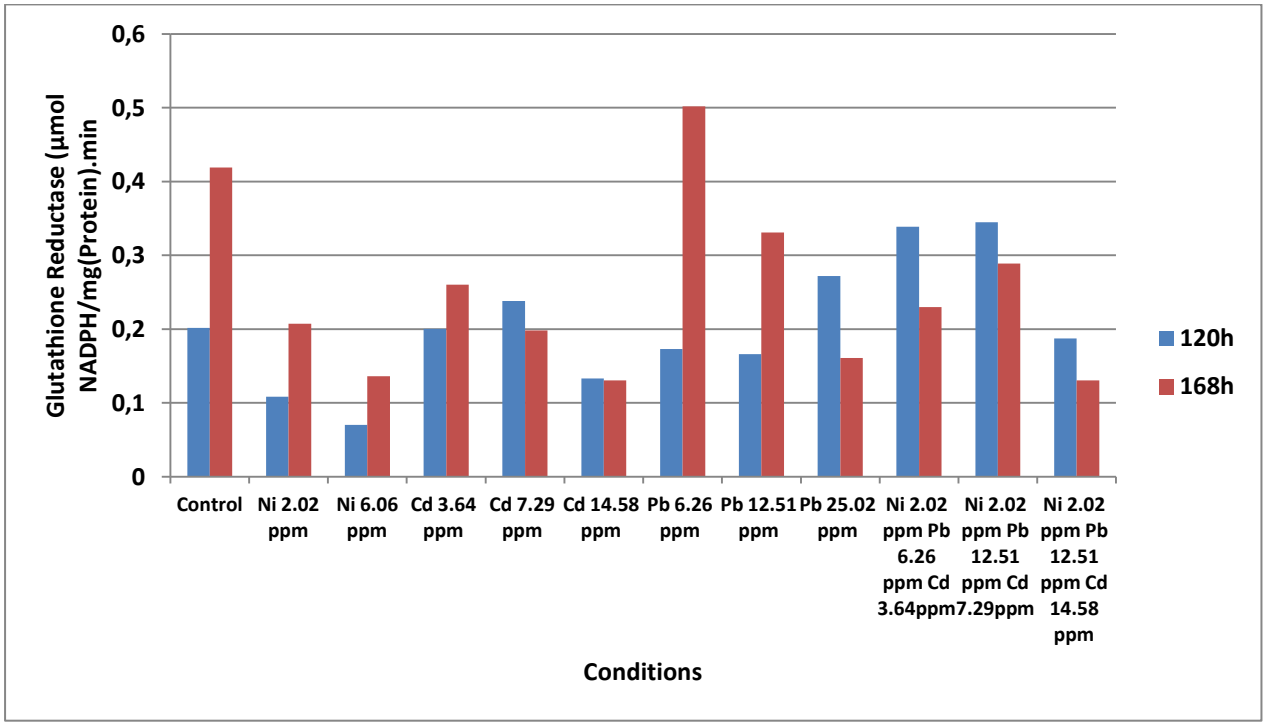

Figure 4.4.2.1.: Enzyme Activity of Glutathione Reductase at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at both time points (n=1)

4.4.3. Enzyme Activity of Peroxidase

Ni^{2+} exposure resulted to lower enzyme activity of peroxidase for the highest Ni^{2+} concentration tested in this study at 120h time point. Also, there is an indication of reduced activity at 168h as well (*Figure 4.4.3.1., Table 4.4.3.2.*). At the time point of 120h, 7.29 ppm and 14.58 ppm of Cd^{2+} treatments exhibited higher enzyme activity than control whereas the most profound reduction was shown for Cd^{2+} 3.64ppm (*Figure 4.4.3.1., Table 4.4.3.1.*). No clear effects were recorded for Pb^{2+} exposure (*Figure 4.4.3.1.*). The most profound effect of the tertiary mixtures was recorded for the condition Ni 2.02 ppm, Cd 7.29 ppm, Pb 12.51 ppm, at 120h time point, whereas a reduction in enzymes activity was shown (*Figure 4.4.3.1., Table 4.4.3.1.*). Peroxidase seems to have high correlation along with the increased metal content of lead at the single treatments (*Table A2.5.*), although this has to be proved with significance.

Table 4.4.3.1.: Effect of single and tertiary metal mixtures on the enzyme activity of Peroxidase at 120h of exposure

Conditions	**µmol oxidized pyrogallol/mg(protein).min**	**Standard deviation**
Control 120h	2,494594263	0,193492821
Ni 2.02 ppm 120h	2,26906435	0,562753951
Ni 6.06 ppm 120h	0,734717743	0
Cd 3.64 ppm 120h	1,494404472	1,670700835
Cd 7.29 ppm 120h	4,284800118	3,112371333
Cd 14.58 ppm 120h	3,599296875	2,190039716
Pb 6.26 ppm 120h	2,460794372	0,365643191
Pb 12.51 ppm 120h	1,94711393	1,003996522
Pb 25.02 ppm 120h	3,699543728	0,355202789
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	3,596560356	1,504555238
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	1,466005507	0,282296913
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	3,511438276	0,499257844

Table 4.4.3.2.: Effect of single and tertiary metal mixtures on the enzyme activity of Peroxidase at 168h of exposure

Conditions	**µmol oxidized pyrogallol/mg(protein).min**	**standard deviation**
Control 168h	4,852280497	3,965449399
Ni 2.02 ppm 168h	3,273432513	1,172375612
Ni 6.06 ppm 168h	3,033672052	0
Cd 3.64 ppm 168h	1,679191974	0,845075786
Cd 7.29 ppm 168h	3,837143233	0,076913824
Cd 14.58 ppm 168h	4,661920469	1,016143191
Pb 6.26 ppm 168h	7,141958202	4,491420755
Pb 12.51 ppm 168h	3,424719777	1,009828667
Pb 25.02 ppm 168h	3,41177852	2,674907873
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	3,334325555	0,263200043
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	3,994587038	2,212712366
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	3,517084421	0,505094373

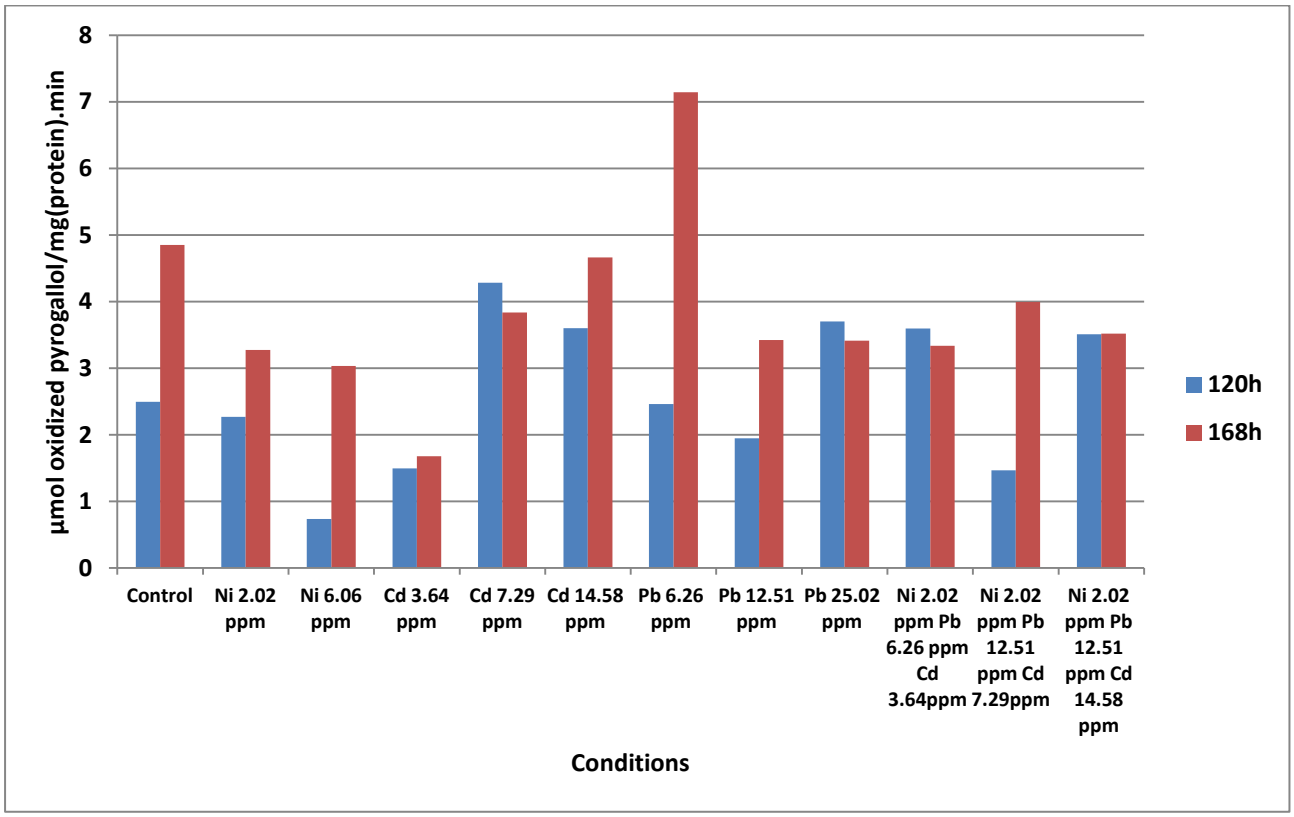

Figure 4.4.3.1.: Enzyme Activity of Peroxidase at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at 120h (n=1).

4.4.4. Enzyme Activity of Proline enzyme Pyrroline 5 – carboxylate synthetase (P5CS)

For 168h of exposure, both of Ni^{+2} treatments seem to result to higher activity than control (*Figure 4.4.4.1., Table 4.4.4.2.*). For 120h of exposure, only 3.64 ppm Cd^{2+} (9.51±0.75) was shown to have an effect of higher activity than control (4.51±0.15) whereas for 168h of exposure all treatments showed higher values comparing to control (*Figure 4.4.4.1.,Tables 4.4.4.1., 4.4.4.2.*). Exposure to Pb^{2+} resulted in a reduction of enzymes activity at 120h time point, whereas no clear results were observed for 168h time point (*Figure 4.4.4.1., Tables 4.4.4.1., 4.4.4.2.*). Similar results with Pb^{2+} behavior were observed for exposes to mixtures of metals (*Figure 4.4.4.1., Tables 4.4.4.1., 4.4.4.2.*). The progressive increase of P5CS content at 168h under cadmium exposure seems promised, although this must be proved with statistical significance (*Figure 4.4.4.1., Table 4.4.4.2., Table A2.4.*).

Table 4.4.4.1.: Effect of single and tertiary metal mixtures on the enzyme activity of P5CS at 120h of exposure

Conditions	**µmol p5c/mg(protein).min**	**Standard deviation**
Control 120h	4,510279452	0,152278749
Ni 2.02 ppm 120h	3,002371671	0,931182169
Ni 6.06 ppm 120h	2,770708759	0
Cd 3.64 ppm 120h	9,51374715	0,75546791
Cd 7.29 ppm 120h	3,961305285	0,017751186
Cd 14.58 ppm 120h	3,43107566	0,45300122
Pb 6.26 ppm 120h	3,387604552	0,472033817
Pb 12.51 ppm 120h	2,658738887	0,674909142
Pb 25.02 ppm 120h	2,958776114	0,547557091
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	3,67263	0,737684974
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	2,738742434	0,767702661
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	2,665501314	0,32616279

Table 4.4.4.2.: Effect of single and tertiary metal mixtures on the enzyme activity of P5CS at 168h of exposure

Conditions	µmol p5c/mg(protein).min	Standard deviation
Control 168h	2,286682088	0,865487126
Ni 2.02 ppm 168h	3,642414456	8,590811877
Ni 6.06 ppm 168h	3,869034115	0
Cd 3.64 ppm 168h	3,474604663	0,104512416
Cd 7.29 ppm 168h	4,158393538	0,887502166
Cd 14.58 ppm 168h	5,744813111	2,405244773
Pb 6.26 ppm 168h	2,87773924	1,03306361
Pb 12.51 ppm 168h	1,881243177	2,2221173
Pb 25.02 ppm 168h	2,796240424	1,212019019
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	2,292624079	0,257344265
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	3,184152052	1,921246053
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	2,188240573	0,169707516

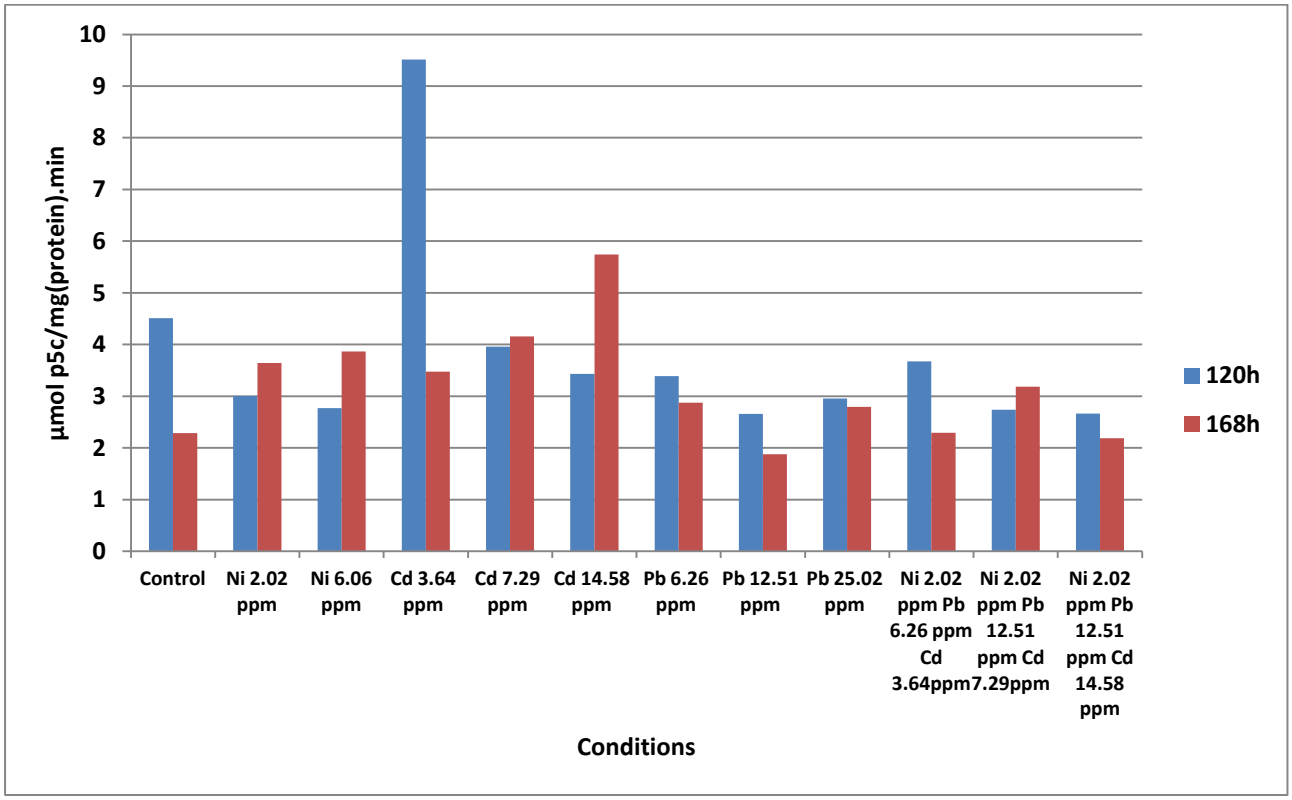

Figure 4.4.4.1.: Enzyme Activity of Pyrroline 5 – carboxylate synthetase at 120h and 168h of exposure to pollutants. Different letters indicate significant differences between the treatments and control (*α=0.05*). About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.4.5. Enzyme Activity of Catalase

At 120h of exposure to Ni^{2+} a reduction of catalase activity was observed for both Ni^{2+} levels whereas no clear effects were observed for 168h time point compared to control (*Figure 4.4.5.1., Tables 4.4.5.1., 4.4.5.2.*). No clear results were observed for Cd^{2+} exposure (*Figure 4.4.5.1.*). Exposure to 12.51ppm of Pb^{2+} seems to result in reduction of catalase activity at both time points (*Figure 4.4.5.1., Tables 4.4.5.1., 4.4.5.2.*). No clear effects were observed on enzymes activity due to exposure to mixtures (*Figure 4.4.5.1., Tables 4.4.5.1., 4.4.5.2.*). .). The progressive increase of catalase content at 168h under cadmium exposure seems promised, although this must be proved with statistical significance (*Figure 4.4.5.1., Table 4.4.5.2., Table A2.4.*).

Table 4.4.5.1.: Effect of single and tertiary metal mixtures on the enzyme activity of Catalase at 120h of exposure

Conditions	**μmol H_2O_2/mg(protein).min**	**Standard deviation**
Control 120h	154,7356965	23,0826556
Ni 2.02 ppm 120h	104,9380964	17,93446071
Ni 6.06 ppm 120h	67,81261536	0
Cd 3.64 ppm 120h	200,9313433	95,90459691
Cd 7.29 ppm 120h	181,0466635	26,50266784
Cd 14.58 ppm 120h	106,214096	22,9921873
Pb 6.26 ppm 120h	150,0801307	19,6246077
Pb 12.51 ppm 120h	78,35677544	22,43816337
Pb 25.02 ppm 120h	127,9779091	19,01477633
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	111,4219531	14,80368936
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	125,7153238	8,172786138
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	182,4969352	76,9643731

Table 4.4.5.2.: Effect of single and tertiary metal mixtures on the enzyme activity of Catalase at 168h of exposure

Conditions	**µmol H2O2/mg(protein).min**	**Standard deviation**
Control 168h	171,0278485	64,44329962
Ni 2.02 ppm 168h	138,414752	74,40991366
Ni 6.06 ppm 168h	162,7540246	0
Cd 3.64 ppm 168h	134,41389	32,75903836
Cd 7.29 ppm 168h	146,7667124	3,246866465
Cd 14.58 ppm 168h	195,9719335	39,85201516
Pb 6.26 ppm 168h	220,3640004	116,6808736
Pb 12.51 ppm 168h	104,7790186	8,988382706
Pb 25.02 ppm 168h	167,8073866	41,11373918
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	154,5216287	36,89288063
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	126,3164858	78,84944871
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	105,4159015	9,778002467

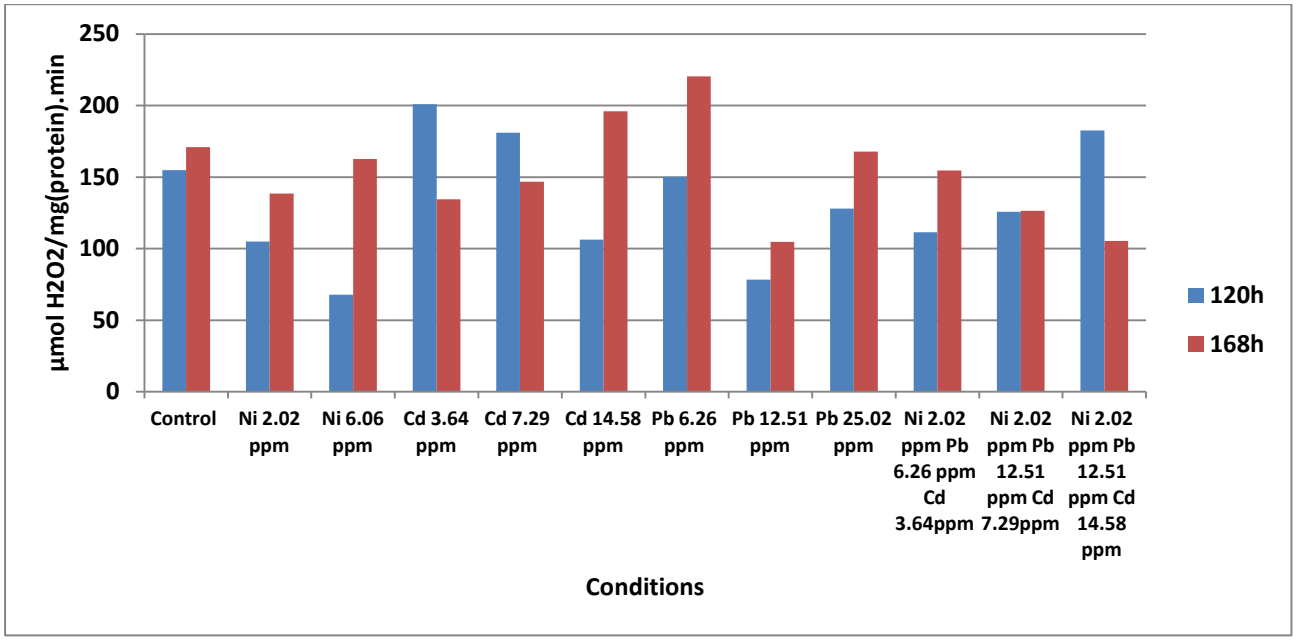

Figure 4.4.5.1.: Enzyme Activity of Catalase at 120h and 168h of exposure to pollutants (n=2). About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.4.6. Enzyme activity of Dehydroascorbate Reductase (DHAR)

Due to very high variation of the observed DHAR activity of both the control and the Ni^{2+} exposed samples the data obtained for Ni^{2+}effects is unclear (*Figure 4.4.6.1.*). The major effect of Cd^{2+} exposure to DHAR activity was observed for Cd 3.64 ppm at 120h (*Figure 4.4.6.1., Table 4.4.6.1.*). The only significant effect of Pb^{2+} exposure was maybe for Pb 12.51 ppm at 120h (*Figure 4.4.6.1., Table 4.4.6.1.*). Inconclusive results for DHAR activity were also observed for metal mixtures (*Figure 4.4.6.1.*), although there is a high correlation with cadmium content at the tertiary mixtures at the time point of 120h (*Table A2.9.*).

Table 4.4.6.1.: Effect of single and tertiary metal mixtures on the enzyme activity of Dehydroascorbate reductase at 120h of exposure

Conditions	**nmol AsA/mg(protein).min**	**Standard deviation**
Control 120h	146,4795228	118,4511768
Ni 2.02 ppm 120h	84,032208	6,629408109
Ni 6.06 ppm 120h	177,7469323	0
Cd 3.64 ppm 120h	684,8140518	781,7974084
Cd 7.29 ppm 120h	154,592627	18,50484556
Cd 14.58 ppm 120h	149,1718953	134,9964298
Pb 6.26 ppm 120h	138,153359	48,23968823
Pb 12.51 ppm 120h	58,21256448	36,26710115
Pb 25.02 ppm 120h	105,1888303	27,95402977
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	114,6323012	44,77541072
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	111,6029066	9,322524756
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	202,9463511	42,95501221

Table 4.4.6.2.: Effect of single and tertiary metal mixtures on the enzyme activity of Dehydroascorbate reductase at 168h of exposure

Conditions	**nmol AsA/mg(protein).min**	**stdv**
Control 168h	142,9574483	94,25283516
Ni 2.02 ppm 168h	171,2400009	123,9012148
Ni 6.06 ppm 168h	180,3188986	0
Cd 3.64 ppm 168h	200,0139931	177,9086348
Cd 7.29 ppm 168h	127,9824108	18,75996745
Cd 14.58 ppm 168h	134,9824419	6,068320775
Pb 6.26 ppm 168h	169,8780939	118,1248766
Pb 12.51 ppm 168h	135,7658289	17,5925333
Pb 25.02 ppm 168h	136,4405282	2,938799443
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	120,0327616	27,51129696
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	175,0324546	202,6714715
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	77,49557251	22,63030968

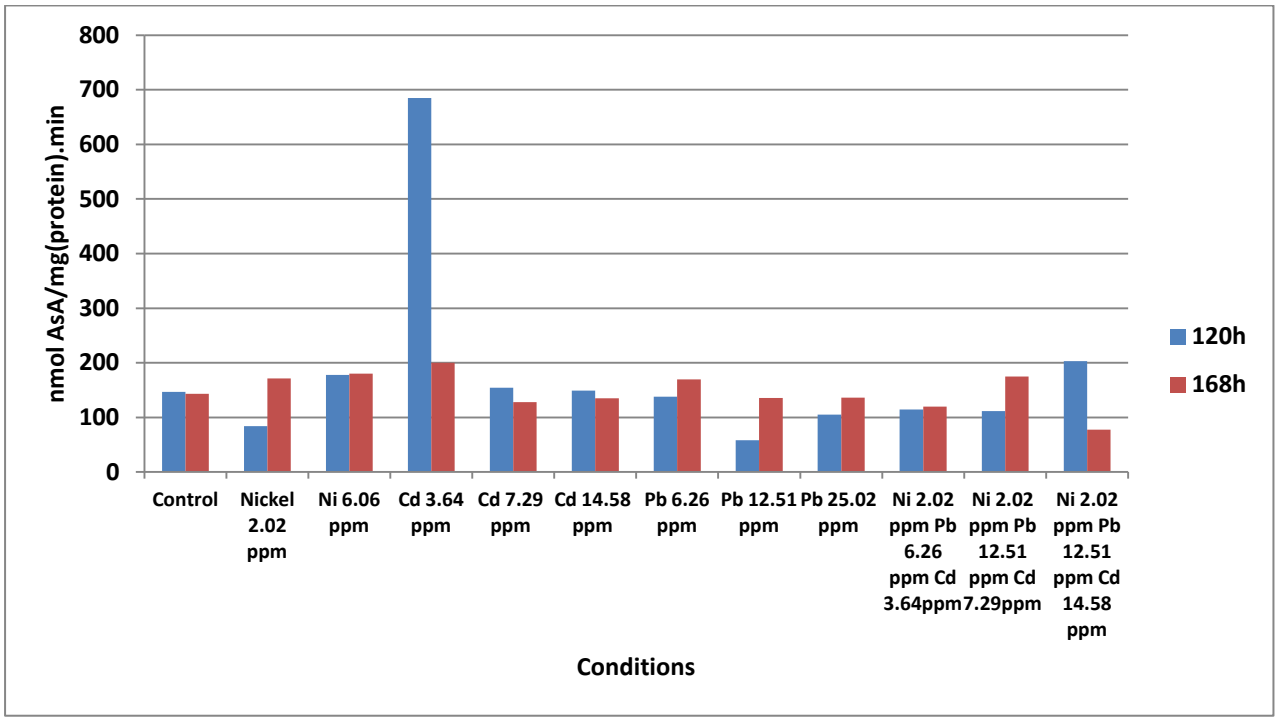

Figure 4.4.6.1.: Enzyme Activity of Dehydroascorbate Reductase (DHAR) at 120h and 168h of exposure to pollutants. Different letters indicate significant differences between the treatments. (*a=0.05*). About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.4.7. Enzyme Activity of Glutathione S – Transferase

Exposure to Ni^{2+} caused a general decrease in GST activity compared to control, for both time points (*Figure 4.4.7.1., Tables 4.4.7.1., 4.4.7.2.*). Exposed to Cd^{2+} did not seem to affect the GST activity. The increase shown for Cd 3.64 ppm at 120h is questionable due to high variation in the repetition results (*Figure 4.4.7.1., Table 4.4.7.1.*). The data obtained for the exposure of the cells to Pb^{2+} and to mixtures of metals do not show any clear effect on GST activity as well (*Figure 4.4.7.1.*).

Table 4.4.7.1.: Effect of single and tertiary metal mixtures on the enzyme activity of Glutathione S - Transferase at 120h of exposure

Conditions	**µmolGSH-CDNB conj./mg(protein).min**	**Standard deviation**
Control 120h	0,078231112	0,016452378
Ni 2.02 ppm 120h	0,048017769	0,01050331
Ni 6.06 ppm 120h	0,045476189	0
Cd 3.64 ppm 120h	0,195840198	0,195721273
Cd 7.29 ppm 120h	0,075479631	0,008696512
Cd 14.58 ppm 120h	0,063366751	0,025760868
Pb 6.26 ppm 120h	0,067935593	0,019530347
Pb 12.51 ppm 120h	0,042636453	0,030071555
Pb 25.02 ppm 120h	0,082089642	0,011170523
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	0,095817999	0,024020111
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	0,067319674	0,001527588
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	0,171886312	0,12361405

Table 4.4.7.2.: Effect of single and tertiary metal mixtures on the enzyme activity of Glutathione S – Transferase at 168h of exposure

Conditions	**µmol GSH-CDNB conj./mg(protein).min**	**Standard deviation**
Control 168h	0,102514363	0,079988349
Ni 2.02 ppm 168h	0,079369886	0,002737506
Ni 6.06 ppm 168h	0,055664137	0
Cd 3.64 ppm 168h	0,082889492	0,007213206
Cd 7.29 ppm 168h	0,066444943	0,000412998
Cd 14.58 ppm 168h	0,063037703	0,009173062
Pb 6.26 ppm 168h	0,17175316	0,130857651
Pb 12.51 ppm 168h	0,077364418	0,010334291
Pb 25.02 ppm 168h	0,058760563	0,017897989
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	0,076043394	0,007528634
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	0,082438993	0,04680132
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	0,046591139	0,00556028

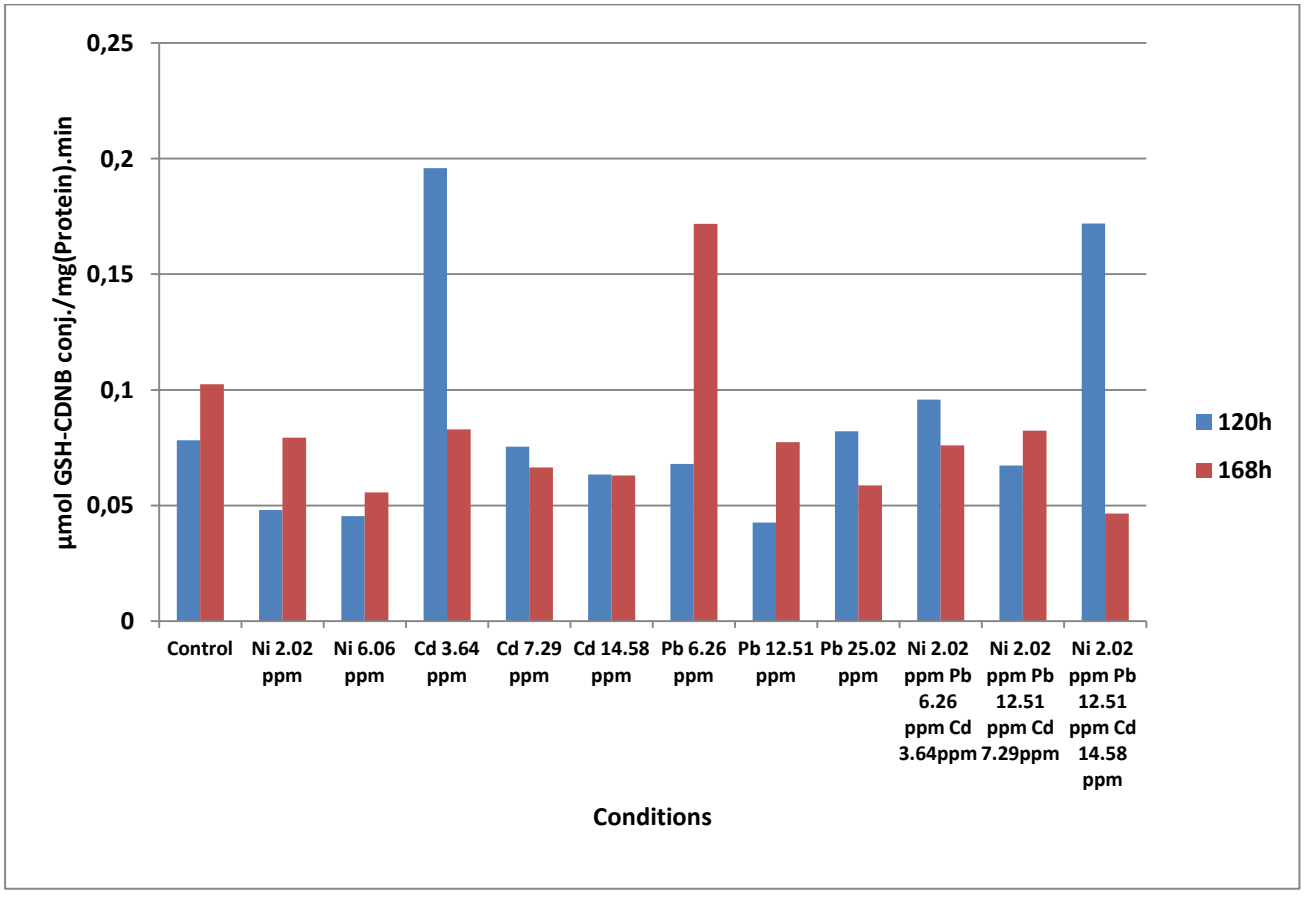

Figure 4.4.7.1.: Enzyme Activity of Glutathione S - Transferase at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.4.8. Superoxide Dismutase

Exposure of the cells to Ni^{2+} pollution caused a decrease to superoxide dismutase activity. This behavior seems to become more obvious to the level of Ni^{2+} pollution increases for both time points (*Figure 4.4.8.1., Tables 4.4.8.1., 4.4.8.2.*). The same trend, a reduction of enzyme activity versus metal concentration seems to hold for Cd^{2+} exposure at 168h time point. In contrast, the results for 120h time limit are inconclusive (*Figure 4.4.8.1., Table 4.4.8.1.*). Exposure to the two higher levels of Pb^{2+} pollution seems to cause reduction to superoxide dismutase activity at 168h time point whereas no such conclusion can be made for 120h time point (*Figure 4.4.8.1., Tables 4.4.8.1., 4.4.8.2.*). The experiment with the mixtures gave inconclusive results (*Figure 4.4.8.1.*).

Table 4.4.8.1.: Effect of single and tertiary metal mixtures on the enzyme activity of Superoxide dismutase at 120h of exposure

Treatments	**Unit SOD/mg protein.min**	**Standard deviation**
Control 120h	3,426216022	2,444359464
Ni 2.02 ppm 120h	1,864731642	0,784986452
Ni 6.06 ppm 120h	0,313021703	0
Cd 3.64 ppm 120h	1,43718452	2,03248584
Cd 7.29 ppm 120h	4,234764889	2,492848189
Cd 14.58 ppm 120h	2,060796812	0,001959999
Pb 6.26 ppm 120h	2,650444716	1,58897041
Pb 12.51 ppm 120h	3,215916766	3,584807335
Pb 25.02 ppm 120h	4,891976021	0,989812103
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	6,696754461	2,332154128
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	6,088360994	0,953248355
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	3,241625262	0,872503789

Table 4.4.8.2.: Effect of single and tertiary metal mixtures on the enzyme activity of Superoxide dismutase at 168h of exposure

Treatments	Unit SOD/mg protein.min	Standard deviation
Control 168h	8,120869019	8,250923069
Ni 2.02 ppm 168h	3,571163018	3,031364004
Ni 6.06 ppm 168h	2,128596594	0
Cd 3.64 ppm 168h	4,378379969	4,050658468
Cd 7.29 ppm 168h	3,428851688	0,148092508
Cd 14.58 ppm 168h	2,91719139	0,138818478
Pb 6.26 ppm 168h	8,255069458	3,041819913
Pb 12.51 ppm 168h	5,878599313	3,71885177
Pb 25.02 ppm 168h	2,741966583	0,236258823
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	4,129334207	2,865152586
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	5,769479896	3,706085128
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	2,406915789	0,557872244

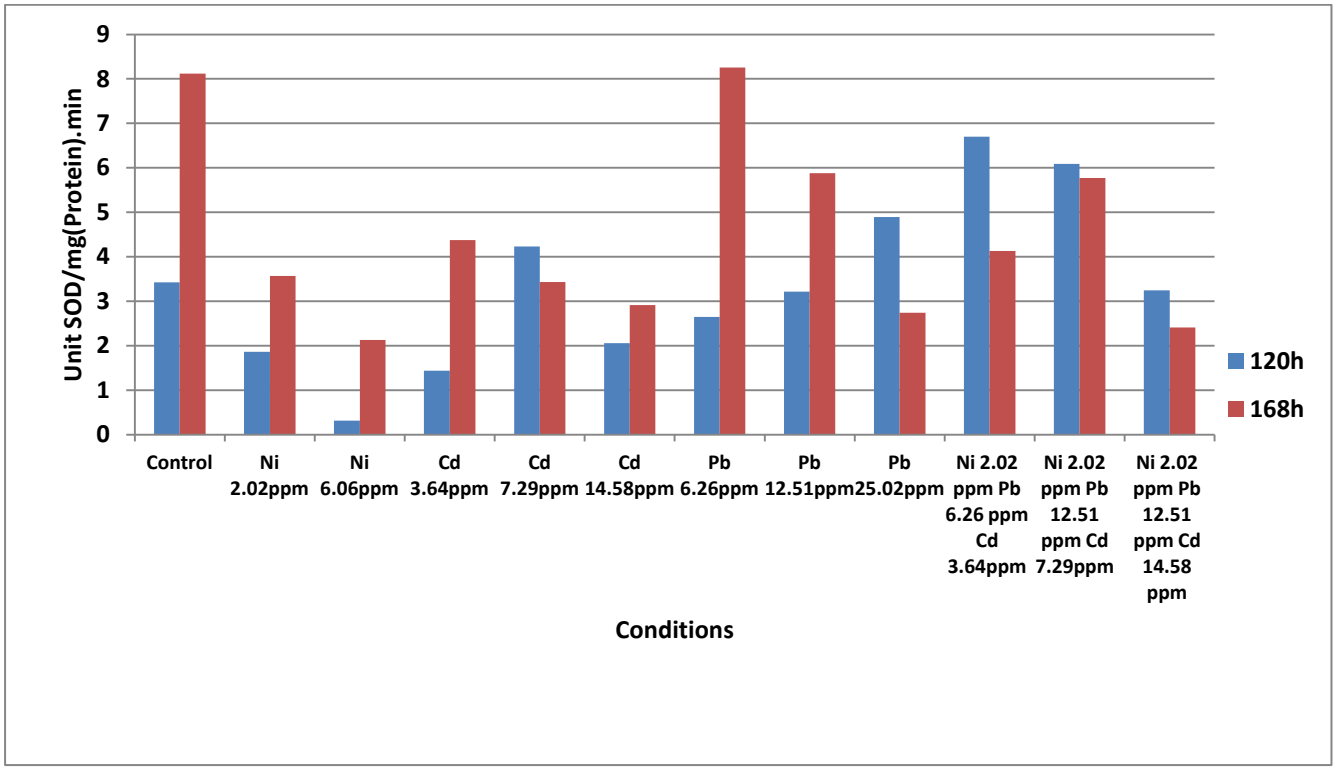

Figure 4.4.8.1.: Enzyme Activity of Superoxide Dismutase at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.5. Metabolites

4.5.1. Total non – enzymatic antioxidant activity

According to *Figure 4.5.1.1,* indicates that the condition of 6.06 ppm Ni^{2+} for 120h results to high content of total non – enzymatic antioxidant activity. There is also an indication of higher non enzymatic antioxidant activity of Ni^{2+} 6.06 ppm sample at 168h. However, this result needs to be considered as potential due to the large error bars shown in the graph (*Figure 4.5.1.1.*). Exposure to Cd^{2+} seems to cause a reduction of non – enzymatic antioxidant activity, at 120h which is roughly unchanged in all three levels of pollution. In contrast the graph in *Figure 4.5.1.1.* shows the opposite behavior for Cd^{2+} pollution at 168h, however, this result should be considered with caution due to the large dispersion of the obtained data.

The most significant change in non enzymatic antioxidant activity due to Pb^{2+} pollution was obtained for the highest Pb pollution level at 168h time point, whereas the other conditions did not give remarkable changes (*Figure 4.5.1.1., Table 4.5.1.2.*). Finally, metal mixtures had rather limited effects on non – enzymatic antioxidant activity, except the third condition at 120h, shown in *Figure 4.5.1.1.,Table 4.5.1.1.*.

Table 4.5.1.1.: Effect of single and tertiary metal mixtures on the total non – enzymatic antioxidant activity at 120h of exposure

Conditions	μmol trolox/1 mil cells	Standard deviation
Control 120h	82,62269825	4,82206742
Ni 2.02 ppm 120h	65,58832476	14,76819905
Ni 6.06 ppm 120h	1114,086339	1527,34645
Cd 3.64 ppm 120h	58,89284383	12,08634039
Cd 7.29 ppm 120h	45,84069179	3,399674167
Cd 14.58 ppm 120h	60,38670827	2,973860791
Pb 6.26 ppm 120h	78,41457804	28,50944831
Pb 12.51 ppm 120h	65,24964194	2,622766362
Pb 25.02 ppm 120h	87,16386758	32,67707977
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	62,35868691	11,41645342
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	66,78701221	2,264936268
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	124,2021394	41,25473792

Table 4.5.1.2.: Effect of single and tertiary metal mixtures on the total non – enzymatic antioxidant activity at 168h of exposure

Conditions	µmol trolox/1 mil cells	Standard deviation
Control 168h	40,73781068	6,070223488
Ni 2.02 ppm 168h	49,00464027	4,930167341
Ni 6.06 ppm 168h	164,9107557	154,2362653
Cd 3.64 ppm 168h	153,1636618	151,9724151
Cd 7.29 ppm 168h	187,9037556	223,1431039
Cd 14.58 ppm 168h	171,2917501	201,4510172
Pb 6.26 ppm 168h	46,60735739	15,25420846
Pb 12.51 ppm 168h	46,70974057	6,936728602
Pb 25.02 ppm 168h	22,51058838	9,645331656
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	39,56928218	2,618831575
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	34,17029701	5,868578638
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	46,2102047	6,479876181

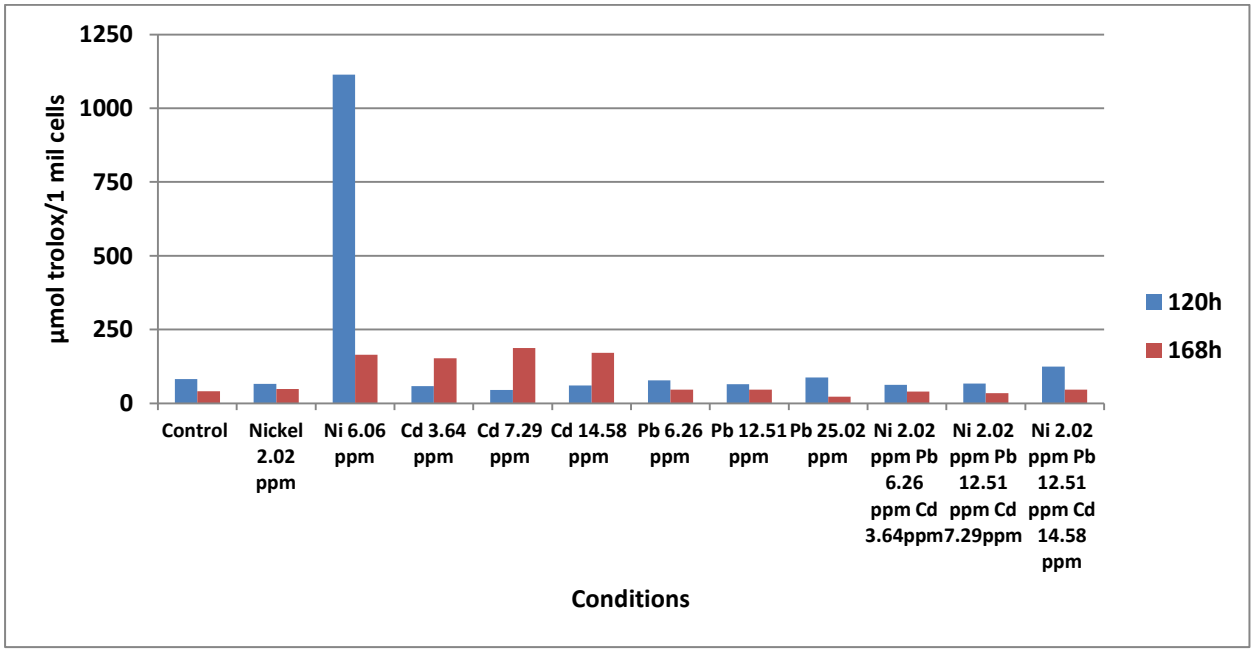

Figure 4.5.1.1.: Content of Total non – enzymatic antioxidant activity of *Chlamydomonas reinhardtii* at 120h and 168h of exposure pollutants.

4.5.2. Polyphenols

The most significant positive effect on polyphenol biosynthesis was observed for the highest level of Ni^{2+} pollution, for both time points (*Figure 4.5.2.1., Tables 4.5.2.1., 4.5.2.2.*). The results from all other conditions were either with no changes, or inconclusive due to very large dispersion of the data (*Figure 4.5.2.1.*). A significant decrease was observed for Cd^{2+} 7.29ppm at 120h comparing to control (*Figure 4.5.2.1., Table 4.5.2.1.*).

Table 4.5.2.1.: Effect of single and tertiary metal mixtures at the content of polyphenols at 120h of exposure

Conditions	**mgGA/ 1 million cells**	**Standard Deviation**
Control 120h	0,011989583	0,001404003
Ni 2.02 ppm 120h	0,01137642	0,003904655
Ni 6.06 ppm 120h	0,152352457	0,208838932
Cd 3.64 ppm 120h	0,007708057	0,000339074
Cd 7.29 ppm 120h	0,006274275	0,000282143
Cd 14.58 ppm 120h	0,009088717	0,000990318
Pb 6.26 ppm 120h	0,009549954	0,003399034
Pb 12.51 ppm 120h	0,010551716	0,002795766
Pb 25.02 ppm 120h	0,014144384	0,006911072
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	0,008362073	0,000747635
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	0,007991561	0,000296499
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	0,014995298	0,005406806

Table 4.5.2.2.: Effect of single and tertiary metal mixtures at the content of polyphenols at 168h of exposure

Conditions	**mgGA/ 1 million cells**	**Standard Deviation**
Control 168h	0,005968003	0,000588693
Ni 2.02 ppm 168h	0,007001898	0,000403321
Ni 6.06 ppm 168h	0,1534164	0,032957239
Cd 3.64 ppm 168h	0,02311123	0,023624964
Cd 7.29 ppm 168h	0,035028484	0,042191536
Cd 14.58 ppm 168h	0,025774828	0,031923778
Pb 6.26 ppm 168h	0,00697239	0,003233904
Pb 12.51 ppm 168h	0,005555033	0,001162006
Pb 25.02 ppm 168h	0,005062701	0,000594038
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	0,005982917	0,000187594
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	0,005092885	0,000728395
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	0,006233735	0,001746278

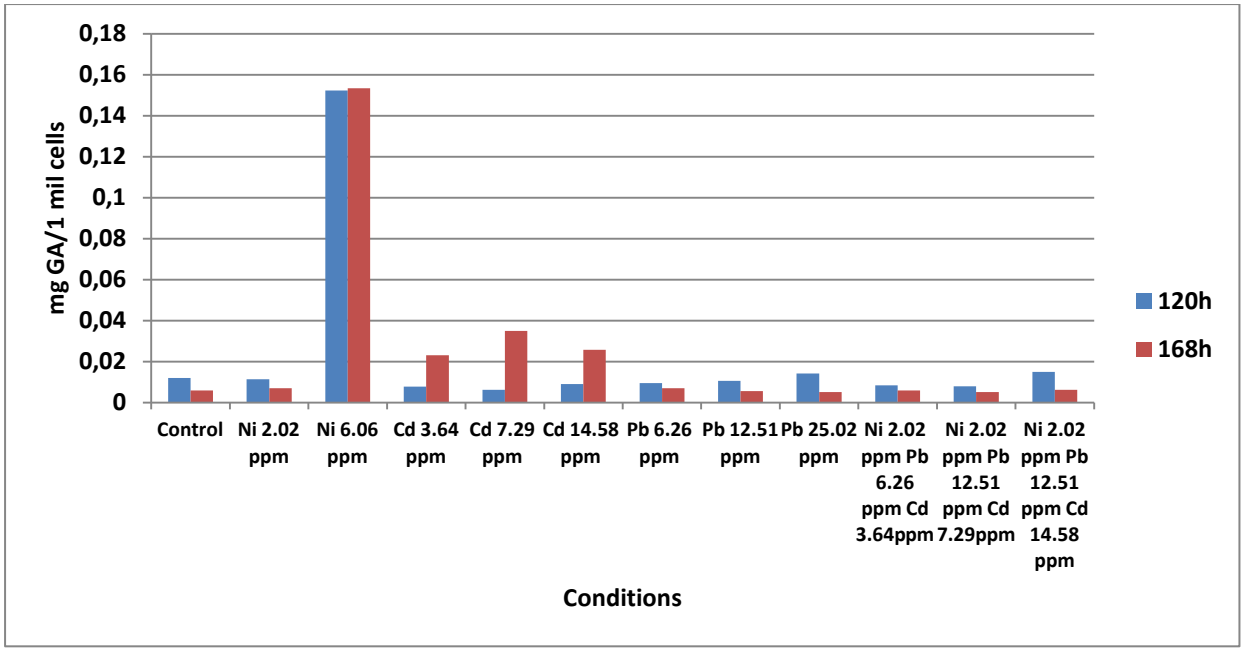

Figure 4.5.2.1.: Polyphenols content of *Chlamydomonas reinhardtii* at 120h and 168h of exposure to pollutants.

4.5.3. Malondialdehyde (MDA)

The lipid peroxidation measurements (as expressed with malondialdehyde formation) in all exposed samples did not lead to conclusive results, either because no major changes were observed (i.e. for mixtures, *Figure 4.5.3.1.*), or to large dispersion of the data, or both.

Table 4.5.3.1.: Effect of single and tertiary metal mixtures at the content of Malondialdehyde at 120h of exposure

Conditions	nmol(MDA)/1mil cells	Standard Deviation
Control 120h	1,041188028	0,440400559
Ni 2.02 ppm 120h	1,118342996	0,10960073
Ni 6.06 ppm 120h	14,96219952	20,36461342
Cd 3.64 ppm 120h	0,641090693	0,130436711
Cd 7.29 ppm 120h	0,588198482	0,133137569
Cd 14.58 ppm 120h	0,91480014	0,172932783
Pb 6.26 ppm 120h	0,879601204	0,352744025
Pb 12.51 ppm 120h	1,28954063	0,221848113
Pb 25.02 ppm 120h	1,26865479	0,465409275
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	0,949316015	0,088730123
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	0,829176252	0,006230299
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	1,701604264	0,851996045

Table 4.5.3.2.: Effect of single and tertiary metal mixtures at the content of Malondialdehyde at 168h of exposure

Conditions	nmol(MDA)/1mil cells	Standard deviation
Control 168h	0,583807755	0,099275821
Ni 2.02 ppm 168h	0,634807432	0,03539967
Ni 6.06 ppm 168h	2,510799071	2,579500552
Cd 3.64 ppm 168h	2,421178887	2,513031928
Cd 7.29 ppm 168h	3,082564896	3,578961861
Cd 14.58 ppm 168h	2,409036984	2,885454742
Pb 6.26 ppm 168h	0,654823174	0,28351308
Pb 12.51 ppm 168h	0,673249036	0,106960062
Pb 25.02 ppm 168h	0,488459575	0,09602979
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	0,7085829	0,001425055
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	0,61458311	0,033608394
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	0,700820594	0,102401823

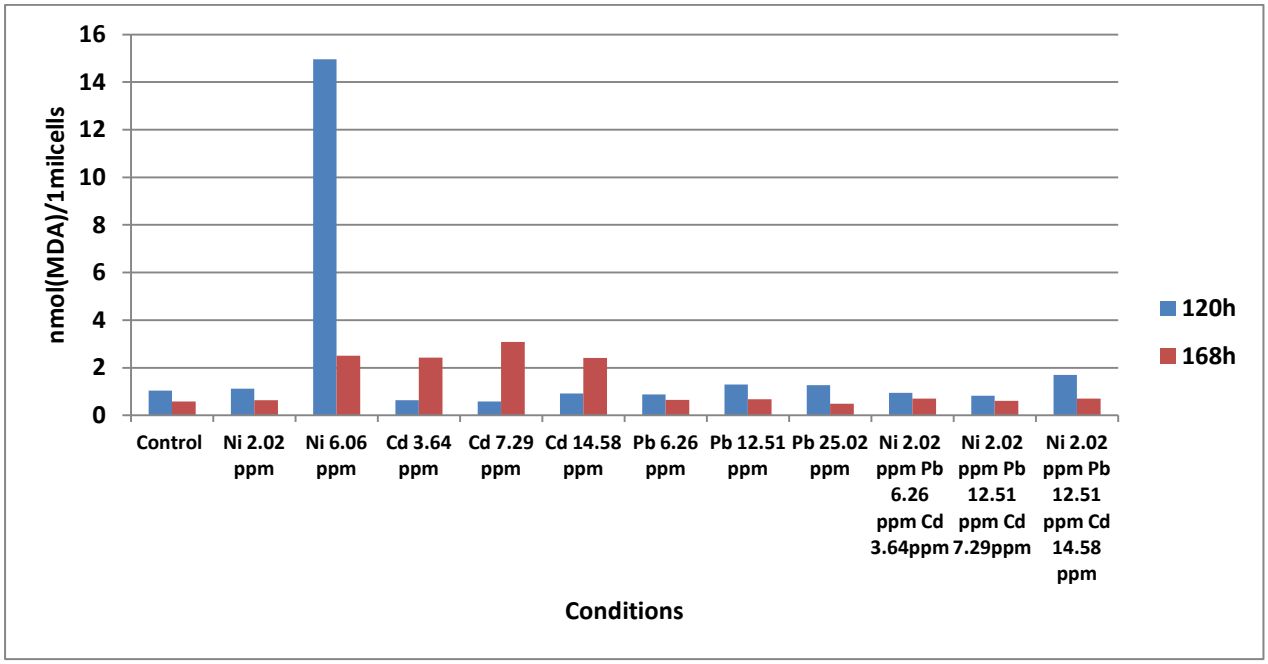

Figure 4.5.3.1.: Malondialdehyde (MDA) content of *Chlamydomonas reinhardtii* at 120h and 168h of exposure pollutants.

4.6. Secondary Metabolites

4.6.1. Chlorophyll *a*

At 120h and 168h time points of exposure to 2.02 ppm Ni^{2+}, chlorophyll a content is was measured, with no significant change to control. At 120h of exposure to 6.06 ppm Ni^{2+}, there was no change for the chlorophyll a content however at 168h of exposure there was a significant decrease (*Figure 4.6.1.1., Tables 4.6.1.1., 4.6.1.2.*). Exposure to Cd^{2+} did not cause any significant changes to chlorophyll a content of the cells (*Figure 4.6.1.1.*). The most significant changes caused by Pb exposure were observed for Pb 25.02 ppm where a slight

increase was measured at 120h and a larger decrease at 168h (*Figure 4.6.1.1., Tables 4.6.1.1., 4.6.2.2.*). The only change in chlorophyll a content due to mixture exposure could be considered at the 168h increase for the first condition and the decrease for the last condition, as shown in *Figure 4.6.1.1.* and *Table 4.6.1.2.*.

Table 4.5.6.1.: Effect of single and tertiary metal mixtures at the content of Chlorophyll *a* at 120h of exposure

Condition	**ChlA (µg/gFW)**	**Standard Deviation**
Control 120h	38,25061903	0,212673559
Ni 2.02 120h	40,15105714	3,616456762
Ni 6.06 ppm 120h	38,40100195	0
Cd 3.64 ppm 120h	52,18666392	7,162992856
Cd 7.29 ppm 120h	31,27995892	5,831986539
Cd 14.58 ppm 120h	39,08220899	2,595749254
Pb 6.26 ppm 120h	42,43983323	22,82430467
Pb 12.51 ppm 120h	32,64151509	9,329753701
Pb 25.02 ppm 120h	45,99774354	9,061000285
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	40,00436545	2,612560324
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	38,75060964	5,959091265
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	33,71332817	15,54994273

Table 4.5.6.2.: Effect of single and tertiary metal mixtures at the content of Chlorophyll *a* at 168h of exposure

Conditions	**ChlA (µg/gFW)**	**Standard Deviation**
Control 168h	36,43846019	8,819097855
Ni 2.02 ppm 168h	48,4843051	11,79724008
Cd 3.64 ppm 168h	34,28635878	3,853017229
Cd 7.29 ppm 168h	43,82679271	11,96534124
Cd 14.58 ppm 168h	27,88241776	18,77961235
Pb 6.26 ppm 168h	45,76735221	1,849462722
Pb 12.51 ppm 168h	44,33783729	3,356069276
Pb 25.02 ppm 168h	19,45555592	13,82105863
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	57,7140629	4,187959874
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	31,7878346	15,33391923
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	23,62910409	18,64321735

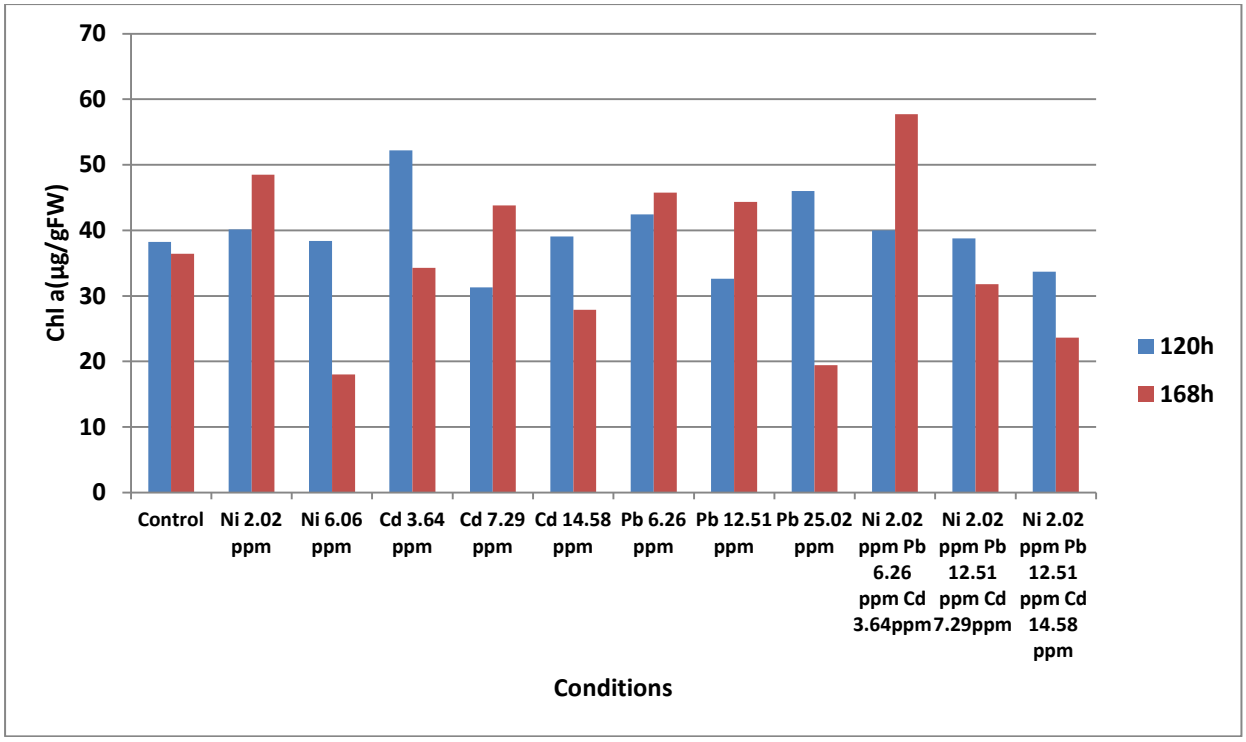

Figure 4.6.1.1.: Chlorophyll *a* content of *Chlamydomonas reinhardtii* at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.6.2. Chlorophyll *b*

The chlorophyll *b* content of the cells, measured at the two time points of 120h and 168h exhibited at the same behavior as chlorophyll a (See paragraph 4.6.1.) for all the conditions (*Figure 4.6.2.1., Tables 4.6.2.1., 4.6.2.2.*). Higher Chl b content was observed at 168h for the condition of Pb^{2+} 6.26ppm and the mixture Ni 2.02ppm, Pb 6.26ppm, Cd 3.64ppm (*Figure 4.6.2.1.,Table 4.6.2.2.*). Lower Chl b content was observed at 120h for the condition of Cd^{2+} 7.29ppm (*Figure 4.6.2.1., Table 4.6.2.1.*).

Table 4.6.2.1.: Effect of single and tertiary metal mixtures at the content of Chlorophyll *b* at 120h of exposure

Condition	**ChlB (µg/gFW)**	**Standard Deviation**
Control 120h	61,49832826	4,390146582
Ni 2.02 120h	60,34830004	15,04229716
Ni 6.06 ppm 120h	55,22533251	0
Cd 3.64 ppm 120h	62,56645725	0,435310131
Cd 7.29 ppm 120h	44,36326942	1,359553724
Cd 14.58 ppm 120h	58,00207615	1,176237815
Pb 6.26 ppm 120h	63,66348401	34,01253002
Pb 12.51 ppm 120h	44,6155903	13,01579827
Pb 25.02 ppm 120h	64,35444414	17,69125475
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	53,65126926	2,082878542
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	58,07880536	8,393052678
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	51,94239094	27,78297912

Table 4.6.2.2.: Effect of single and tertiary metal mixtures at the content of Chlorophyll *b* at 168h of exposure

Conditions	**ChlB (µg/gFW)**	**Standard Deviation**
Control 168h	46,37267046	2,53511797
Ni 2.02 ppm 168h	61,07741483	11,88717051
Cd 3.64 ppm 168h	42,6763571	4,576835749
Cd 7.29 ppm 168h	52,12726546	21,10420047
Cd 14.58 ppm 168h	39,1095651	27,01216348
Pb 6.26 ppm 168h	57,47075694	1,305883567
Pb 12.51 ppm 168h	57,86748999	9,677032023
Pb 25.02 ppm 168h	24,46103683	17,69912606
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	62,12936257	2,380870363
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	42,71446932	13,55992059
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	42,7999109	27,18786816

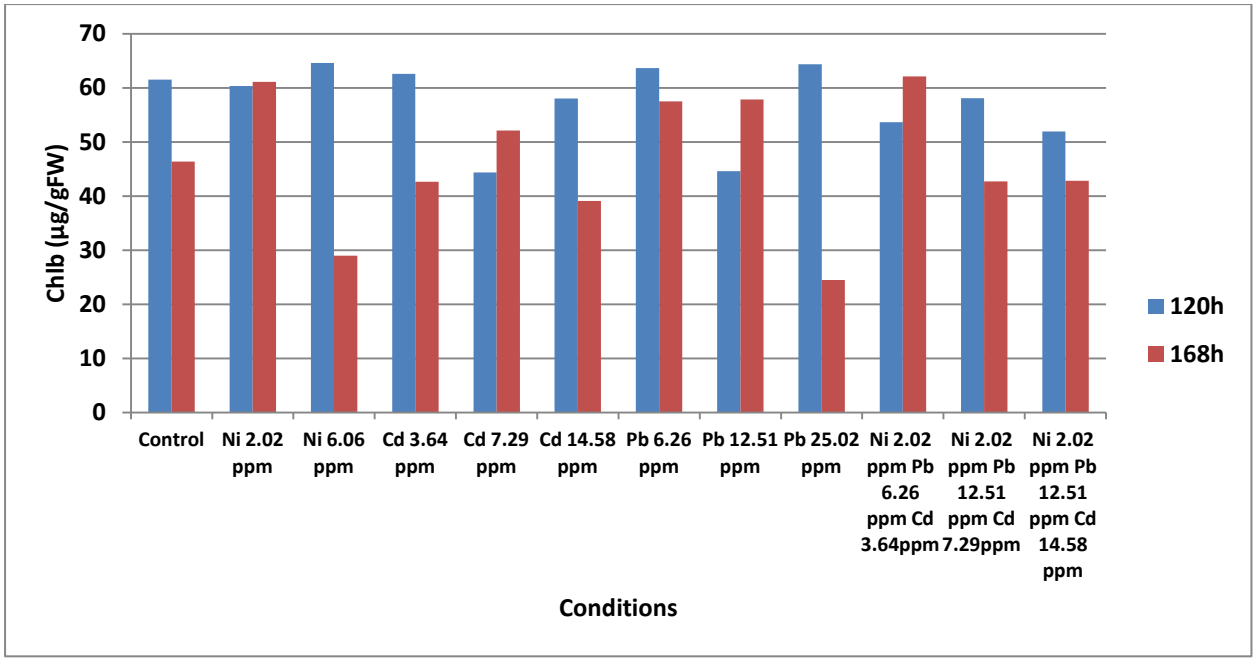

Figure 4.6.2.1.: Chlorophyll *b* content of *Chlamydomonas reinhardtii* at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.6.3. Xanthophylls and Carotenoids

Exposure of the cells to Ni did not seem to have any significant changes to carotenoids content, except the Ni 6.06ppm at 168h where a rather significant change was observed (*Figure 4.6.3.1., Table 4.6.3.2.*). Cd treatments did not affect carotenoid content of the cells (*Figure 4.6.3.1.*). The only noticeable effect of the exposure to Pb was observed at 25.02 ppm at 168h time point, where a decrease of the carotenoids content was observed, which however needs to be considered with caution, due to large dispersion of the averaged values(*Figure 4.6.3.1., Table 4.6.3.2.*). Mixtures of metals had subtle effects on carotenoids content of the cells, with the increase shown at 168h for the first condition shown in *Figure 4.6.3.1.*to be the only noticeable. No significant results were obtained at both time points.

Table 4.6.3.1.: Effect of single and tertiary metal mixtures at the content of Xanthophylls and Carotenoids at 120h of exposure

Condition	**Carotenoids + Xanthophyls (µg/gFW)**	**Standard Deviation**
Control 120h	19,99295384	0,245470187
Ni 2.02 120h	23,21285080	2,168114733
Ni 6.06 ppm 120h	55,22533251	0
Cd 3.64 ppm 120h	26,45547157	6,991864242
Cd 7.29 ppm 120h	20,35230856	4,675325341
Cd 14.58 ppm 120h	24,41569032	2,378290814
Pb 6.26 ppm 120h	23,89058565	10,57861674
Pb 12.51 ppm 120h	19,70926515	5,869391054
Pb 25.02 ppm 120h	27,51890437	4,425928342
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	23,19012442	4,901062649
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	22,41971604	3,814062913
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	24,06150424	11,14389383

Table 4.6.3.2.: Effect of single and tertiary metal mixtures at the content of Xanthophylls and Carotenoids at 168h of exposure

Conditions	**Carotenoids + Xanthophyls (µg/gFW)**	**Standard Deviation**
Control 168h	29,16225464	0,857259579
Ni 2.02 ppm 168h	28,99512226	7,418990341
Cd 3.64 ppm 168h	26,86032847	1,404026392
Cd 7.29 ppm 168h	28,81098331	6,858572786
Cd 14.58 ppm 168h	23,73913651	6,599767953
Pb 6.26 ppm 168h	30,45419217	1,65920739
Pb 12.51 ppm 168h	31,11942625	6,781333323
Pb 25.02 ppm 168h	14,1789289	8,564566814
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	37,48223646	5,031500026
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	26,73424218	5,10101275
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	16,37334987	13,77145032

Table 4.6.3.2.: Effect of single and tertiary metal mixtures at the content of Xanthophylls and Carotenoids at 168h of exposure

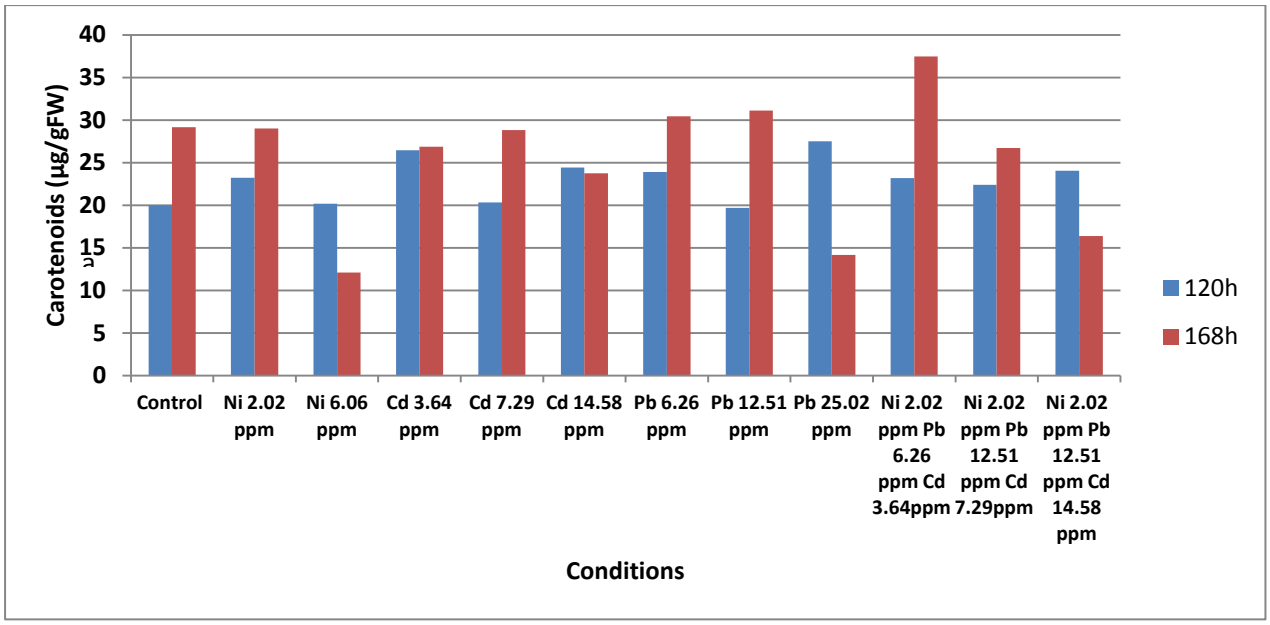

Figure 4.6.3.1.: Carotenoids and Xanthophylls content of *Chlamydomonas reinhardtii* at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at 120h (n=1)

4.6.4. Flavonoids

Exposure to higher concentration of Ni^{2+} 6.06 ppm caused a decrease on flavonoids concentration at both time points (*Figure 4.6.4.1., Tables 4.6.4.1., 4.6.4.2.*). Exposure to cadmium seemed to affect the flavonoid content of the cells at all cadmium levels, but only after 168h of exposure (*Figure 4.6.4.1., Table 4.6.4.2.*). Significant were observed at 168h among the control and the mixture of Ni 2.02ppm, Pb 12.51ppm, Cd 7.29ppm which found to be increased almost three times more comparing to control (*Figure 4.6.4.1., Tables 4.6.4.2., A1.48.*). At the same time point, the same mixture and Ni 2.02ppm, Cd 14.58ppm, Pb 12.51ppm were found to be significant different to the most of the conditions (*Figure 4.6.4.1.*,*Table 4.6.4.2., Table A1.48.*).

Table 4.6.4.1.: Effect of single and tertiary metal mixtures at the content of Flavonoids at 120h of exposure

Conditions	**µmol Q-3-glu equiv/gFW**	**Standard Deviation**
Control 120h	1,2628125	1,473875697
Ni 2.02 ppm 120h	1,43703125	1,013151435
Ni 6.06 ppm 120h	0,41125	0
Cd 3.64 ppm 120h	1,18546875	1,307042691
Cd 7.29 ppm 120h	1,3315625	0,837479594
Cd 14.58 ppm 120h	0,77921875	0,423159214
Pb 6.26 ppm 120h	2,19796875	0,420949506
Pb 12.51 ppm 120h	0,99875	0,322617469
Pb 25.02 ppm 120h	0,86671875	0,913714544
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 120h	2,326875	2,466034899
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 120h	2,351875	2,925654307
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 120h	0,6315625	0,417634943

Table 4.6.4.2.: Effect of single and tertiary metal mixtures at the content of Flavonoids at 168h of exposure

Conditions	**µmol Q-3-glu equiv/gFW**	**Standard Deviation**
Control 168h	2,12375	0,570104842
Ni 2.02 ppm 168h	1,8034375	0,448570864
Ni 6.06 168h	0,683125	0,397747564
Cd 3.64 ppm 168h	1,16359375	0,208817471
Cd 7.29 ppm 168h	1,220625	0,313778634
Cd 14.58 ppm 168h	1,46359375	1,218654343
Pb 6.26 ppm 168h	2,05265625	0,573419405
Pb 12.51 ppm 168h	1,5628125	0,178986404
Pb 25.02 ppm 168h	1,9565625	0,868415516
Ni 2.02 ppm Pb 6.26 ppm Cd 3.64ppm 168h	4,7346875	4,571887282
Ni 2.02 ppm Pb 12.51 ppm Cd 7.29ppm 168h	5,1346875	0,514862125
Ni 2.02 ppm Pb 12.51 ppm Cd 14.58 ppm 168h	3,608125	0,026516504

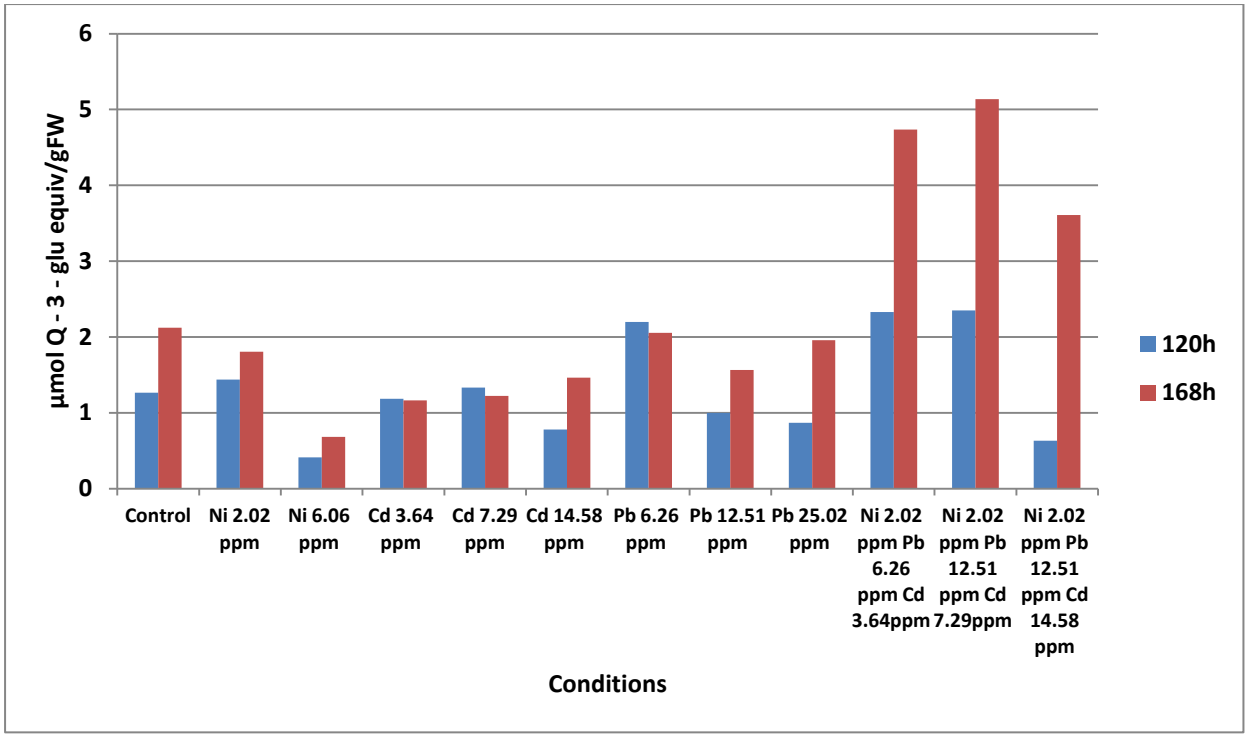

Figure 4.6.4.1.: Flavonoids content of *Chlamydomonas reinhardtii* at 120h and 168h of exposure to pollutants. About the condition of Ni^{2+} 6.06ppm at 120h (n=1).

Chapter 5: Discussion

The study of stress conditions on organisms, e.g. microalgae, focuses on the basis of food chain for the accumulation of pollutants, like heavy metals, which are affected their viability and growth. The use of high advanced technologies like transcript analysis and metabolic analysis offer the advantage of the detection of early effects and subsequently more information of the toxicity mechanism of a stress condition. Model species like the unicellular organism, *Chlamydomonas reinhardtii*, shows to be suitable for determination of biomarkers for the assessment of an integrative approach to the mechanisms of toxicity occurred in specific time points. Finally, at this study, heavy metal pollutants of nickel, cadmium and lead have been included, either as single exposures, either as mixtures.

At this final part, the obtained results will be discussed in different sub – chapters, also will be studied the relation to each other and focus on the goal that we set at the beginning. The questions that must be addressed based on the results are the following:

Do the techniques offer us more and effective information about the mechanism of the metal toxicity?

Do the techniques offer us information for a metal pollution at an earlier stage than the usual endpoints?

Do the specific heavy metal concentrations offer us high added value products?

Growth and viability

Coulter counter is an apparatus for counting and sizing particles suspended in electrolytes. Its use exhibits very good results with high significant results between the treatments. The concentrations of the heavy metal salts were characterized as sub – lethal to the cells, exceptions are Ni^{2+} 8.01 ppm (*Figure 4.1.1.*) and the combined heavy metal mixture Ni^{2+} 6.06ppm, Cd^{2+} 3.64ppm and Pb^{2+} 6.26ppm (*Figure 4.1.4.*) were lethal to the cells and result to death phase. Growth rates of exposed cultures to Cd^{2+} conditions show that all the exposed conditions were significantly higher than control's growth rate (*Figure 4.1.2.*). This result suggests that *Chlamydomonas reinhardtii* can maintain Cd^{2+} in levels that doesn't affect the growth of cells at conditions of 3.64ppm, 7.29ppm and 14.58ppm. All the conditions of exposed cultures to Pb^{2+}, excepting the highest of 25.02ppm, were significantly higher than the control growth rate (*Figure 4.1.3.*). The condition of 25.02ppm Pb^{2+} decreased the cell growth of *Chlamydomonas reinhardtii (Figure 4.1.3.)*. About mixtures, the mixture of Ni^{2+} 2.02 ppm, Cd^{2+} 7.29 ppm, Pb^{2+} 12.51 was significantly higher than control and the mixture Ni^{2+} 2.02 ppm, Pb^{2+} 6.26, Cd^{2+} 3.64 had similar growth rate with the control (*Figure 4.1.4.*). The rest conditions of mixtures had significantly lowest growth rates than control.

Cell accumulation

The range of the values between the treatments can be depended of pH, when the pH is acidic, metals are more toxic free hydrated ions, in alkaline media the precipitation is one of

the reason that toxicity decreases because metals can form insoluble complexes with nutrients. Another one is that direct competition between the metal cations and Ca^{2+}/Mg^{2+} may also cause a reduction to heavy metal accumulation and toxicity.

The use of Inductive coupled plasma spectrometry (ICP - MS) shows statistical significant results among some treatments. Although, according to the figures *4.2.1., 4.2.1., 4.2.3.*, to obtain higher significance, there must be more than two biological replicates. Nevertheless, cadmium cell internal concentration of the mixture Ni 2.02ppm Cd 3.64ppm Pb 6.26 shows a statistical significant increase at both time points comparing to control and Cd^{2+} 3.64ppm single exposure (*Figure 4.2.2.*). This could show that Cd^{2+} shares common metal binding proteins with Ni^{2+} and Pb^{2+}. Ni^{2+} intracellular cell content was increased when Cd^{2+} exposure concentration was raised to 14.58 ppm. In conclusion, Cd^{2+} exposure concentration shares metal binding proteins and metal transporters (*figure 4.2.2.*) with Ni^{2+} and probably they share the same pumpers for entering the cell interior (Synergistic action).

Gene expression

Reverse transcription method was used to determine the expression of associated genes to antioxidant system and probably use the results as markers. Glutathione peroxidase (GPX1) mRNA had been reverse transcribed into cDNA and a qPCR was performed to obtain the quantification of gene expression. The results we obtain shows high statistical significance between the most of the treatments. According to the *Table 4.3.1.*, at 120h of exposure, control was found to be significant different comparing to all Cd^{2+} treatments, to Pb^{2+} 12.51ppm, to the highest concentration of Ni^{2+} and to the mixtures of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm and Ni 2.02ppm, Cd 7.29ppm, Pb 12.51ppm. The condition of Ni^{2+} 6.06ppm exhibits the higher expression according to statistical significance, almost 4 times more comparing to control. Due to toxic effects of Ni^{2+}, Cd^{2+} and Pb^{2+} which are associated with the generation of oxidative stress, glutathione peroxidase plays a crucial role in oxidative defense mechanism of the organism and protects the membranes of the thylacoids from oxidative stress. Glutathione peroxidase plays a crucial role in glutathione metabolism which is responsible for the catalysis of the H_2O production from the degradation of H_2O_2. The overexpression of glutathione peroxidase gene shows that glutathione metabolism probably functions under certain heavy metal stresses. The overexpression of glutathione peroxidase is coming in agreement with the results of *Jamers et al, 2006* where under 8.1μM and 100μM of cadmium at 48h they found an induced expression of glutathione peroxidase 3.4 and 3.5 times increased, respectively. The differences among the most of the treatments have been found significant. Only the highest concentration of lead and mixtures were found with no significant differences. At 168h of exposure, significant differences to control have been found for the conditions of Ni^{2+} 2.02ppm and Pb^{2+} 12.51ppm. About the lowest condition of Ni^{2+}, there was a slight increase of glutathione peroxidase gene expression and for 12.51ppm of Pb^{2+} was a two times decrease comparing to control. Our results come into agreement with the results of *Leisinger et al, 1999*, *Jamers et al, 2006* and *Jamers et al, 2013.* At the last two publications there was overexpression of glutathione peroxidase under the exposure of copper and cadmium, respectively. The differences between the most of the treatments were found significant different. The overexpression of glutathione peroxidase shows that probably glutathione cycle is, probably, active at intensive stresses

and offers protection to the photosynthesis apparatus of the cell. Among the others, at 168h the organism seems to have less response to protect the membranes due to the intensive stress conditions which is proven by the results (*Figure 4.3.1.*).

Enzyme Activity

Studies have been conducted about the enzymes of Ascorbate Peroxidase (APX), Glutathione Reductase (GR), Peroxidase (POX), Superoxide Dismutase (SOD), Dehydroascorbate reductase (DHAR), Catalase and Pyrroline 5 – carboxylate synthetase (P5CS), a key enzyme of proline biosynthesis. The most of them are involved at the ascorbate – glutathione cycle, a part of the plant antioxidant system. The reason that standard deviations are not included at the figures is that the variations were huge and the statistical analysis wasn't possible having two biological replicates. Although, the appendixes A1 and A2 include the tables for p – values and Pearson correlation analysis. According to *Figure 4.4.1.1.,* the quantification of ascorbate peroxidase enzyme activity didn't offer significant differences compare to control, even the control has great variation between the two biological replicates. According to *Figure 4.4.2.1.,* glutathione reductase enzyme activity is inhibited by the treatment of Ni^{2+} 2.02 ppm at 120h and is being induced by the treatments of Pb^{2+} 25.02ppm, Cd^{2+} 14.58ppm and the mixtures of Ni 2.02ppm, Pb 12.51ppm, Cd 7.29ppm and Ni 2.02ppm,Pb 12.51ppm, Cd 14.58ppm. Cd^{2+} 7.29ppm induced the activity of glutathione reductase, while the highest concentration of Cd^{2+} shows an inhibition. Also, there was a significant difference between the highest concentrations of Pb^{2+} and Cd^{2+}, while the first induces the activity of glutathione reductase and the second inhibits. According to *Figure 4.4.3.1.,* peroxidase which is consisted of many large families, e.g. glutathione peroxidases, activity shows significant differences only between the condition of Ni 2.02ppm, Pb 12.51ppm, Cd 7.29ppm and the single exposure of Pb^{2+} 25.02ppm and the mixture of Ni 2.02ppm, Pb 12.51ppm, Cd 14.58ppm at 120h. The mixture of Ni 2.02ppm, Pb 12.51ppm, Cd 7.29ppm exhibits an inhibition of peroxidase activity while the highest concentration of Pb^{2+} and the highest concentration of the mixtures show an induced activity of the enzyme. About the key enzyme of proline biosynthesis, Pyrroline 5 – carboxylate synthetase, the only significant differences were at 120h, between the control and the lowest condition of Pb^{2+} and the mixture of Ni 2.02ppm, Pb 12.51ppm, Cd 14.58ppm. The results show that there was an inhibition of proline biosynthesis under the exposure of the abovementioned treatments. *Alia et al (1991)* have found that Cd^{2+} is the main inducer of proline accumulation and Zn the weakest. At our quantification (*Figure 4.4.4.1.*), the progressive increase of P5CS content at 168h under cadmium exposure seems promised, although this must be proved by significant changes. At 168h there are some promised results that cadmium concentrations exhibits higher enzyme activity comparing to control. The only significant differences for catalase activity (*Figure 4.4.5.1.*) are among Cd^{2+} 7.29ppm to Pb^{2+} 12.51ppm and Ni 2.02ppm, Pb 12.51ppm, Cd 14.58ppm at 168h. Cd^{2+} 7.29ppm show higher enzyme activity than the other two conditions. When cadmium concentration was increased at the mixture, the enzyme activity of catalase was reduced. About DHAR activity (*Figure 4.4.6.1.*), there were significant differences among Ni^{2+} 2.02ppm and Cd^{2+} 7.29ppm at 120h. Ni^{2+} 2.02 ppm exhibits greater inhibition than Cd^{2+} 7.29ppm which induces DHAR activity which plays a crucial role at the ascorbate – glutathione cycle.

Although, control shows big variation and the comparison with the treatments is statistical, very weak. In continue, glutathione S – Transferase (*Figure 4.4.7.1.*) enzyme activity shows significant differences at 168h for the mixture of Ni 2.02ppm, Pb 12.51ppm, Cd 14.58ppm comparing to Ni^{2+}2.02ppm, Cd^{2+} 7.29ppm, Cd^{2+} 3.64ppm and the mixture of Ni 2.02ppm, Pb 6.26ppm, Cd 3.64ppm. The enzyme activity of Glutathione S – Transferase of Ni^{2+} 2.02ppm found significant different comparing to Cd^{2+} 7.29ppm at 168h. The condition of Ni^{2+} 2.02ppm exhibits higher enzyme activity comparing to the abovementioned conditions, which lead to the conclusion that Ni^{2+} 2.02ppm cause more intensive oxidative stress than the other treatments. According to *Figure 4.4.8.1.,* superoxide dismutase enzyme activity at 120h showed that Ni 2.02ppm, Pb 12.51ppm, Cd 7.29ppm is significant different comparing to Ni^{2+} 2.02ppm and Cd^{2+} 14.58ppm. The mixture exhibits higher enzyme activity of superoxide dismutase than the single metal exposure.

Metabolites

Non – Enzymatic antioxidant activity measurement had been conducted to search for possible vitamins (Ascorbic acid, *α* - tocopherol), polyamines, alkaloids etc. According to *Figure 4.5.1.1.,* There were only significant differences at the time point of 120h between control and the single metal exposures of Cd^{2+} 7.29ppm and 14.58ppm, and Pb^{2+} 12.51ppm. Additionally, between Cd^{2+} 7.29ppm and Cd^{2+} 14.58ppm, Pb^{2+} 12.51ppm and the mixture of Ni 2.02ppm, Pb 12.51ppm, Cd 7.29ppm. No treatments have been increased more than control with significant difference. At 168h, there was no significant result between the treatments. Comparing to control, the abovementioned conditions were less abundant in non – enzymatic antioxidant compounds. Also, measurements about the class of polyphenols have been conducted and presented at *Figure 4.5.2.1.*. Polyphenols represent a diverse class of compounds including flavonoids (i.e. flavones, flavonols, flavonones, flavononols, chalcones and flavan-3-ols). The only significant differences were between the Cd^{2+} 7.29ppm with control and the mixture of Ni 2.02ppm, Cd 7.29ppm, Pb 12.51ppm at 120h. The abovementioned single exposure was less abundant in polyphenols content comparing to control. Malondialdehyde (MDA) potential biomarker has been measured in order to check the lipid peroxidation level. The data presented at *Figure 4.5.3.1.,* show that the only significant difference was between control and Cd^{2+} 7.29ppm at 120h. Control condition was more abundant at the end product of lipid peroxidation, MDA, than the exposure to Cd^{2+} 7.29ppm.

Secondary Metabolites

Secondary metabolites content like Chl *a,* Chl *b*, carotenoids/xanthophylls and flavonoids were measured in order to find out inhibition or inducement of chlorophyll biosynthesis and possible induced antioxidant activity of flavonoids. Chlorophyll content is often measured to investigate the impact of heavy metals to pigments biosynthesis and photosynthesis mechanism. About Chl *a* and according to *Figure 4.6.1.1.,* the only significant difference observed was between the conditions of Cd^{2+} 3.64ppm and the mixture Ni 2.02 ppm, Cd 3.64ppm, Pb 6.26ppm, whereas the Chl *a* content of the mixture is significantly increased more than the single condition of the lowest Cd^{2+} exposure at 168h. According to growth data, the growth of Cd^{2+} 3.64ppm is more increased than the combination with the other

two metals. The increased content of Chl *a* at the mixture may is an evidence of potential antioxidant activity of Chl *a* at 168h. Chl *b* content was quantified following the same procedure as for Chl *a*. According to *Figure 4.6.2.1.,* Chl *b*, at 120h, shows that the condition of Cd^{2+} 3.64ppm doesn't affect the biosynthesis of Chl *b* comparing to other conditions like Cd^{2+} 7.29ppm and 14.58ppm and the mixture of Ni 2.02ppm, Pb 6.26ppm, Cd 3.64ppm which there is an inhibition of pigments biosynthesis. A significant difference between the control and Cd^{2+} 7.29ppm was observed, at 120h there was a clearly inhibition of Chl *b* biosynthesis comparing to control. At 168h, biosynthesis of Chl *b* is increased comparing to control for the conditions of Pb^{2+} 6.26ppm and the mixture Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm. This increase may refer to a potential antioxidant activity of Chl *b* when the organism is exposed to these concentrations. Significant differences also observed between the condition of Cd^{2+} 3.64ppm and the conditions of Pb^{2+} 6.26ppm and the mixture of Ni 2.02ppm, Cd 3.64ppm, Pb 6.26ppm. The conclusion is that the lowest concentration is more toxic for the Chl *b* than the lowest concentration of Pb^{2+} and the abovementioned mixture. According to *Figure 4.6.3.1.,* no significant changes were observed from carotenoids/xanthophylls quantification for both time points.

Flavonoids quantification didn't offer significant results at 120h due to high variation between replicates (*Figure 4.6.4.1.*). At 168h, there was significant difference between control and the mixture Ni 2.02ppm, Cd 7.29ppm, Pb 12.51 whereas flavonoids content was significantly increased comparing to control, Ni^{2+} 2.02ppm, Ni^{2+} 6.06ppm, Cd^{2+} 3.64ppm, Cd^{2+} 7.29ppm, Pb^{2+} 6.26ppm, Pb^{2+} 12.51ppm and Pb^{2+} 25.02ppm. The mixture Ni 2.02ppm, Cd 14.58ppm, Pb 12.51ppm was significantly more abundant in flavonoids than the abovementioned conditions, exceptions were Pb^{2+} 6.26ppm and Pb^{2+} 25.02ppm (*Figure 4.4.6.1.*). Increased flavonoids biosynthesis offers maintenance of membranes integrity by preventing the access of harmful molecules to the hydrophobic region of the bilayer, including free radicals which can affect membrane structure.

Chapter 6: Conclusion

Initially, the experiment must be repeated once or even twice to obtain statistically significant results. Nevertheless, there were some promised results from the measurements. About internal metal content, synergistic effects were observed on the accumulation of each of the three metals in the presence of the other two. The enzyme activities of both pyrroline-5-carboxylate synthase and catalase were dependent to the pollutant metal and its level. It's well known that chloroplasts and their arrangement represent a common target of toxic substances in algae and higher plants (*Carginale et al, (2004)., Nacorda et al, (2007)., Basile et al, (2008)*). Gene expression quantification obtained via reverse transcription method offered enough significant differences about the response of *Chlamydomonas reinhardtii* to Ni^{2+}, Cd^{2+}, Pb^{2+} concentrations and combinations of them in mixtures. This leads to the conclusion that at 120h glutathione peroxidase may offer critical assistance for the protection of the chloroplast and subsequently for the photosynthesis apparatus and in general, the survival of the organism. At 120h, among the control and Cd^{2+} 7.29ppm and 14.58ppm and Pb^{2+} 12.51ppm the content of non – enzymatic antioxidant activity remained significant without changes. This is coming in contrast to *Nowicka et al, 2016* who have exposed *Chlamydomonas reinhardtii* to chronic stress induced by Ag, Cd, Cr, Cu, and Hg ions and they have found an increased level of prenyllipid antioxidants. Although, a critical point is that the determination of *Nowicka et al, 2016* was performed using two weeks old cultures.

Chapter 7: Future Perspectives

At the future perspectives, the study of metal transporters for biomaterials research will follow. The construction of knock out lines for specific genes and analyze them with a molecular and biochemical approach. Especially, this study will focus what influence has the absence of a specific gene (e.g. a gene involved in biosynthesis of histidine, asparagine, proline or methionine). At last, an additional work on third or even fourth biological replicate of thesis experiment to obtain more statistical significant results.

Chapter 8: Literature

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Abbrevations

AFS: Allele frequency spectrum

Ag: Silver

APX: Ascorbate Peroxidase

As: Arsenic

Ba: Barium

Bi: Bismuth

BSA: Bovine Serum Albumin

CAT: Catalase

CBLP*: Chlamydomonas* beta subunit-like polypeptide

CCC1: Ca (II) – Sensitive Cross – complementary

Cd: Cadmium

CDF: Cation Diffusion Facilitator

cDNA: Complementary DNA

Co: Cobalt

CPX1: Coproporphyrinogen III oxidase

Cr: Chromium

CRD1: Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase 1

CTR : Cu transporter

Cu/ZnSOD: Copper/Zinc dependent superoxide dismutase

Cu: Copper

CYC6: Cytochrome c6

Cys: Cysteine

DHA: Dehydroascorbate

DHAR: Dehydroascorbate reductase

dNTP: Set consists of four deoxynucleotides (dATP, dCTP, dGTP, dTTP)

DOF: DNA – Binding with one finger

DTT: Dithiothreitol

EC_{50}: Half maximal effective concentration

Ehmt – 1: Histone-lysine N-methyltransferase

EMEP: European Monitoring and Evaluation Programme

EtOH: Ethanol

FeSOD: Iron - dependent superoxide dismutase

FPN: Ferroportin

FRAP: Ferric Reducing Antioxidant Power

FTR: Fe Transporter

GC: Guanine/Cytocine

GPX: Glutathione Peroxidase

GR: Glutathione reductase

GSA: Glutamic –γ – semialdehyde

GSH: Glutathione

GSSG: Oxidized Glutathione

GSSH: Glutathione disulfide

GST: Glutathiones S-Transferase

Hg: Mercury

HSP 70B: Heat Shock Protein 70B

ICP – AES: Inductively coupled plasma atomic emission spectroscopy

ICP – MS: Inductively Coupled Plasma Mass Spectrometry

MDA: Malondialdehyde

MDHA: Monodehydroascorbate

MES: 2-[N-Morpholino]ethanesulfonic acid

Mn: Manganese

MnSOD: Manganese-dependent superoxide dismutase

Mo: Molybdenum

MQ water: Milliq water

MSWI: municipal solid waste incinerator

NADPH: Nicotinamide adenine dinucleotide phosphate

Ni: Nickel

NRAMP: Natural Resistance – Associated Macrophage Proteins

OD: Optical Density

OECD: Organisation for Economic Co-operation and Development

OLIGO $(dt)_{15}$: Primer is designed to initiate synthesis of a cDNA from total RNA in a reverse transcription reaction

OSPAR: Convention for the Protection of the Marine Environment of the North-East Atlantic

P2C: Pyrroline 2 – carboxylate

P5C: pyrroline 5 – carboxylate

P5CR: P5C reductase

P5CS: Pyrroline 5 – carboxylate synthetase

Pb: Lead

PC: Phytochelatin

PCR: Polymerase chain reaction

POX: Peroxidase

PSI: Photosystem I

PUFA: polyunsaturated fatty acids

PVC: Polyvinyl chloride

ROS: Reactive Oxygen Species

RT – PCR: Real Time Polymerase chain reaction

Sb: Antimony

Se: Selenium

SGR: Specific growth rate

Sn: Tin

SOD: Superoxide Dismutase

SPHMT1: *Schizosaccharomyces pombe* heavy metal tolerance factor 1

TAgP: Tris – Acetate – Glycerophosphate

TAP: Tris – Acetate – Phosphate

TBA: Thiobarbituric acid

TCA: Trichloroacetic acid

TNO: The Netherlands Organisation for applied scientific research

TNT: 2, 4, 6 – trinitrotoluene

TPC: Total Polyphenol Content

TROLOX: 6 – Hydroxy – 2,5,7,8, Tetramethylchromane – 2 – carboxylic acid

U: Uranium

UDP: Uridine diphosphate

UNEP: United Nations Environmental Protection

ureG: Urease accessory protein

USGS: United States Geological Survey

V: Vanadium

VIT1: Vacuolar Iron Transporter 1

WHO: Worldwide Health Organization

ZIP: Zrt -, Irt – like proteins

Zn: Zinc

α – OAT: Ornithinea – α – aminotransferase

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