PHYSIOLOGICAL RESPONSE OF *SCENEDESMUS QUADRICAUDA* (TURPIN) DE BRÉBISSON TO LEAD (Pb), NITRATE (NO_3) AND PHOSPHATE (PO_4)

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AUGUST, 2014

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A THESIS SUBMITTED TO THE SCHOOL OF POST-GRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF
MASTER OF SCIENCE DEGREE IN BIOLOGICAL SCIENCES

DEPARTMENT OF BIOLOGICAL SCIENCES,
FACULTY OF SCIENCES
AHMADU BELLO UNIVERSITY, ZARIA,
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NOVEMBER, 2014

DECLARATION

I declare that the work in this project Physiological Response of *Scenedesmus quadricauda* **(**Turpin**)** de Brébisson to Lead (Pb), Nitrate (NO_3) and Phosphate (PO_4) was carried out by me in the Department of Biological Sciences. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Promise Chimdirim KALU _____ _____

Signature Date

CERTIFICATION

This thesis entitled "PHYSIOLOGICAL RESPONSE OF *SCENEDESMUS QUADRICAUDA* **(**TURPIN**)** DE BRÉBISSON TO LEAD(Pb), NITRATE(N) AND PHOSPHATE(P)" by Promise Chimdirim KALU (Msc/Scie/02554/2010 – 2011) meets the regulations governing the award of degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contributions to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to God almighty for his mercies and grace, my family and all those in the field of science.

AKNOWLEDGEMENTS

First and foremost, the honour, glory and praise goes to Almighty God, the knower of my first and last words and deeds and for giving me the strength to do this work.

For the priceless guidance provided to me by the members of my supervisory committee comprising Dr. A.M. Chia and Dr. W.S Japhet, I remain eternally grateful to you.

My thanks go to the lecturers and staff of the department of Biological Sciences, who in one way or the other supported me during the course of this work.

I will not fail to appreciate my colleagues and friends – Egbeagu U, Ramadan M, Ogundayo E, Osazee E, Yunusa S, Ejemole K, Odoh U and Musa A for their support all through the research period.

I say a big thank you, to the family of Mr. Frankly Ogbonnaya.

My profound gratitude goes to members of my family – my parents, my siblings (Uzochukwu, Nneoma, Amarachi, Chibuike, Ruth, Egoudo, Nkechi and uncle Chijoke) for making my stay in this school financially and morally worthwhile.

To members of the Post Graduate Fellowship and church of the Chapel of Redemption and all those who in one way or the other had played a role during the course my study, I say a big thank you.

ABSTRACT

A study on the physiological response of *Scenedesmus quadricauda* to Lead(Pb), Nitrate and Phosphate was conducted by examining their interactive effects on the growth, chlorophyll *a*, dry weight, phenotypic plasticity, cell counts and antioxidant enzyme activities during a 18-day period. The results showed that at lower nitrate (1.8×10^{-5} and 1.8×10^{-6}) and phosphates (1.2×10^{-6} and 1.8×10^{-7}) concentrations, there was a decrease in the biomass production compared to the control (1.8×10^{-4}). The exposure to Pb caused a significant reduction in biomass production of the microalga. The reduction in biomass production had a linear positive relationship with increasing Pb concentration. An increase in the activities of Superoxide dismutase, Peroxidase, Catalase and Glutathione-S-transferase was observed when the microalga was grown under limited nitrate and phosphate concentrations. The activities of the enzymes were further increased when nutrient limitation was combined with Pb stress. These results show that the severity of Pb pollution in aquatic ecosystems will vary depending on the concentrations of important structural nutrients like N and P. This means that even very low Pb concentrations can exert significant toxicity to microalgae when the concentrations of major nutrients are limiting in water.

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CHAPTER ONE

1.0INTRODUCTION

1.1 Background of the Study

Microalgal growth and survival are controlromiseled by prevailing changes in their physical and chemical environment. Abiotic factors such as temperature, light intensity, pH, nutrients and pollutants affect the physiology, biomass production and biochemical composition of algae (Gressler *et al*., 2011, Chia*et al.*,2012). Trace metals contamination in aquatic system continues to increase globally due to increased industrial activities (Ruangsomboon and Wongrat, 2006). Lead (Pb) is a very toxic heavy metal ion that negatively impacts on the environment (Gupta and Rastogi, 2008). Process industries, such as battery manufacturing, metal plating and finishing are also key sources of Pb pollution. Its mobilizationin the environment has greatly increased as a result of major anthropogenic emissions and poor waste disposal that result in its accumulation in food webs (Raungsomboon *et al*., 2008). Pb toxicity to microalgae is well reported in literature. Heng *et al*. (2004) reported that Pbcould inhibit the growth of *Anabeana flosaquae*by 50% at 0.15 and 1µg mL^{-1}. The high toxicity of Pbat very low concentration was further confirmed by Lasheen (1990) who reported a growth inhibition of algae even at 0.05µg mL^{-1}. The dose dependent manner of growth inhibition was also confirmed by Fargasova (1999) who studied the effects of Cd, Cu, Zn, Pband Feon the green alga *Scenedesmus quadricauda*. Fathi *et al*. (2000) further confirmed that these metals induced concentration dependent reduction in the cell count of *Scenedesmus bijuga* and *Anabaena spiroides.* Substitution of heavy metals for Znin some metabol-enzymes could be the possible reason for the growth promotion at low metal concentrations (El-Sheekh *et al., 2003).* The total chlorophyll

content of *Cladophora fracta* significantly decreased when the exposure time and Pb concentration were increased. Pb at high concentrations destroyed chloroplasts of *C. fracta*, which then inhibited chlorophyll biosynthesis (Chantana *et al.*, 2005).

A number of trace metals are used by living organisms to stabilize protein structures, facilitate electron transfer reactions and catalyze enzymatic reactions (Ash and Stone, 2003). For instance, copper (Cu),zinc (Zn) and iron (Fe) are essential as constituents of the catalytic sites of several enzymes (Allan, 1997; Torres *et al.*, 2008). Other metals, however, such as lead (Pb), mercury (Hg) and cadmium (Cd) may displace or substitute for essential trace metals and interfere with proper functioning of enzymes and associated cofactors (Torres *et al*., 2008; Qian *et al*., 2009; Bajguz, 2011). These metals however occur at low or very low (~ 10^{-9} - 10^{-12} mol L^{-1}) concentrations in aquatic ecosystems. The exposure of microalgae to trace metals occurs in mainly chronic exposure, e.g, exposure to low concentrations of trace metal for long periods of time (Bere *et al.*, 2012). This can lead to metabolism modification/adaptation and persistence of the cells, enabling them to survive or eliminate the algae that are unable to adapt themselves to the new conditions. Finally, this will affect the biodiversity of the environment (Chia *et al.*,2012; Chia *et al.*, 2013).

The antioxidative responses to Pb stress are different between algal species (Wu *et al.*, 2009). This also relates to the damage caused by reactive oxygen species (ROS), known as oxidative stress. Thus, algal tolerance to heavy metal pollution is likely to depend on the defense responses that prevent oxidative damage. Metals can break the oxidative balance of the algae, inducing antioxidant enzymes such as Superoxide dismutase (SOD), Glutathione perioxidase (GPX), Glutathione S-transferase (GST), Peroxidase (POD),

Catalase (CAT) and Ascorbate Peroxidase (APX). The amount of oxidized proteins in the algal cells thus indicates the severity of the stress (Okamoto *et al*., 1996). Oxidative damage could be caused either by means of increasing the cellular concentration of reactive oxygen species (ROS) (Macfarlane and Burchett, 2001) or by reducing the cellular antioxidant capacity (Sies, 1999). Various mechanisms such as production of heavy metal binding factors and proteins, exclusion of toxic heavy metals from cells by ion-selective metal transporters and excretion or compartmentalization have been proposed for reducing heavy metal toxicity to organisms (Gharieb and Gadd, 2004). Algal tolerance to heavy metal, however, seems to be dependent highly on the defense responses against the possible oxidative damages (Pinto *et al*., 2003), exudation capacity of chelating compounds, active efflux of metal ions by primary ATPase pumps and reduced uptake (Gaur and Rai, 2001). High level of Pb causes inhibition of enzyme activities, alterations in membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005).

Pb inhibits the activity of enzymes at cellular level by reacting with their sulfhydril groups. High Pb concentration also induces oxidative stress by increasing the production of ROS in microalgae (Reddy *et al.,* 2005). At higher doses, Pb strongly suppresses some enzyme activities of *D.bijuga*metabolism. Metal toxicity is usually accompanied by an inhibition of protein production by algae (Kumar *et al.,* 2010). Several studies have shown that oxidative stress is one of the mechanisms of Pb in microalgae and alternation in enzyme activity such as SOD and GPX, changes in the concentration of antioxidant molecules such as GST in Pb exposed microalgae (Wang *et al*., 2001). SOD activity is an important component of cellular antioxidant system that protects the cell from the harmful effect of O_2^- and its derivatives. Inhibition of SOD activity by Pb can lead to decreased scavenging

of ROS and result in oxidative damage. Peroxidase activity represents the first protective response for small adjustment in H_2O_2 concentrations under normal physiological conditions (Wang *et al*., 2001). Catalase is an important enzyme in the antioxidant defense system protecting microorganisms from oxidative stress. Glutathione can diminish heavy metal toxicity in algal cells (Gowrinathan and Rao, 1992; Amal and Mostafa,1998). Several antioxidant enzymes such as SOD, CAT, GPX, GST etc, protect DNA from oxidative stress. Therefore, exposure of microalgae to Pb alters the antioxidant defense mechanism.

Nutrients are one of the most important factors regulating phytoplankton growth in natural and artificial systems (Chia, 2012). Changes in nutrient regimes can have drastic effects on aquatic ecosystems (Chia *et al*., 2009, 2011a, 2011b, 2012, 2013). Microalgae remove nitrogen and phosphorus from water mainly by uptake into algal cells (Aslan and Kapdan, 2006; Garcia *et al*., 2006). The most widely studied microalgae species for nitrogen and phosphorus removal are *Scenedesmus*(Shi *et al*., 2007), *Chlorella* (Hernandez *et al*., 2006), and *Spirulina* (Olguín *et al*., 2003). For example, phosphorus (P) is critical to the support of life but in excess it can cause eutrophication, which results in severe aquatic environmental impacts. It is well reported that algal blooms are caused by excess of nutrients. Phosphorus, mostly in the form of phosphates, is often reported as the limiting nutrient in the growth of algae (Butzler 2002; Chia, *et al*., 2009, 2012). In biogeochemical cycles, phosphorus (P) is considered as one of the most critical single factor that acts upon its maintenance. Nitrogen (N) is another important nutrient of structural and metabolic functions required in copious amounts by microalgae. It enters phytoplankton based food chain mostly in the form of ammonium or nitrate ions (Chia *et al*., 2011a,2012;2013;).

Nitrates released into tropical waters can come from sediments and/or ground water. Sewage from homes/industries and fertilizers from agricultural lands continuously increase the load of N and P into aquatic systems (Chia *et al*., 2011a).

Nutrients are usually spatially and temporally dynamic in terms of both concentrations and speciation in aquatic environments, and sometimes may be patchily distributed (Beardall *et al*., 2001). Microalgae suffer a series of metabolic acclimations in response to nutrient availability in addition to other environmental conditions that can be referred altogether as physiological plasticity. These oftenresult in variations of the cellular composition, considering macromolecules such as carbohydrates, lipids, amino acids, nucleic acids, pigments and proteins (Berdalet *et al*., 1994; Latasa and Berdalet, 1994; Zhao*et al*., 2009). Changes in these molecules and their specific ratios, including chlorophyll/protein, chlorophyll/RNA, RNA/DNA, protein/carbohydrate under different nutrient conditions can help to indicate the algal physiological state and nutritional status (Lai *et al*., 2011; Chia *et al*., 2012; 2013). Phytoplankton pigment abundance or composition has also been suggested as indicators of the physiological status of phytoplankton (Stolte, *et. al*., 2000; Lai *etal*., 2011).The availability and uptake of trace metals including Pb are influenced by the concentrations of nutrients like P and N (Sivakumar *et al*., 2010). The presence of nutrient replete conditions aids microalgae to cope with the toxicity of trace metals in the environment (Sathya and Balakrishnan, 1988; Serra *et al*., 2010).

1.2 Statement of the Problem

Heavy metals in the aquatic environment are bioaccumulated by organisms either passively from water or by facilitated uptake. Aquatic organisms varied in their physiology as to the

maintaining internal metal concentrations to ones that cannot. Metal pollution is considered hazardous to biological systems because of their oxidative and carcinogenic potential. Recent studies showed that metals can act as carcinogens through oxidative mechanisms, generating free radicals and reactive oxygen species (ROS), which attack and damage DNA and important enzymatic proteins. The toxicology and carcinogenicity of many heavy metals is another important environmental concern of the scientific community (Chang *et al*., 1996; Bal *et al*., 2002).

The primary response of an organism to an oxidative stress condition includes the activation of antioxidant enzymes, and the use of water-soluble antioxidant compounds (Foyer *et al*., 1997; Noctor and Foyer, 1998; Smirnoff, 2000). Oxidative stress is the production and accumulation of reduced oxygen intermediates such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals that can damage protein and DNA. An antioxidant is a molecule that inhibits the oxidation of other molecules and these are substances that (either directly or indirectly) protect cells against adverse effects of xenobiotics, drugs, carcinogens, and toxic radical reactions.

Insufficient levels of antioxidants or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Microalgae are the basis of food chain and food web in all aquatic ecosystem, they are major producers in the ecosystem. Adverse effects on microalgae caused by natural or anthropogenic phenomena can directly or indirectly affect organisms at higher trophic levels and, finally, the integrity of entire ecosystems. The production of reactive oxygen species is also present in aquatic ecosystem and oxidative stress is an important component of the stress response in microorganisms exposed to

variety of stress as a result of changes in environmental conditions such as thermal stress, exposure to pollution.

These stresses lead to a series of changes in microalgae resulting in reduce biomass production and development by affecting molecular, biochemical, and physiological, processes (Wang *et al*., 2003; Sonia and Bruno, 2012). Organisms respond in different ways to heavy metal ion stress including exclusion, chelation, compartmentalization, and expression of stress protein genes.

Lead (Pb) poses serious environmental risk on the ecosystem, the aquatic ecosystem is not left out; high doses of Pb in the water affect the physiological and biochemical composition of microalgae. Excessive amount (≥0.01mg/l) affects biochemical composition and physiology of microalgae. In general, Pb, effects are more pronounced at higher concentrations and continuance. In some cases, lower concentrations can stimulate metabolic processes and the enzymes involved in those processes (Gomes 2011; Sonia and Bruno 2012).This way, being one of the main sources of environmental pollution, previous studies have shown that Pb inhibits metabolic processes such as nitrogen assimilation, photosynthesis, respiration, water uptake, and transcription. In fact, Pb causes two types of unfavorable processes in biological systems:

Firstly, Pb inactivates several enzymes by binding with their SH-groups. Secondly, Pb ions can lead to oxidative stress by intensifying the processes of reactive oxygen species (ROS) production. These processes are mutually connected and stimulate each other by destructively affecting cell structure and metabolism, resulting in a possible decreased efficiency of oxidation-reduction enzymes or the electron transport system leading to fast production of ROS in the cell (Mroczek-Zdyrska and Wójcik, 2012;Sonia and Bruno,

2012) . Organisms have different defense strategies to cope with the toxicity of heavy metals. The primary defense strategy consists in avoiding the metal entry into the cell by excluding or binding it to a cell wall. The secondary defense system is composed of various antioxidants to combat the increased production of ROS caused by metals (Sonia and Bruno, 2012).

Clearly, microalgae response to Pb contamination is a key research problem, and a special effort is being undertaken in seeking factors affecting the reduction of Pb absorption or toxicity in microalgae (Sonia and Bruno, 2012; Mroczek-Zdyrska and Wójcik, 2012)].

1.3 Justification

It is recognized that stress-induced changes at the ecosystem level are of concern. However, such changes are generally too complex and are often omitted from the list of indicators used for early detection and prediction of environmental stress (Depledge *et al.*, 1993). However, experimental evidences suggest that at least in part, it induces oxidative stress in algae (Pinto *et al*., 2003). Many heavy metal ions have a direct influence on various physiological and biochemical processes of microalgae. As the growth reflects the metabolism of the cell, it has been used as a key indicator of the toxicity of heavy metal ions in micro organisms (Carr *et al*., 1998) and it depends on the proper functioning of various metabolic processes, such as photosynthesis, respiration and nutrient uptake, *etc.* (Tripathi and Gaur, 2006). Growth inhibition in microalgae is related to the amount of heavy metal ions bound to the algal cell surface, in some cases, to the amount of intracellular heavy metal ions (Franklin *et al*., 2000, 2002a; Ma *et al*., 2003) and to the chemical nature of the heavy metal ions (Tripathi and Gaur, 2006). Hence, investigations

into variations in physiological characteristics of populations of organisms including microalgae are important. Changes in growth, biomass production, biochemical composition and antioxidant responses to stress serve as important biomarkers because they change between species, in addition to environmental conditions.

Lead pollution in Nigeria has started to increase due to indiscriminate pollution of substances such as batteries , metal plating and finishing these are key sources of Pb pollution and also in recent times the Pb poisoning cases in some states in Northern Nigeria(Zamfara, Sokoto) due to gold mining activities in the states (CDC, 2010). Limiting nutrients (Nitrates and Phosohates) poses serious risk to Nigeria aquatic ecosystem as result of the rise in agricultural activities**(**Nwachukwu, 2012). *Scenedesmusquadricauda*microalgae is now an important species in the Nigeria freshwater. Reports on phycological information in similar freshwater bodies are available Kadiri 2002:Mustapha 2010 Onyema *et al*., 2010: Mustapha 2010: Adejare and James 2010).

Even though, metal toxicity to algae has been thoroughly studied in the past couple of decades (Tripathi *et al*., 2000), a comprehensive description about the mechanism underlining the toxicity is yet to be fully elaborated and understood. Toxicity of a metal seems to be related to cell surface interactions or to intracellular accumulation (Morlon *et al*., 2005). Most studies have considered the influence of either metals or nutrients on the physiological response of microalgae. Very few investigations have considered the combined effects of Pb (at lethal and sub-lethal concentrations/environmentally relevant concentrations) and nutrient limitation (N and P). In many aquatic systems, growth of

phytoplankton is not only P and/or N limited (or in excess), but also controlled by the presence of metals (Spijkerman and Wacker, 2011). This in turn poses a risk to the survival of herbivores that depend on phytoplankton, which is due to P limitation and Pb toxicity (Lukas *et al*., 2011).

This implies that the physiological state of algae is a function of several environmental processes acting simultaneously in an additive, antagonistic or synergistic way. To date, little is known about the antioxidant response of freshwater microalgae to Pb at varying N and P concentrations. Therefore, this area of research still requires further investigations as the responses may vary among different microalgae species. This study will contribute to the current understanding of the responses of freshwater microalgae to the effect of Pb, N and P.

1.4 Aimand Objectives

To determine the effect of Pb at different N and P concentrations on the growth, biomass production and antioxidant response of *D. quadricauda.*

The objectives of this study were to determine:

I. The effect of Pb in combination with N and P on the growth and biomass production of *D. quadricuada*;

II. The changes in phenotypic plasticity of *D. quadricauda* as a function of different Pb, N and P combinations.

III. The interactive effect of Pb and N, and Pb and P on the biochemical composition and antioxidants response of the microalga *D. quadricauda*.

1.5 Hypotheses

The research hypotheses for this study are that:

1. Pb in combination with N and P does not significantlyaffect the growth and biomass production of *D. quadricauda.*

2. Phenotypic plasticity of *D. quadricauda* is not influenced by different concentrations of Pb, N and P.

3. Pb does not significantly interact with N and P to determine the biochemical composition and antioxidant response of *D. quadricauda.*

CHAPTER TWO

2.0LITERATURE REVIEW

2.1 Microalgae *Scenedesmus quadricauda*

Scenedesmus is a ubiquitous organism, and frequently is a dominant microalga in freshwater lakes and rivers (Borowitzka and Borowitzka, 1998). The green algae are among the most common and taxonomically diverse of the chloroccalean genera, within which over 200 species, and almost 1200 infra-specific taxa, have been identified (John *et al.*, 2002).*Scenedesmus quadricauda* is one of the green algae, classified to:

Division: Chlorophyta

Class: Chlorophyceae

Order: Chlorococcals

Family: Scenedesmaceae

Genus: *Scenedesmus*

Species*: quadricauda* ……. (Al-Saai and Sulaiman 2006).

Usually, this alga is found in fresh water and would be with coenobium, the number of cells about 2- 16 cells, arranged in one or two rows, may some of terminal cells contain processes or spikes. The cells are oval or lunar and have one wall plastid with one starch center (Al-Saai and Sulaiman 2006). *Scenedesmus* grows, reproduces and survives like any other living organism. It is a sensitive alga . *Scenedesmus* is sensitive to cation concentrations and its growth can be affected by high concentrations of heavy metal, even at very low concentration of this metal, its survival is threatened. Most of the research focuses on involving the algae with concentrations of organic and inorganic compounds. *Scenedesmusquadricauda* is interesting for its colonial form, structure and reactions with

heavy metals andpollutants. *Scenedesmus* are small, non-motile algal cells that are easily recognized by their oval shape and their tendency to form orderly looking chains of cells called cneobium. They are a cosmopolitan species, meaning they can be found wherever conditions are acceptable and they are common throughout the world. They can float individually but they are usually found in colonies of 2-16, and the two end cells of the chain have spines typically around 200 micrometers in length. They are autotrophic, meaning they get their carbon supply from CO_2 using photosynthesis. *Scenedesmus*free float near the surface where they can get access to light. They form small colonies that may be of advantage in terms of resource gathering and use, as well as protection from predation and environmental stressors(Al-Saai and Sulaiman 2006). They have been observed to preferneutral, pH, temperatures slightly over room, and a slight concentration of iron(Al-Saai and Sulaiman 2006).

2.2 Heavy Metals in the Environment

Metals occur naturally, and several of them are essential components of global ecosystems. They are present in the environment with a wide range of oxidation states and coordination numbers, and these differences are related to their toxicity. Metals such as copper (Cu) and zinc (Zn) are essential to life, whereas others such as lead (Pb) and mercury (Hg) are not known to perform a useful biochemical function (Allanr 1997). Environmental pollution by metals became extensive as mining and industrial activities increased in the late 19th and early 20th century. The currentworldwide mine production of Cu, Cadmium (Cd), Pb, and Hg is considerable (Kennish 1996). These pollutants, ultimately derived from a growing number of diverse anthropogenic sources (industrial effluents and wastes, urban

runoff, sewage treatment plants, boating activities, agricultural fungicide runoff, domestic garbage dumps, and mining operations), have progressively affected more and more different ecosystems (Macfarlane and Burchett 2001).It appears that human activity is often closely linked with toxic pollution. In one study comparing a developed with an undeveloped seawater bay in Massachusetts (Pospelova *et al.,* 2002), pollution in the developed area caused a drastic decline in phytoplankton species richness as determined from analyses of dinoflagellate cysts in the sediment. Interestingly, this study concluded that 72% of the variability in species richness could be attributed to increases in copper. A phytotoxicity test, evaluating the inhibition of photosynthesis in the green alga *Chlamydomonas* by heavy metals (Hg, Cu, Pb, and Cd) leaching from salt mine wastes,showed that inhibition varied from about 100% (Hg) and 80% (Cd) to almost 0% for Pb (Wundram *et al.*, 1996).Heavy metals are usually present at low concentrations in oceanic surface waters originating from atmospheric transport and upwelling. Higher levels occur in coastal waters, however, because of river runoff. Close to urban centers, pollution is associated with sewage outlets (Wickfors and Ukeles 1982; Rebhun and Amotz 1984), but levels are also elevated near extensive areas of industry (Cotte´-Krief *et al.*, 2000, Bu-Olayan *et al.,* 2001, Esser and Volpe 2002).

Interestingly, nutrient availability (in particular, nitrogen) dramatically increases the ability of algae to accumulate heavy metals (Wang and Dei 2001a, b), suggesting that agricultural runoff into fresh water and coastal areas will greatly increase the entry of heavy metals into the food chain. In the natural environment, organisms living in chronically polluted sites are exposed to low concentrations of metals for long periods. In other cases, organisms may be abruptly exposed to high levels of metals upon the outfall of a pollutant in coastal

waters. Though toxic at high concentrations, Cu and Zn are micronutrients essential for the activity of many enzymes and part of molecules playing key roles in photosynthetic electron transport (Raven *et al.,* 1999), as distinct from Hg and Pb, which are toxic but non-essential elements. The storage of metals by cellular detoxifying mechanisms makes them available for assimilation by the biota and biomagnifications along the aquatic food chain, with the potential for producing adverse effects throughout the marine environment. In this context, standard biotoxicity tests are useful tools to determine the effects of pollutants on cell growth and viability. These tests are especially important for evaluating the potential impact of pollution on aquatic ecosystems, aiming to prevent possible injuries to the biota by establishing maximum tolerable levels of toxicants (Laube *et al.,* 1980; Chapman *et al.,* 1996). The mechanism underlying toxicity of heavy metals is not always clear. More generally, however, heavy metal toxicity is related, at least in part, to the oxidative stress induced in living systems (Robinson*et al.,* 1994; Okamoto and Colepicolo 1998; Adonaylo and Oteiza 1999; Livingstone 2001; Wang and Shi 2001). Heavy metals can promote oxidative damage both by directly increasing the cellular concentration of reactive oxygen species (ROS) (Winterbourn 1982) and by reducing the cellular antioxidant capacity (Sies 1999). In contrast to higher plants, antioxidant response to oxidative and environmental stress of algae still needs to be extensively investigated at the molecular level (Reed and Gada 1990; Rodriguez-Ariza *et al.,* 1991; Okamoto*et al.,* 1996) as it is not always the same for all organisms and pollutants. In recent years it is interesting to note the development of programs using microorganisms for water and wastewater treatment, heavy metal control in natural waters and industrial waste streams, and even biological detoxification. For example, potential tools for bioremediation of Chromium

(Cr) pollution using algae have been described (Cervantes *et al.*, 2001), and it seems clear that different species of algae accumulate metals tovarious degrees (Jordanova *et al.*, 1999). Many parameters affect the accumulation of heavy metals from solution by *Chlorella vulgaris* (Bajguz 2000; Lo´pez- Sua´rez *et al.*, 2000). Likewise, the biological concentration factor (defined as Cb/Cw, where Cb is the metal concentration in the biota expressed as mmol or mg/mL of dry weight and Cw is the metal concentration in water given in mmol or mg/mL) for Cu, Pb, Cd, and Hg in *Porphyra* spp. and *Enteromorpha* spp. changed seasonally in field conditions and was specific for each metal reproducibly over several years (Vasconcelos and Leal 2001). The use of algae in bioremediation depends on their ability to survive potentially toxic treatments. However, algae have been used to identify areas of trace metal contamination because of the absorption and toxicity of heavy metals (Muse *et al.*, 1999; Yu *et al.*, 1999). To reconcile these observations, it must be noted that accumulation of heavy metals is not always related to their toxicity. Heavy metal tolerance has been demonstrated for the green algae *Chlorella* and *Scenedesmus*. In particular, *Scenedesmus acutus* strains tolerant to Cr were also tolerant to Cu but not to Zn (Abd-El-Monem *et al.*, 1998). To understand tolerance and to maximize the potential application of algae to bioremediation, it is essential to understand why heavy metals are toxic and how algae can defend against them.

2.3 Effect of Trace Metals on the Physiology of Microalgae

Trace elements are transferred between trophic levels, becoming bioaccumulated (biomagnified, or increasing in concentration with each trophic step) or biopurified (decreasing in concentration with each trophic step) depending on the elemental species

and particular pathway through the food web (Sankaran, 2012). Microalgae are sensitive indicators of environmental change and, as the basis of most freshwater and marine ecosystems, are widely used in the assessment of risk and development of environmental regulations for metals (Levy *et al*., 2007). There are a remarkable number of investigations demonstrating the toxic effects of heavy metals on different species of algae (El-Sheekh *et al*., 2003; Osman *et al*., 2004; Fathi *et al*., 2005; Akira *et al*., 2005; Muwafq and Bernd, 2006; Anne *et al*., 2006).

Heavy metal constitutes a stress factor for many microbes where their concentrations increased as a consequence of a wide range of agricultural and industrial activities. De Filippis and Pallaghy (1994) reportedthat some trace metals are required for cell growth, the high concentrations of trace metals can exert toxic effects on metabolic machinery of algae. The phenomenon of the growth of algae subjected to heavy metals stress was clarified (Fathi and Falkner, 1997; Fathi, 2002; 2005; Fathi *et al*., 2008).It is recognized that stress-induced changes at the ecosystem level are of concern. A probable solution to this problem lies in the effective characterization of distress signals at the molecular and cellular levels (biomarkers) that can provide early warning prognostics of reduced performance (Moore *et al.*, 2004; Torres *et al.*, 2008). Doing this, possible linkages to higher ecological levels are possible and precise prediction of its consequences can be made. Typically, biomarkers are defined as quantitative measures of changes in the biological system that can be related to exposure to the toxic effect of environmental chemicals (Torres *et al.*, 2008). According Torres *et al*., (2008) the use of the term *biomarker* or *biomarker response* is often restricted to cellular, biochemical, molecular or

physiological changes that are measured in cells, body fluids, tissues, or organs within an organism that are indicative of environmental stress. An observation of biochemical composition variation like total proteins, carbohydrates and lipids will implicate the effect of metals on microalgae. Metal toxicity is usually accompanied by an inhibition of protein production by algae (Kumar *et al.,* 2010). However, the production of proteins and antioxidant enzymes by microalgae when exposed to different metal concentrations vary depending on their degree of tolerance and species. It has generally been shown that exposure to metals leads to increases in 18:1 content, alteration of the relative proportions of 18:2 and 18:4 (with the changes being metal-specific) in microalgae (Gushina and Harwood, 2006a; Pinto *et al.,* 2011). Heavy metals constitute a very heterogeneous group of elements, which greatly differ in their chemical properties, biological function and how an alga respond to a particular metal will depend on the:

i. the form of the metal in water or chemical speciation (Rijstenbil and Gerringa, 2002);
ii. the influence of other ions especially cations such as Na^{+}, Ca^{2+} and other trace metals as well as nutrients (Struck *et al.*, 1997);
iii. the inherent tolerance (or sensitivity) of the algal cell (Csonto *et al*., 2001);
iv. the physiological condition of the algal cell (Gaur and Rai, 1994).

Heavy metalions can cause plasma membrane depolarization and acidification of the cytoplasm (Cardozo *et al*., 2002; Conner and Schimid, 2003). In fact, cell membrane injury is one important effect of heavy metal ions that may lead to the disruption of cellular homeostasis. A chain of metabolic events, beginning with the respiration and photosynthesis and continuing with uptake and assimilation of nutrients, dilution of

intracellular level of the heavy metal ions, *etc*. seems to play an important role in balancing the cellular homeostasis, regardless of whether they are strongly or weakly correlated with the algal growth (Tripathi and Gaur, 2006). Membrane injuries seem to be common in algal and cyanobacterial response to metal toxicity (Rangsayatorn *et al*., 2002).

Heavy metal ions (such as Pb^{2+}) are capable of binding to thylakoid membrane resulting in the alteration of the ultrastructure of thylakoids, which would eventually deteriorate the routine functions of thylakoids (Heng *et al*., 2004). There are some reports on the destruction of the chloroplast by heavy metal ions at higher concentrations (Lamaia *et al*., 2005). In fact, it is well known that Cddisorganizes chloroplast causing the damage of photosynthetic pigments (Leborans and Novillo, 1996). As a consequence of this, the photosynthetic activity could severely be affected, causing the growth inhibition or complete death of the cells. Biosynthesis of phycocyanin and carotenoid could also be affected by the heavy metal ions (Atri and Rai, 2003). In addition, heavy metal ions could interrupt routine metabolic processes by competing for the protein binding sites; activate enzymes and various biological reactive groups, causing poor or no growth. The presence of heavy metal ions in the growth medium could induce the activity of the peroxidase that is involved in the degradation of indole acetic acid (IAA), a hormone widely known for its ability of stimulating plant growth and multiplication. Some heavy metal ions may inhibit enzymes in the cytoplasm such as esterase and ß-D-galactosidase (Franklin *et al*., 2001). Taking into account of all the previous reports, it could be suggested here that heavy metal ions can inhibit the growth of microalgae in different ways, which depend on the species, the metal types and the condition in the growing media. Morlon *et al*., (2005) investigated the cellular growth and intracellular concentrations of selenite in the unicellular green alga

Chlamydomonas reinhardtii and concluded that toxicity is mainly linked to intracellular accumulation of selenite. For all the experimental parameters (except lipid content), namely protein, carbohydrate, and photosynthetic-pigment levels, uptake and assimilation of nitrate, photosynthetic O_2 evolution and respiration and growth of *Scenedesmus*sp., were decreased by Cu and Zn (Tripathi and Gaur, 2006). They concluded that almost constant lipid level in *Scenedesmus*sp. under metal stress might be due to the tolerance of the lipid metabolism enzymes or to a simultaneous decrease (or in-crease) in the activities of enzymes involved in the production and consumption of lipids Siripornadulsil, *et al*., (2002) observed that exposure of *Chlamydomonas* to lower toxic levels (50 to 100molL^{-1}) of Cd resulted in reduced growth rates and chlorophyll content per cell (70% less chlorophyll per cell) and increased lipid peroxidation and GSH oxidation. The mode of toxicity of copper has been investigated in several studies with microalgae (Wilde *et al*., 2006).

Growth is widely used in assessing the toxicity of heavy metals to microalgae, because it is the metabolic process most frequently and apparently affected. Most of the studies with microalgae (*Chlorella*, *Chlamydomonas*, *Scenedesmus*and *Pseudokirchneriella* sp.) have shown that the increase of metal toxicity with the increase of pH is a result of decreased competition between the metal ion and H^+ at the cell surface (Franklin *et al*., 2000; De Schamphelaere *et al*., 2003). However, some studies have shown that the increase of metal toxicity with the decrease of pH is due to the predominance of the free metal ion at low pH (Rai *et al*., 1993). Morlon *et al*., (2005) stated that toxicity is primarily related to intracellular accumulation, while, Wilde *et al*., (2006) stated that Zn-induced inhibition is not related to the intracellular Zn concentration, but to extracellular Zn. Furthermore,

Hassler and Wilkinson (2003) reported that in the case of Zn uptake in *Chlorella kesslerii*, neither Zn concentration in the solution nor surface-bound Zn was a good predictor of Zn uptake fluxes. Wilde *et al*., (2006) reported that Cu has no effect on other cell functions such as photosynthesis, respiration, ATP production, electron transport and membrane ultrastructure, though it inhibits the cell division of *Chlorella* sp. However, Wong *et al*., (1994) reported Cu-induced structural alterations in thylakoid membranes of *Chlorella* sp. and inhibition of photosynthesis. Although many plants and algae respond to heavy metal stress with increased steady state levels of Pro, Siripornadulsil *et al*., (2002) reported that *Chlamydomonas* does not accumulate pro in response to Cd exposure. Their results are consistent with earlier investigations on wild-type *Chlamydomonas* strains, where carboxylate groups accounted for nearly half of all Cd binding sites (Adhiya *et al*., 2002). The chlorophylls level in *Olisthodiscus luteus* decreased to 86.7% after 180 hr exposure to 0.5 mg/l $Cd^{2+.}$ In addition, high Cd concentrations reduced cell sizes and caused adecrease in growth rate (Fernandez-Leborans and Novillo, 1996). Nassiri *et al*., (1997) found no growth inhibition at Cd concentrations < 1mg/l, but *Tetraselmis suecica* had 10, 30 and 50% growth inhibition, after 4 days in solutions contained 2, 5 and 10 mg/l Cd respectively. Arif, (1998) reported effects of Cd, Cu, cobalt(Co),mercury(Hg), lithium(Li), manganese(Mn), molybdenum(Mo), nickel(Ni) and Pbwith different concentrations on growth, chlorophyll, DNA and protein contents of *Chlorella vulgaris*. The highest growth inhibition was caused by Hgwhile the least caused by Mo. In addition, all the used heavy metals reduced total chlorophyll and protein contents, which increased by increasing the heavy metals concentrations. Toxic effects of Cu, Ni,Pb and Zn on photosynthesis and motility of *Chlamydomonas reinhardtii* were observed at concentrations ranged from 0.1

to 2.0 mg/l and Cuhad the highest effect (Danilov and Ekelund, 2001). Exploring the fundamental physiological relationship between nutrient and metal uptake in algae is important in piecing together a biogeochemical picture of environments where nutrient and metal enrichment co-occur. Algae play an intrinsic role in the cycling of nutrients and metals by sequestering elements from their environment and transferring them down into sediments and up through food webs.

Currently, the majority of aquatic ecosystem are increasingly influenced by anthropogenic activities (Halpern *et al.*, 2008) making the co-occurrence of metal and nutrient enrichment commonplace in coastal environments (Sankaran, 2012).

2.4Lead (Pb)

Lead (Pb) is a silvery-white highly malleable metal. Among its physical properties, at normal environmental conditions this metal is present in solid state; it is dense, ductiles, and very soft with poor electrical conductivity when compared to most other metals. The chemical symbol for lead, Pb, is an abbreviation of the Latin word *plumbum*, meaning soft metal.

Pb is rarely found in native form in nature but it combines with other elements to form a variety of interesting and beautiful minerals. Galena, which is the dominant Pb ore mineral, is blue-white in color when first uncovered but tarnishes to dull gray when exposed to air (Johnson, 1998).Not so long ago, Pb had a widespread use in all anthropogenic activities, for instance, leaded paints, automobile batteries (as lead oxide), ammunitions, molten Pb as coolant, leaded glass, crystal, and fossil fuels. Until recently, tetraethyl lead (TEL) was commonly used in petrol fuels as an inexpensive additive used

since 1920. Even today Pb is still used in protective coatings with applications for radiation shielding in medical analysis (Vural and Duydu, 1995; Prengaman, 2003). According to Ma (1996)and Rossato*et al*, (2012), one way of exposure of organisms to Pb is via the food chain. It has long been recognized that the heavy metal accumulation in soil may result in potential health risk to organisms (Zhuang *et al.,* 2009).

In general, Pb effects are more pronounced at higher concentrations and continuance. In some cases, lower concentrations can stimulate metabolic processes and the enzymes involved in those processes (Gomes, 2011).Pb negatively influences growth by reducing the uptake and transport of nutrients in microalgae, such as Calcium (Ca), Iron (Fe), Magnesium (Mg), Manganese (Mn), P, and Zn (Sónia and Bruno 2012). Thus, Pb interferes with several physiological and biochemical processes (Gomes, 2011).

2.5 Reactive Oxygen Species (ROS) and Oxidative Stress

A number of different ROS, including the superoxide anion (O_2), hydrogen peroxide (H_2O_2), singlet oxygen (O_2), and the hydroxyl radical (OH^-), occur transiently in aerobic organisms. These species are normal byproducts of oxidative metabolism and pose a constant threat to all aerobic organisms (fig.1.1).

Although some of them may function as important signaling molecules that alter gene expression and modulate the activity of specific defense proteins, all ROS can be extremely harmful to organisms at high concentrations. ROS can oxidize proteins, lipids, and nucleic acids, often leading to alterations in cell structure and mutagenesis (Halliwell and Gutteridge 1999).

Production of ROS constitutes a particularly severe threat to photosynthetic organisms, because a common biological source of O_2^- is the single-electron reduction of molecular oxygen by electron transport chains. Indeed, because of the intense electron flux in their microenvironment, which also contains elevated oxygen and high metal ion concentrations, the mitochondria and chloroplasts of photosynthetic organisms are cell compartments highly susceptible to oxidative

injury. Paradoxically, trace metals play key roles in photosynthetic electron transport in thylakoids of O_2-evolving organisms, participating in enzymes that remove ROS such as ascorbate peroxidase (APX) (Fe), Fe-superoxide dismutase (SOD) (cyanobacteria, higher plants, and most algae), and Cu-Zn-SOD (some algae and higher plants). In addition, they are part of

essential components to the photosystems (Fe) or mobile electron carriers such as the iron-containing cytochrome and the copper-containing plastocyanin (Raven *et al.,* 1999).

Many environmental factors can induce oxidative stress in the cell by generation of O_2^-, therefore, modulation of antioxidant levels constitutes an important adaptive response to withstanding adverse conditions. Indeed, maintenance of a high antioxidant capacity in cells has been linked to increased tolerance against different kinds of environmental stress (Pedrajas *et al.,* 1993; Dat *et al.,* 1998; Thomas *et al.,* 1999). The intoxication with pollutant metals induces oxidative stress because they are involved in several different types of ROS-generating mechanisms (Stohs and Bagchi 1995). For example, transition metals (such as Fe and Cu) participate in the well-known Haber-Weiss cycle, producing OH from O_2 (Winterbourn 1982). Metals without redox capacity (such as Cd, Pb and Hg) can enhance the pro-oxidant status by reducing the antioxidant glutathione (GSH) pool,

activating calcium- dependent systems and affecting iron-mediated processes. These heavy metals can also disrupt the photosynthetic electron chain, leading to O^2 and O^2 (1Dg) production (Asada and Takahashi 1987). Thus, algal tolerance to heavy metal pollution in the environment is likely to depend heavily on defense responses that prevent oxidative injury.

Organisms have developed a wide range of protective mechanisms that serve to remove ROS before they can damage sensitive parts of the cellular machinery. These can be conveniently divided into low molecular weight compounds such as GSH, phenolics, ascorbate, flavonoids, tocopherols, and carotenoids, as well as enzymatic catalysts of high molecular weight. Carotenoids are widely distributed naturally occurring pigments found in bacteria, yeast, algae, plants, animals, and humans (Britton *et al.*, 1995). With regard to the high molecular weight compounds, aerobic organisms express a battery of enzymes that contribute to the control of cellular ROS levels. Superioxide dismutase, Catalase, Glutathione peroxidase, Ascorbate perioxidase, Lipid peroxidase, Glutathione reductase, Thioredoxin, and Peroxiredoxin are considered the main natural antioxidant enzymes (Rice-Evans *et al.*, 1996; Asada, 1999). Peroxiredoxin catalyses the breakdown of alkyl hydroperoxides into water and their corresponding alcohols (Rouhier and Jacquot 2002). Catalase and Glutathione peroxidase catalyze the production ofH_2O fromthe degradation of $H_20_2$and ROOH, respectively, whereas Ascorbate perioxidase reduces H_2O_2 to H_2O using ascorbate as electron donor (Fridovich, 1997). Superioxide dismutase, which catalyzes the disproportionation of O_2 to O_2 and H_2O_2, has been called the cell's first line of defense against ROS (Hassan and Scandalios, 1990). This is because O_2^- is a precursor to several other highly reactive species, so that control over the steady state O_2^-concentration by SOD

constitutes an important protective mechanism (Fridovich, 1997). Three major SOD isoforms have been described in eukaryotic photosynthetic organisms (Asada, 1999): a CuZnSOD located in the thylakoid membranes and cytosol of higher plants, certain dinoflagellates, and charophycean green algae; a MnSOD isoform found within the mitochondria; and FeSOD isoform in the chloroplast stroma. Indeed, FeSOD is considered to be the major O_2^- scavenger in chloroplasts, whereas MnSOD is the most active scavenger in mitochondria (Fridovich, 1997). Interestingly, SOD is induced by its substrate (Colepicolo *et al.*, 1992; Allen and Tresini 2000), and thus activation of specific SOD isoforms can serve as an indicator of the cell compartment experiencing pollutant- induced O_2^-levels. Although SOD genes have been isolated from many different species, the FeSOD isoform has been reported from only a few. Recently, Okamoto *etal.*(2001) reported the isolation and molecular cloning of the FeSOD isoform from the marine dinoflagellate *Lingulodinum polyedrum*, and the changes in expression of this enzyme during algal growth have been described (Sigaud-Kutner *et al.*, 2002). Finally, as might be expected on the basis of the involvement of SOD in mitigating the effects of light, SOD activity increases after the exposure of *Gracilariopsis tenuifrons* to visible light (Rossa *et al.*, 2002).

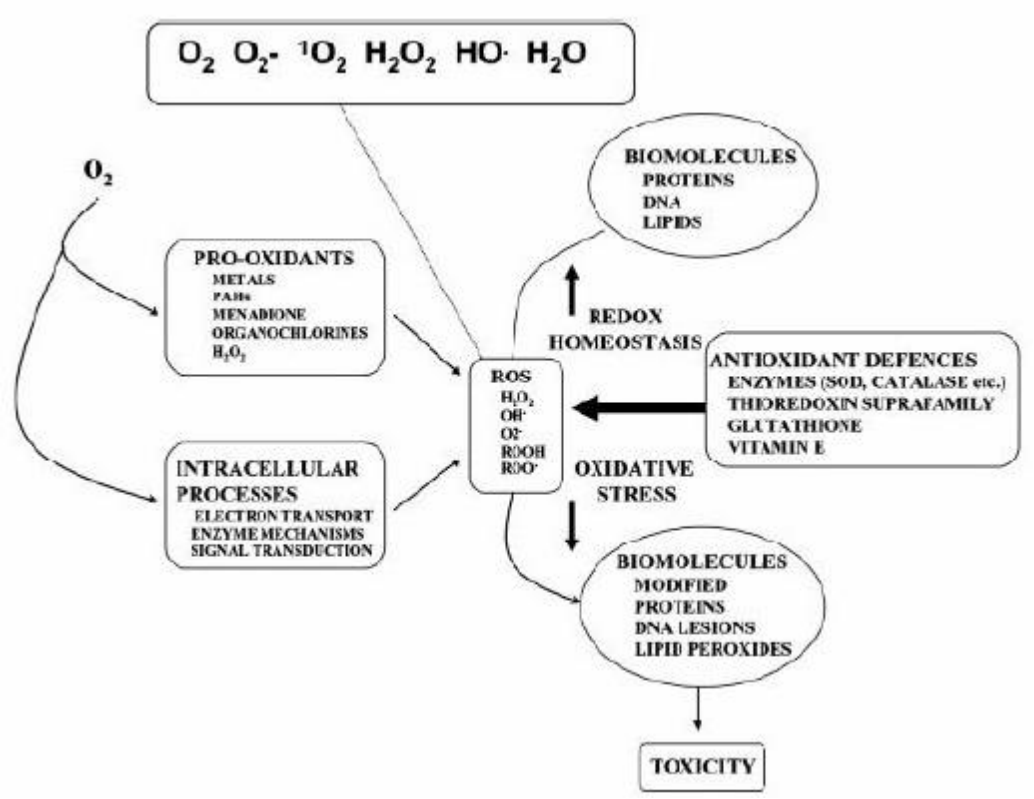


Figure 1.1: Oxidative stress is elicited by ROS derived by a univalent reduction of O_2. ROS can be induced by endogenous or external sources such as heavy metals, PAHs, polyaromatic hydrocarbons. (*Source:* Dowling and Sheehan, 2006).

2.6Algal Responses to Heavy Metals

Toxic metal ions are able to cross membranes, and several possible mechanisms have been suggested to account for their transport (Van Ho *et al.*, 2002; Zalups and Ahmad 2003). One type of mechanism is described as molecular mimicry, whereby metals either compete for binding to multivalent ion carriers (such as Ca channels) or, after binding to low molecular weight thiols (such as cysteine), enter the cell by active transport (e.g. using amino acid transporters). In another type of mechanism, metals bound to chelating proteins

(such as metallothioneins) may enter the cell by endocytosis. The heavy metals can cause membrane depolarization and acidification of the cytoplasm (Cumming and Gregory 1990; Cardozo *et al.,* 2002; Conner and Schimid 2003), and in fact, membrane injury is one important effect of metal ions that may lead to disruption of cellular homeostasis. Thus, cellular adaptations such as exudation of chelating compounds and active efflux of metal ions by primary ATPase pumps can provide some degree of metal tolerance (Cumming and Gregory 1990; Rosen 1996).

The relationship between ROS and heavy metal toxicity and the effects of heavy metal on ROS metabolism in algae are varied (Adonaylo and Oteiza 1999). In the marine dinoflagellate *L. polyedrum*, heavymetals cause increased oxidation of proteins and lipids; increased levels of SOD, APX, and carotene; and a decreased GSH content (Okamoto and Colepicolo 1998; Okamoto *et al.,* 2001a). Interestingly, pollutant treatment of this organism induces the MnSOD and FeSOD isoforms, whereas the activity of the cytosolic CuZnSOD isoform is not significantly altered. These cellular responses may involve transcriptional control, because exposure of *L. polyedrum* to toxic metals results in an increased level of the FeSOD mRNA (Okamoto *et al.,* 2001). In any event, the induction of both organelle isoforms suggests that both mitochondrial and chloroplast electron transport systems are affected by heavy metals. Increased SOD activity after treatment with Cd was noted both in the Prasinophycean *Tetraselmis gracilis* (Okamoto *et al.,* 1996) and in the diatom *Ditylumbrightwellii* (Rijstenbil *et al.,* 1994), but cellular responses can differ in other algae. In the green unicellular alga *Selenastrum capricornutum,* increased APX activity has been observed (Sauser *et al.,* 1997), whereas a decreased GSH redox ratio has been reported in the green macroalga*Enteromorpha prolifera* (Rijstenbil *et al.,*1998) and

freshwater macrophyte *Ceratophyllum demersum* (Devi and Prasad 1998). Nagalakshmi and Prasad (2001) observed increases in APX, SOD, and GPX activities in *Scenedesmus bijugatus* exposed to different copper concentrations (0–100 mM). Moreover, they also observed a progressive depletion of GSH content in the cells with increasing concentrations of Cu. The alteration of the equilibrium between synthesis and utilization of GSH was attributed to its antioxidant role or its use as precursor in the synthesis of phytochelatins (Nagalakshmi and Prasad 2001). Clearly, the general theme is an increase in antioxidant defense mechanisms, although the particular players involved may vary in different organisms. Regarding the effects of metals on macroalgae, In outdoor pond cultures of the red alga *Gracillaria tenuistipitata*, additions of Cu have been used to decrease epiphytism by the green macroalga *Enteromorpha intestinalis*, because *G. tenuistipitata* is more resistant to Cu than the green alga (Haglund *et al.*, 1996). In the red algae *Mastocarpus stellatus* and *Chondrus crispus*, a correlation was found between the generallevel of ROS metabolism, oxidative and general-stress tolerance (Collen and Davison 1999). Although toxic at high concentrations, the effects of Cu could be attenuated under chronic conditions because it is a micronutrient essential for the activity of several enzymes, including SOD.Copper plays a dual role in the metabolism of photosynthetic organism. It is both a micronutrient, for example, as an important part of oxidases (e.g. cytochrome oxidase and amino oxidases) and in electron transport chain components (e.g. plastocyanin), but it is also highly toxic (Fernandes and Henriques 1991). This duality extends to the generation of ROS as well. Not only is Cu an important part of the reactive oxygen scavenging system (e.g. CuZnSOD), but it is also able to cause oxidative stress through increased production of ROSvia its toxic effects on

photosynthesis. Cd has no known metabolic function in macroalgae and, in contrast to Cu, cannot contribute to OH^- formation in the Fenton reaction (Halliwell and Gutteridge 1999). However, Cd^{2+} has been shown to be a co-factor in a carbonic anhydrase of the diatom *Thalassiosira weissflogii* (Lane and Morel 2000), whereas in higher plants Cdcauses disturbances in growth, photosynthesis, ion- and water transport, and general decreases in enzyme activities due to reactions of Cd with thiol groups (Prasad 1995). Although probably not essential for growth of macroalgae, Cd is readily taken up. The uptake mechanism is not known but is partially light dependent (Hu *et al.*, 1996) and requires protein synthesis. When the levels of ROS formed exceed the ability of the antioxidant system to cope with them, damage to cellular compounds occurs. Thus, increased levels of oxidized proteins and lipids are indicative of a state of oxidative stress. Comparisons between the biological effects of Cu and Cd indicate that the former is proportionally more efficient in causing oxidative stress than the latter, whose effects probably lean more toward non oxidative stress invoking reactions. It has been suggested that Cu (as well as Pb and Zn) interacts directly with the thylakoid membranes in the chloroplast, whereas Cd and Nickel (Ni) interferes with other metabolic processes in plants (Szalontai *et al.*, 1999). Because Cu primarily triggers oxidative stress in the chloroplasts, an increase in chloroplastic APX and SOD levels is probably an efficient way to avoid the detrimental effects of this metal. An increase in the activity of these antioxidant enzymes reduces the concentrations of O_2 and H_2O_2 formed in the chloroplast, thereby reducing the risk of OH formation through cycling between Cu and Cu^{2+}. Indeed,increases in peroxidase activity are regarded as a reliable indicator of stress or potential phytotoxicity of heavy metals, the increases in peroxidase activity being a response to an increase in peroxides, disruption of

the plasma membrane by lipid peroxidation, and the ROS produced by heavy metal accumulation (Macfarlane and Burchett 2001). The induction of antioxidants in response to enhanced ROS production is generally proportional to the duration and severity of the stress applied to algal cultures (Okamoto *et al.*, 1996). For example, enhanced levels of cellular antioxidants could allow cells to acclimatize to increased steady-state concentrations of ROS during chronic stress. In contrast, the abrupt generation ofhigh levels of ROS over a short period of acute stress will usually exceed the total antioxidant capacity.

Acute stress, which lowers both the GSH pool and depletes NADPH levels, will thus provoke rapid oxidation of the ascorbate pool. A decrease in ascorbate availability will as a result limit not only APX but also all peroxidase activity. To investigate this further, the O_2 uptake and the GSH pool in metal-treated cells can be monitored. A useful estimate of oxidative stress is the content of GSH relative to its oxidized form GSSG (Sies 1999). Increased O_2 uptake is alsoan useful index of oxidative stress because it is often associated with formation of O_2 , H_2O_2, and peroxy radicals, during reduction of O_2 by components of electron-transport chains (Halliwell and Gutteridge 1999). Interestingly, some dinoflagellate algae have developed an important defense mechanism: encystment. This diverse group of unicellular organisms constitutes a major fraction of marine phytoplankton, and their contribution to the ocean's primary production can be considerable (Lignell *et al.,* 1993; Rao and Pan 1993). These organisms differ from other algae in many respects, such as photosynthetic capacity, growth rate (Tang 1996), cell division, and ultrastructural and biochemical properties of the nucleus (Taylor 1987; Rizzo 1991). For instance, nitrogen deficiency or low temperatures are able to trigger encystment

of the freshwater dinoflagellates *Peridiniumcinctum* and *Peridinium willey* (Chapman and Pfiester 1995). When exposed to pollutant metals (Hg, Cd, Pb, and Cu), the dinoflagellate *L. polyedrum* may exhibit cell death or cyst formation depending on the dose (Okamoto *et al.*, 1999, Okamoto and Colepicolo 2001).

In summary, the regulation and induction of antioxidants takes place as a response to differentkinds of pollutant stress. In many cases, a prompt induction of antioxidant enzymes is critical to control the steady-state levels of ROS and thus avoid the ensuing oxidative damage. This type of defense mechanism is especially important within sub-cellular sites highly prone to oxidative stress such as chloroplasts and mitochondria.

2.7Antioxidant Defense System

Microalgae and plants have different defense strategies to cope with the toxicity of heavy metals. The antioxidant defense system is essentially constituted by Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX), Glutathione (GSH), Ascorbate (vitamin C) among others. These species are distributed through the cell and are present in vacuoles and chloroplasts in higher amountsfollowing distribution for the main antioxidant components was suggested by Scandalios (Scandalios, 2005; Sónia and Bruno 2012): 73% in the vacuole (Ascorbate, Glutathione, and Peroxidase); 17% in chloroplasts (Carotenoids, α-tocopherol, Ascorbate, aAcorbate peroxidase, Glutathione, Glutathione reductase, Cu/Zn-SOD, Monodehydroascorbate Radical reductase, and Dehydroascorbate reductase); 5% in the cytosol (Ascorbate peroxidase, CuZn-SOD, Catalase, Peroxidase, Gutathione, Ascorbate, Glutathione reductase, and Monodehydroascorbate, Radical reductase); 4% in the apoplast (Peroxidase and Ascorbate); 1% in the mitochondria

(Catalase, Glutathione, Glutathione reductase, Mn-SOD, Monodehydroascorbate and Radical reductase) and peroxisomes (Catalase; Cu/Zn-SOD). Besides the antioxidative system, stress proteins (also called heat-shock proteins, HSPs) are also activated in microalgae species under adverse conditions (Wang and Gu, 2011), and the accumulation of some organic compounds in microalgae such as polyamines (diamine, putrescine, triamine spermidine, and tetramine spermin) and L-proline play significant roles in microalgae adaptation to a variety of environmental stresses (Tatar and Gevrek, 2008; Wei*et al.,* 2009).

2.7.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD), the cell's first line of defense against ROS, catalyzes the disproportionation of O_2 to O_2 and H_2O_2 (Ken et*al.,* 2005). Since O_2 is a precursor to several other highly reactive species, control of this free radical concentration by SOD constitutes an important protective mechanism (Fridovich, 1997). The activation of specific SOD isoforms (FeSOD in chloroplasts, MnSOD in mitochondria, and CuZnSOD in the cytosol) can serve as a biomarker for cells that are experiencing pollutant-induced increases in O_2 levels (Barros *et al.,* 2005; Murthy *et al.,* 2005). Although SOD genes have been isolated from many different species, the FeSOD isoform has been reported from only a few microalgae (Okamoto *et al.,* 2001b).

2.7.2 Catalase (CAT)

Catalase (CAT, EC 1.11.1.6) are tetrameric heme containing enzymes with the potential to directly dismutate H_2O_2 into H_2O and O_2 and are indispensable for ROS detoxification during stressed conditions.CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert 6 million molecules of H_2O_2 to H_2O and O_2 per minute.

Catalase is important in the removal of H_2O_2 generated in peroxisomes by oxidases involved in ß-oxidation of fatty acids, photorespiration and purine catabolism. Among the antioxidant mechanisms developed by cells to maintain redox homeostasis, the antioxidant enzyme catalase is one of the most efficient enzymes in degrading hydrogen peroxide (H_2O_2) in water and oxygen (Lesser, 2006) and has been extensively studied (Chelikani *et al*., 2004). Catala se enzymes are predominantly located in peroxisomes of the cells (Bonnineau *et al.*, 2010). Due to its relatively low affinity for H_2O_2, Catalase is mainly involved in the removal of excess H_2O_2 during important oxidative stress (Mittler, 2002). As a change in redox balance can be dramatic for the cell, changes in CAT level may reflect environmental disturbances. Catalase inhibition may suggest the presence of disturbances that reduce the cell's ability to cope with oxidative stress, while an increase in CAT activity may suggest an active response of cells to oxidative stress. Both CAT inhibition and activation provide useful information to detect environmental disturbances and estimate cell resistance to oxidative stress.

Catalase activity may be inhibited by different factors, such as a very high concentration of H_2O_2 (Lardinois *et al.,* 1996) or an excess of light. Moreover, environmental perturbations (osmotic stress, changes in temperature) can reduce the usually high turnover of CAT and, therefore, decrease its activity (Lesser, 2006). CAT activity, like other antioxidant enzyme activities, may complement the information obtained by classical endpoints (growth, mortality) and be successfully used in toxicity assessment in algae (Geoffroy *et al*., 2004; Qian *et. al.,* 2008, 2009;Contreras *et al.,* 2009; Liu and Xiong 2009). For example, Dewez *et al.,* (2005) pointed out that CAT was a more sensitive biomarker of fludioxionil toxicity than photosynthetic parameters in *Scenedesmus obliquus* (Dewez *et al.,* 2005). In addition,

Geoffroy *et al.,* (2004) highlighted the higher sensitivity of CAT than other antioxidant enzymes (Ascorbate peroxidase, Glutathione reductase) in *Scenedesmus obliquus* exposed to the herbicide flumioxazin.

2.7.3 Peroxidase

Peroxidase (EC 1.11.1.11), like catalase, catalyze the reduction of H_2O_2 to H_2O, but they require a source of electrons that subsequently becomes oxidized (Micheal, 1996).

2.7.4 Glutathione S-transferases (GST)

Glutathione-S-transferases (GSTs) E.C.2.5.1.18is a family of intracellular enzymes with the main function in detoxification processes by catalyzing the conjugation of tripeptide glutathione (GSH) with some endogenous toxic metabolites and many environmental contaminants (Egaas *et al.,* 1995). The plant glutathione transferases also known as glutathione S-transferases are a large and diverse group of enzymes which catalyse the conjugation of electrophilic xenobiotic substrates with the tripeptide glutathione (GSH; g-gluecysegly).

Noctor *et al.,* (1998) reported that GSTs have the potential to remove cytotoix or genotoxic compounds, which can react or damage the DNA, RNA and proteins. In fact, GSTs can reduce peroxides with the help of GSH and produce scavengers of cytotoxic and genotoxic compounds.

2.8Effect of Nutrients on Microalgal Biochemical Composition

Under nutrient limitation microalgae exhibit variations in biochemical composition, that depends on the type of limiting nutrient and degree of limitation (Kilham*et al.,* 1997). Inhibition of this enzyme reflects the natural deletorious effect of toxicants on microalgae

and represents a real ecological interest considering that algae are involved in the primary step of the food chain. High concentrations of nutrients, such as phosphates and nitrates, have been reported by several authors to reduce the toxicity of heavy metals (Haglund *et al*., 1996; Florence, 2007).

Phosphorus (P) is an essential nutrient for algal nucleic acid and ATP production among other essential molecules, and it is often the growth limiting nutrient for phytoplankton in aquatic systems (Spijkerman, 2008; Spijkerman and Wacker, 2011). However, nitrogen deficiency also affects other aspects of cell physiology, including a decrease in chlorophyll and proteins. This affects the cells ability to photosynthesize and results in decreased growth rates.

2.9 Effect of Nutrient to Protein

Proteins are the primary effectors molecules that can be affected by environmental, physiological or pathological conditions. Protein hydrolysis results in peptides being released that may possess beneficial biological activity in the organism. These so-called 'bioactive peptides' include particularly those with antioxidant activity, able to inhibit the oxidation of biomolecules (Medina *et al.*, 2010).

CHAPTER THREE

3.0MATERIALS AND METHODS

3.1 Unialgal Culture

All experiments were conducted with axenic cultures of the green alga *Scenedesmus quadricauda*. The microlga was isolated from an unprotected freshwater pond that receives surface runoff from different sources during rainfalls (Chia *et al.*, 2011; Chia and Musa 2014; Chia *et al.*, 2014).

Stock cultures was maintained in Organisation for Economic Co-operation Development (OECD) medium without EDTA as shown in table 3.1 (OECD, 1984) under batch conditions.The growth medium pH was adjusted to 7±0.2 before autoclaving and maintained throughout the experimental period due to the sem-icontinuous culture system, which consisted of 4 days of culture medium replacement in the experiment. Prior to inoculation, the culture was sterilized through autoclaving at 121^{o}C for 30 min and inoculated after 24 h. Cultures were kept under controlled conditions of light intensity (150 µmol m^{-2} s^{-1}), and temperature (22±1 °C). The reagents used for culture media preparations were of analytical grade and were obtained from Signma Aldrich (USA)..

3.1.1 Lead, Phosphates and Nitrates experiments

These experiments were performed using the selected culture medium, Organisation for Economic Co-operation Development (OECD). Phosphorus (P) was provided as KH_2PO_4 at 1.2×10^{-7},$1.2\times10^{-6}$and 1.2×10^{-5}(control) (Table 3.2), and Nitrogen (N) was provided as $NaNO_3$ at 1.8×10^{-6},1.8×10^{-5}and1.8×10^{-4}(control) (Table 3.3) concentrations. N and P were altered one at a time, not simultaneously, and when necessary, Pb was added as $PbCl_2$ at

Table 3.1: Organisation for Economic Co-operation Development (OECD) medium composition

	Stock solution	Concentration (mgL^{-1})
1.	$NaNO_3$	15
2.	$MgCl_2.6H_2O$	12
3.	$CaCl_2.7H_2O$	18
4.	MgSO.7H2O	15
5.	KH_2PO_4	1.6
6.	$NaHCO_3$	50
7.	Micronutrients	
	$ZnCl_2$	3×10^{-3}
	H_3BO_3	0.185
	$MnCl_2.4H_2O$	0.145
	$NaMoO_3.2H_2O$	7×10^{-3}
	$CuCl_2.2H_2O$	10^{-5}
	$CoCl_2.6H_2O$	1.5×10^{-5}

Table 3.2: Experimental combinations of Pb and P to which *Scenedesmus quadricaud*was exposed

P($MolL^{-1}$)	No Pb	$3.6\times10^{-8}MolL^{-1}$(Pb1)	$3.6\times10^{-7}MolL^{-1}$(Pb2)
$1.2x10^{-5}$(P1)	P1	Pb1P1	Pb2P1
$1.2x10^{-6}$ (P2)	P2	Pb1P2	Pb2P2
$1.2x10^{-7}$(P3)	P3	Pb1P3	Pb2P3

Each experimental condition was tested in triplicate.

Table 3.3: Experimental combinations of Pb and N to which *Scenedesmus quadricauda* was exposed.

$N(MolL^{-1})$	No Pb	$3.6\times10^{-8}MolL^{-1}$(Pb1)	$3.6\times10^{-7}MolL^{-1}$(Pb2)
1.8×10^{-4}	N1	Pb1N1	Pb2N1
1.8×10^{-5}	N2	Pb1N2	Pb2N2
1.8×10^{-6}	N3	Pb1N3	Pb2N3

Each experimental condition was tested in triplicate.

3.6 $\times 10^{-8}$mol/L and 3.6×10^{-7}mol/L. The chosen N and P concentrations represent replete and limited concentrations in aquatic ecosystems that are environmentally relevant (Reynolds, 2006) . The Pb concentrations used in this study represent concentrations that are usually found in the aquatic environment and the maximum permissible levels by USEPA (2014) of 0.015mg L^{-1} approximately (3.6×10^{-8}mol/L) *i.e* the control.

The N, P and Pb experiments were done using semi-continuous system in 500mL polycarbonates (Nalgene, USA) containing 200mL of sterile culture medium. Sterile techniques were used throughout to minimize contamination. Batch cultures were acclimated to the specific nutrient condition to be tested. This was performed through culture transfers at exponential growth phase, which ensured that algal metabolism reflected the culture media conditions. Culture transfers continued until steady growth rate was observed. At this stage, growth rates did not significantly differ between culture transfers ($P>0.05$) and *S. quadricauda* was considered to be acclimated to the N and P concentrations. The total experimental period for the semi-continuous cultures was 16 days , when sampling for growth , chlorophyll *a*, phenotypic plasticity, protein and antioxidant enzyme determination was done.

Free Pb ions concentrations were obtained through calculations using the chemical equilibrium software MINEQL+ 4.62.3 for nitrate and phosphate experiments (Table 3.4 and 3.5) (Environmental Research Software, Hallowell, ME, USA).

Table 3.4: Pb speciation in the growth media as calculated using MINEQL^{+} software. Where Pb1 = 3.6x10^{-8} mol L^{-1} Pb and Pb2 =3.6x 10^{-7} mol L^{-1} Pb, N = nitrogen, pPb and pLabile are negative natural log values for free Pb ion and labile Pb concentrations.

Nominal NO_3^{2-}(mol L^{-1})	Measured NO_3^{2-} (mol L^{-1})	Pb speciation			
		Nominal	Measured	pPb2	pLabilePb
1.8x10^{-4}	1.2×10^{-4}	No Pb		-	-
1.8x10^{-4}	1.2×10^{-4}	3.6x10^{-8} M Pb	9.9×10^{-7}	2.05x10^{-10}	2.67x10^{-8}
1.8x10^{-4}	1.2×10^{-4}	3.6x10^{-7} M Pb	1.4×10^{-5}	8.73x10^{-10}	1.94x10^{-7}
1.8x10^{-5}	8.8×10^{-6}	No Pb		-	-
1.8x10^{-5}	8.8×10^{-6}	3.6x10^{-8} M Pb	9.9×10^{-7}	2.05x10^{-10}	2.67x10^{-8}
1.8x10^{-5}	8.8×10^{-6}	3.6x10^{-7} M Pb	1.4×10^{-5}	8.73x10^{-10}	1.94x10^{-7}
1.8x10^{-6}	4.7×10^{-7}	No Pb		-	-
1.8x10^{-6}	4.7×10^{-7}	3.6x10^{-8} M Pb	9.9×10^{-7}	2.05x10^{-10}	2.67x10^{-8}
1.8x10^{-6}	4.7×10^{-7}	3.6x10^{-7} M P	1.4×10^{-5}	8.73x10^{-10}	1.94x10^{-7}

Table 3.5: Pb speciation in the growth media as calculated using MINEQL^{+} software. Where Pb1 = 3.6x10^{-8} mol L^{-1} Pb and Pb2 = 3.6x 10^{-7} mol L^{-1} Pb, P = phosphorus, pPb and pLabile are negative natural log values for free Pb ion and labile Pb concentrations.

Nominal PO_4^{3-} (molL^{1})	Measured PO_4^{3-} (mol L^{-1})	Pb speciation			
		Nominal	Measured	pPb2	pLabilePb
1.2x10^{-5}	4.8×10^{-6}	No Pb		-	-
1.2x10^{-5}	4.8×10^{-6}	3.6x10^{-8} M Pb	9.9×10^{-7}	2.05x10^{-10}	2.67x10^{-8}
1.2x10^{-5}	4.8×10^{-6}	3.6x10^{-7} M Pb	1.4×10^{-5}	8.73x10^{-10}	1.94x10^{-7}
1.2x10^{-6}	3.6×10^{-6}	No Pb		-	-
1.2x10^{-6}	3.6×10^{-6}	3.6x10^{-8} M Pb	9.9×10^{-7}	2.05x10^{-10}	2.67x10^{-8}
1.2x10^{-6}	3.6×10^{-6}	3.6x10^{-7} M Pb	1.4×10^{-5}	8.73x10^{-10}	1.94x10^{-7}
1.2x10^{-7}	2.6×10^{-6}	No Pb		-	-
1.2x10^{-7}	2.6×10^{-6}	3.6x10^{-8} M Pb	9.9×10^{-7}	2.05x10^{-10}	2.67x10^{-8}

3.1.2 Growth and Biomass Determinations

3.1.2.1 Absorbance

For biomass and growth kinetics determinations, on each sampling day, aliquots were taken. Monitoring of growth was done according to Liu *et al*., (2008) using optical density at 500 nm with a Sherwood colorimeter 257 (Sherwood scientific Ltd. Cambridge, England). Growth rates were determined using natural logarithmic transformed regression growth curves.

$$\mu = \frac{\log (ABS(t2)\ (t2-t1)^{-1}}{a \times ABS(t1)} \quad (1)$$

where

$$a = \frac{Tvol - Rvol}{Tvol} \quad (2)$$

Note:ABS = absorbance, t = time, Tvol = total volume and Rvol = removed/replaced volume

3.1.2.2Dry weight

Dry weight quantification was done using Whatman GF/C filters (5 cm diameter) that was dried for 2 h at 60°C, cooled in desiccators and weighed. This filter was used for filtering 20 mL of the samples. The filters was dried again for 2 h at 60°C, cooled in desiccators for at least 15 min and weighed to the nearest mg.

Dry weight was calculated in mg L^{-1} DW as the difference between the initial dry weight of the filter without microalgal biomass and the final weight of the filter with the retained microalgal biomass.

3.1.2.3 Chlorophyll a determination

Chlorophyll extraction was done using acetone according to Shoaf and Lium, 1973 method. 5mL of the algal sample was filtered through a cellulose acetate filter paper, which was placed in a 5mL plastic centrifuge tube containing acetone (3 mL). The container wrapped with foil paper and the extract was then refrigerated at -20^{o}Cfor 3h. After refrigerating, the supernatant was removed. Chl*a*quantification was done using absorbance measured at 653 and 666 nm with a UV-VIS Spectrophotometer (B.bran scientific and instrument company, England). Pigment concentrations were determined using the following equations provided by Németh (1998) and Wellburn (1994):

Chl *a*(mg L^{-1}) = 17.12 . A_{666} -8.86. A_{653}

3.1.2.4 Density (cells mL^{-1})

Direct microscopic counts using a Zeis microscope was used to determine cell density with the aid of an improved Neubauer haemocytometer.

3.1.3Phenotypic plasticity

The morphological effects of Pb, N and P on the phenotypic plasticity of *Scenesdesmus quadricauda* was evaluated by quantifying the number of cells per coenobium on each experimental treatment and controls after 96h exposure (Lombardi *et al*., 2007). Coenobium was classified into four categories: four-celled coenobium, three-celled coenobium, two-celled coenobium and free cells (absence of coenobium) under each treatment condition.

3.1.4Proteinextraction

For the analysis of protein and all the antioxidant enzymes, the following extraction procedure was adopted.

The centrifugation of 5ml of the cultures at 10000 rpm for 15 minutes, the supernatant discarded. 1.5ml of 0.05M of phosphate buffer (pH 7.8) was added, squashed on ice using glass rods and centrifuged at 10000 rpm for 15 minutes. The supernatant obtained was used for totalprotein determination and antioxidant enzymes assay.

3.1.4.1 Total protein determination

To determine the total intracellular protein content of the microagal cells, the procedure of BRADFORD (1976) with bovine serum albumin (BSA) as protein standard was used. To every 500µl of the supernatant, 2.5mL of Bradfordreagent (0.01% Coomassie blue, 47% ethanol, and 85% phosphoric acid) prepared just prior to the assay, was added and allowed to stand for 5 min at room temperature. The absorbance of the solution was then read at 595 nm. The BSA used as standard was prepared in phosphate buffer (pH 7.2) solution with concentrations ranging from 0 to 400 µg mL^{-1}. These value was used to plot the total protein standard calibration curve (fig.3.1).

3.1.5 Antioxidants enzyme determination

The enzymic antioxidants that were analysed are superoxide dismutase, catalase, peroxidase and glutathione S-transferase.

3.1.5.1 Assay of Superoxide Dismutase (SOD)

The activity of superoxide dismutase was assayed spectrophotometrically by the method of Misra and Fridovich (1972) with some modifications.

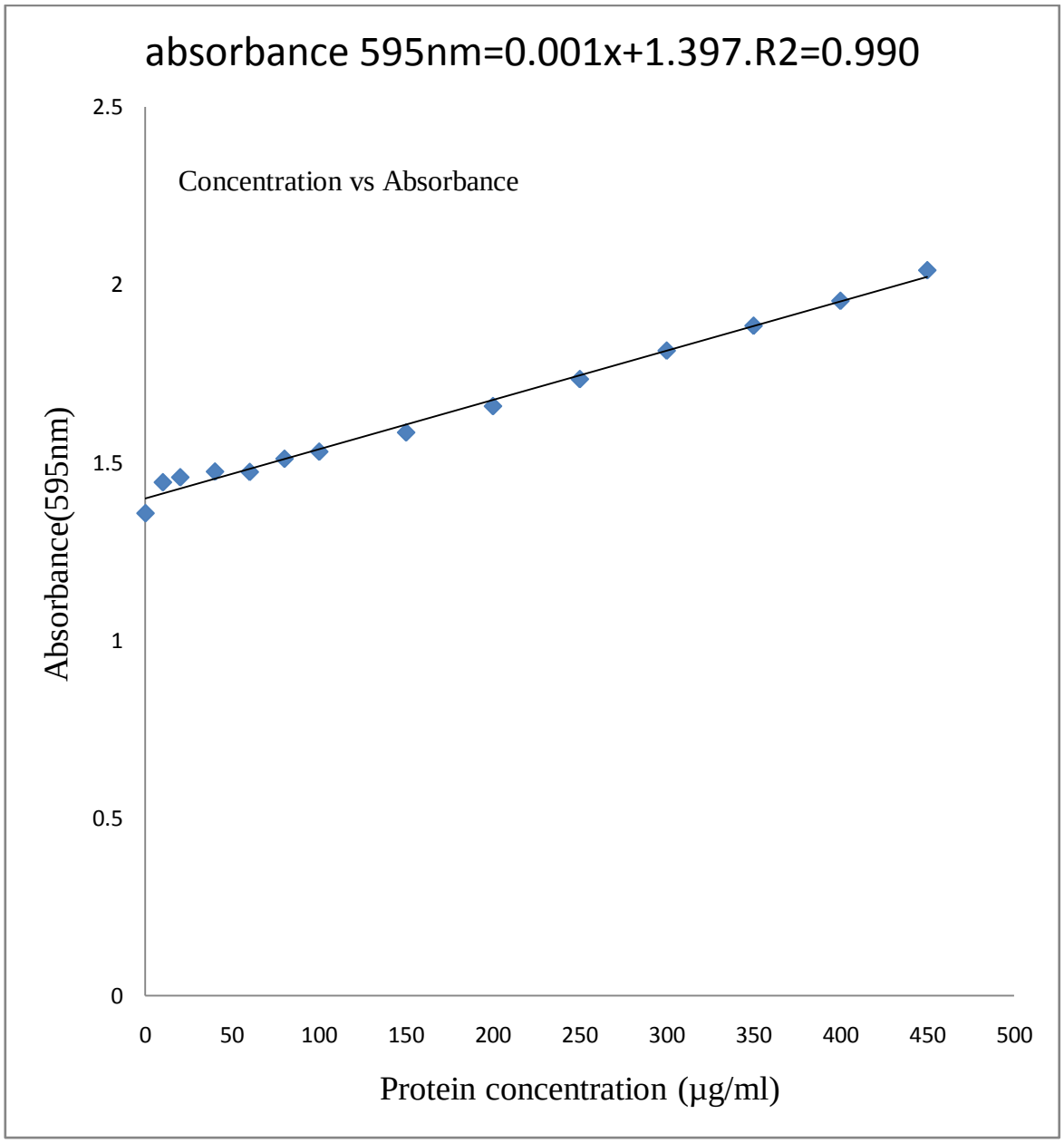


Figure 3.1: Protein calibration curve used in this study

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of Nitroblue tetrazolium (NBT) reduction, the extent of which was assayed spectrophotometrically at 600nm.

Reagents

1. Potassium phosphate buffer (67 mM, pH 7.8)

2. Methionine (450 μM)

3. Riboflavin (53 mM)

4. Nitro Blue Tetrazolium (NBT) (840 μM)

Procedure

The incubation medium contained, in a final volume of 3.0 ml, 6.7 mM potassium phosphate buffer (pH 7.8), 45 μM methionine, 5.3 mM riboflavin, 84 μM NBT. The amount of homogenate added to this medium was kept below one unit of enzyme to ensure sufficient accuracy.

The tubes wereplaced in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. After exposure to light for 10 minutes, the reduced NBT was measured spectrophotometrically at 600nm. Superoxide dismutase activity was determined by calculating the percentage inhibition of NBT as follows: The maximum reduction was observed in the absence of the enzyme.

$$\% \text{ inhibition} = \frac{\text{Absorbance of experimental sample - Absorbance of control sample}}{\text{Absorbance of control sample}} \times 100$$

One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT.

3.1.5.2 Assay of catalase

Catalase activity in the algal samples was determined by adopting the method of Luck, (1974).

Principle

The Ultra-violet light absorption of hydrogen peroxide can be easily measured between 230– 250 nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity can be estimated by this decrease in absorption.

Reagents

1. Phosphate buffer: 0.05M (pH 7.2)

2. Hydrogen peroxide in phosphate buffer (0.036%w/w)

Procedure

The methodology is described as follows:

Pipetted (in milliliters) the following reagents into suitable cuvettes**.**

Test Blank

Reagent A (50 mM Phosphate Buffer) -------- 3.0 ml

Reagent B (0.036% H_2O_2 solution 2.9 ml -------

Prepared in reagent A)

Monitored the absorbance until constant and recorded the absorbance at 240 nm on spectrophotometer.

Then add,

Reagent C (Catalase extract) 0.1 ml ---------

The reagents were immediately mixed by inversion and recorded the absorbance at 240 nm.

The catalase activity was calculated as follows:

$$\text{Catalase activity(units)} = \frac{3.45}{\text{ml enzyme} \quad (\text{Min})(0.1)}$$

Where,

3.45 correspond to the decomposition of 3.45 micromoles of hydrogen peroxide in a 3.0 ml of reaction mixture.

0.1= Volume of enzyme used (in milliliters)

One enzyme unit was calculated as the amount of enzyme required to change the absorbance at 240 nm by 0.05 units.

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme.}}$$

3.1.5.3 Assay of Peroxidase

The activity of peroxidase in the algal samples was assessed by themethod of Reddy *et al*., (1995) with some modifications.

Principle

Peroxidase catalyses the conversion of H_2O_2 to H_2O and O_2, in the presence of the hydrogen donor pyrogallol. The oxidation of pyrogallol to a coloured product called purpurogalli can be measured spectrophotometrically at 430 nm with the specified time interval. The intensity of the product is proportional to the activity of the enzyme.

Reagents

1. Pyrogallol (0.05 M in 0.1 M phosphate buffer, pH 6.5)

2. H_2O_2 (1% in 0.1M phosphate buffer, pH 6.5)

Procedure

Pyrogallol solution (2.7 mL) and enzyme extract (0.1 mL) was pipetted out into a cuvette. The spectrophotometer will be adjusted to read zero at 430 nm follow by the addition of 0.2mL of 1% H_2O_2 and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes.

The activity of enzyme was then calculated as follows:

$$\text{Peroxidase activity (units/mg)} = \frac{\Delta A_{510}/\text{mins}}{6.58 \times \text{ml enzyme/ml rxn mixture}}$$

Where,

A = Absorbance

3.1.5.4 Assay of Glutathione S-Transferase

The assay of glutathione S-transferase activity was performed by themethod of Habig *et al*., (1974) with some modification.

Principle

Glutathione S-transferase conjugates GSH with CDNB and the extent of conjugation is used as a measure of enzyme activity from the proportionate change in the absorption at 340 nm.

Reagents

1. Chloro-2, 4-dinitrobenzene (CDNB) (1mM in ethanol)

2. Reduced glutathione (1mM in distilled water)

3. Phosphate buffer (0.1M, pH 6.5)

Procedure

The enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer. The assay mixture contains 10µl of GSH, 10µl of CDNB and phosphate buffer in a total volume of 990µl.The reaction was started by the addition of 10µl of enzyme extract to this mixture and the readings was recorded against distilled water blank after every 1minute for minimum of 5minutes. The complete assay mixture without the enzyme served as the control to monitor non-specific binding of the substrates. One unit of GST activity is defined as the nmoles of CDNB conjugated per minute.

The change in absorbance is directly proportional to the GST activity.

$$\Delta A_{340} = \frac{A_{340}\text{(final reading)} - A_{340}\text{(final reading)}}{\text{Reaction time(mins)}}$$

ΔA_{340} /minsample-ΔA_{340}/minblank

Use the above equation for the GST specific activity.

GST specific activity =ΔA_{340} /min×V/€mM×Venzyme (ml)=µmol/ml/min

Where,

€mM(mM/cm)=extinction coefficient for CDNB conjugate at 340nm.

=0.0096.

V= reaction volume.

3.1.6Chemical analyses

3.1.6.1 Lead analysis

Lead (Pb) concentrations in culture media were analyzed using AA240FS (Varian) atomic absorption spectrophotometer (AAS). Sample digestion and analyses were carried out using the procedures described in APHA (1998). The speciation of Pb under the different experimental conditions was determined using the chemical equilibrium software MINEQL^{+} 4.62.3(Environmental Research software, USA). This showed that over 98% of the metal remained as free and labile Pb in solution.

3.1.6.2*Nitrate analysis*

Nitrates concentrations in culture media were determined according to the American Public Health Association (APHA 1998) using the Phenol-disulphonic acid method. In this method, 100ml of water sample was evaporated to dryness and 2ml of phenol-disulphonic acid was added to the residue and left for 10minutes, then 15ml of distilled water was added, followed (fig.3.2).

3.1.6.3*Phosphates analysis*

Phosphates concentrations in culture media were determined according to the American Public Health Association (APHA 1998) using the stannous chloride method. The phosphate content was determined by adding 1ml of ammonium molybdate (deniges reagent) and one drop of stannous chloride solution to 100ml of the culture medium and allowed to stand for 10minutes. Absorbance was taken at 600nm using a spectrophotometer (fig.3.3).

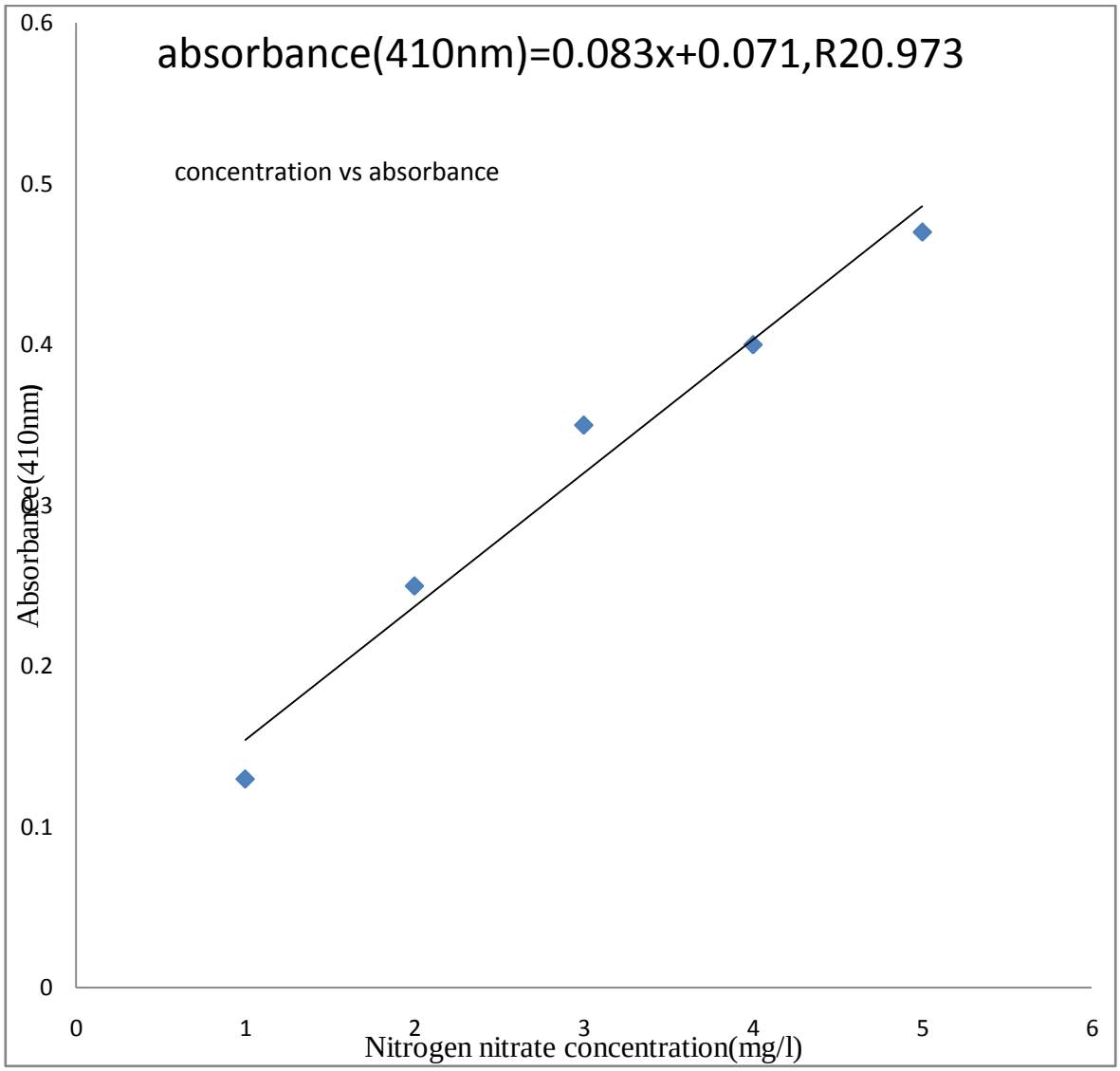


Figure 3.2: Nitrate calibration curve for nitrate determination.

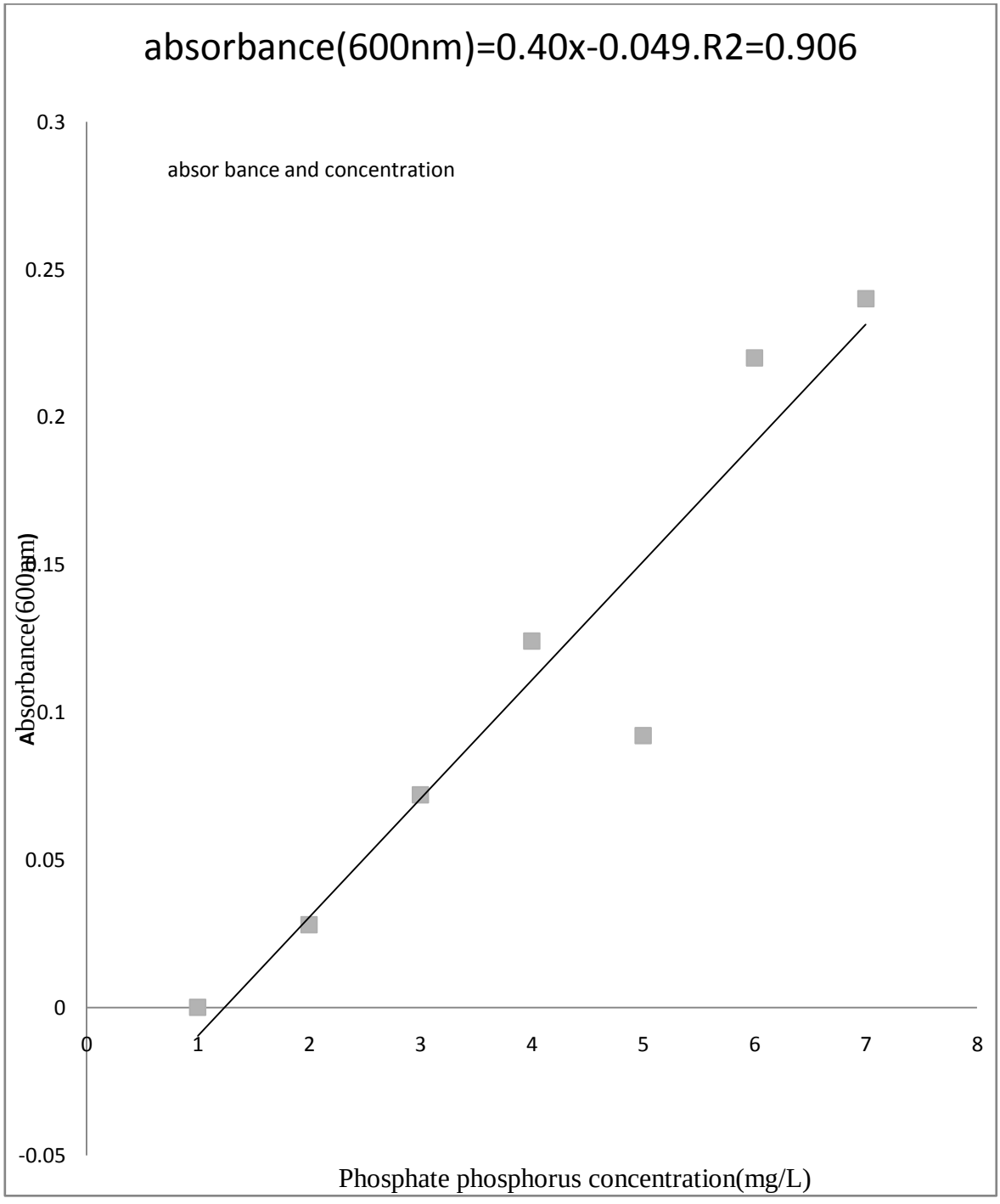


Figure 3.3: Phosphate calibration curve for phosphate determination.

CHAPTER FOUR

4.0 RESULTS

4.1 The effect of Pb and NO_3 on Growth and Biomass Determination of *S. quadricauda.*

S. quadricauda growth rates (Table4.1) decreased under N limitation, although thedifference between the N limitation treatments without Pb was not significant. The addition of Pb at the highest concentration (10^{-7}M) further inhibited the growth rate. Although, the additionof Pb to N replete cultures resulted in lower growth rate, there were no significant (p >0.05)differences in the growth rates under the two Pb exposure concentrations. The changes in growth of the microalga can also be seen in the growth absorbance graph (Fig.4.1).Biomass production results showed that the dry weight, cell density (Fig. 4.1.4) and chlorophyll *a* concentrations generallydecreased with decreasing N in the cultures and also decreased with Pb increase under Nlimitation conditions. The effect of N and Pb on biomass production was significant(p<0.05), while there was no significant interaction between N/Pb on biomass production (Table3). However, at the highest Pb and lowest N treatment, after the second day, chlorophyll *a*production began to gradually decrease with the lowest value obtained on the fourth day (Fig. 4.2).Animportant observation can be seen in the dry weight production results where most of the N/Pbstress conditions showed that the most severe effect of the various treatments was observedwithin the first 48 hours (Fig. 4.3).The number of cells increased throughout the experimental period. The effect of N limitation and Pb stress was significant in cell density,the Pb stress was significant in experimental cultures with high Pb concentrationsand low nutrients concentrations throughout the experimental .(Fig.4.4).

Table 4.1: Specific growth rates of *Scenedesmus quadricauda* as a function of different Lead (Pb) and Nitrate (NO_3) concentrations determined after 96 h incubation.

Nitrogen(M)	NoPb Growth rate	3.6×10^{-8}(Pb1) Growth rate	3.6×10^{-7}(Pb2) Growth rate
1.8×10^{-4}	0.609±0.012	0.380±0.029	0.349±0.004
1.8×10^{-5}	0.567±0.033	0.359±0.024	0.287±0.006
1.8×10^{-6}	0.538±0.019	0.263±0.030	0.153±0.029

Values represent mean and standard deviation for n=3.

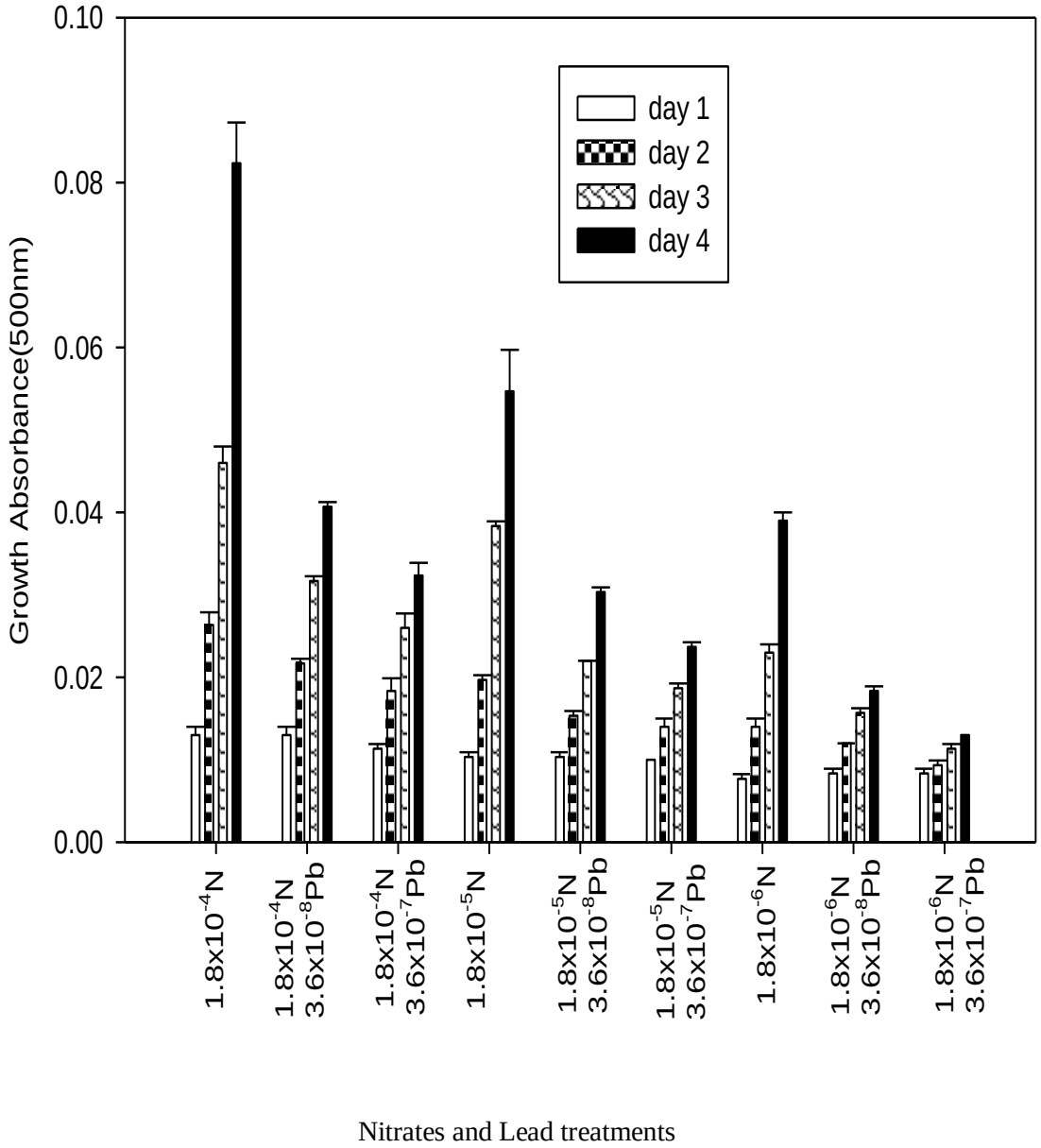


Figure. 4.1: Growth absorbance at 550nm by *Scenedesmus quadricauda* at different Pb and N concentrations.

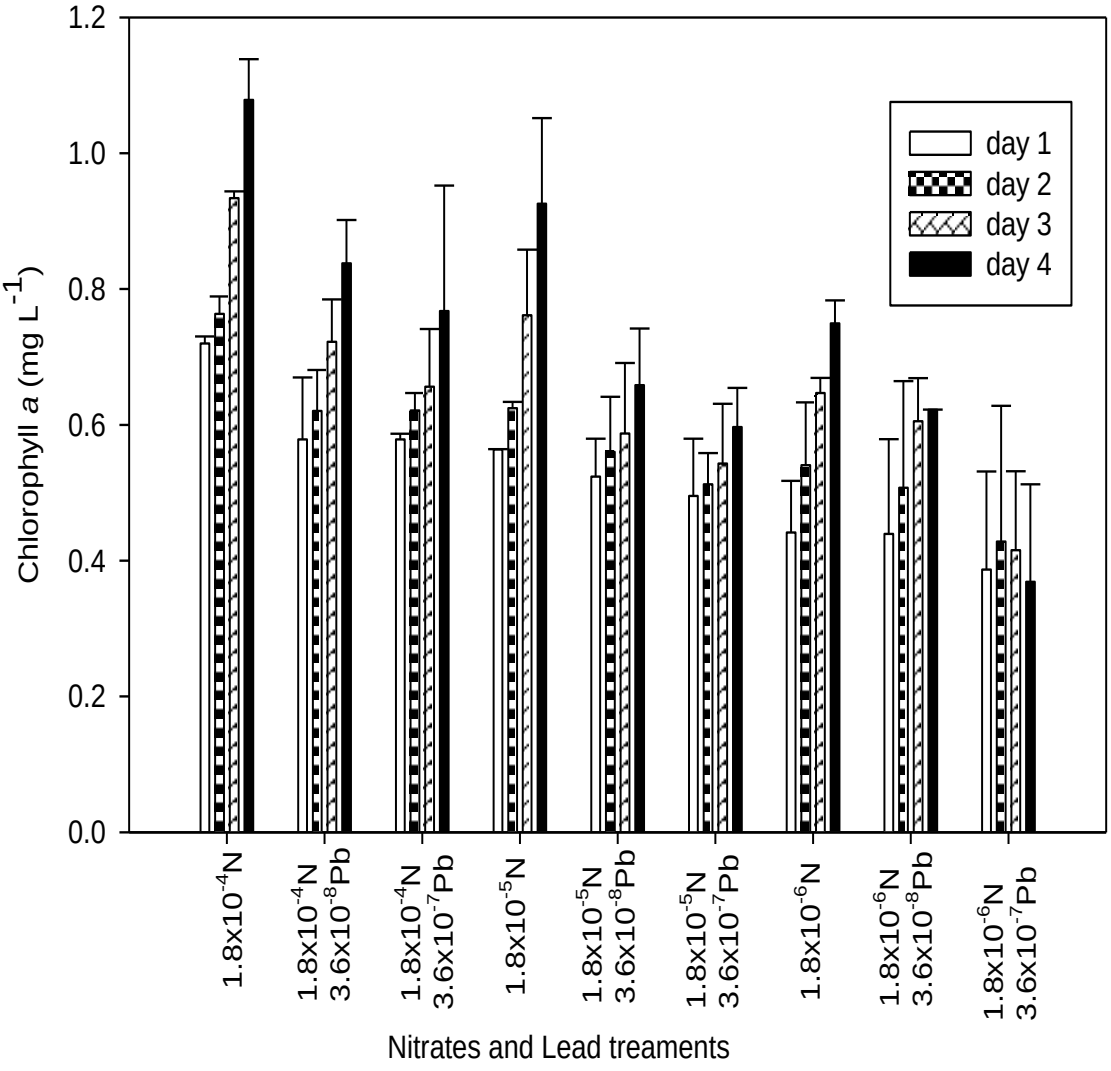


Figure 4.2: Chlorophyll *a* production by *Scenedesmus quadricauda* at varying Pb and N concentrations.

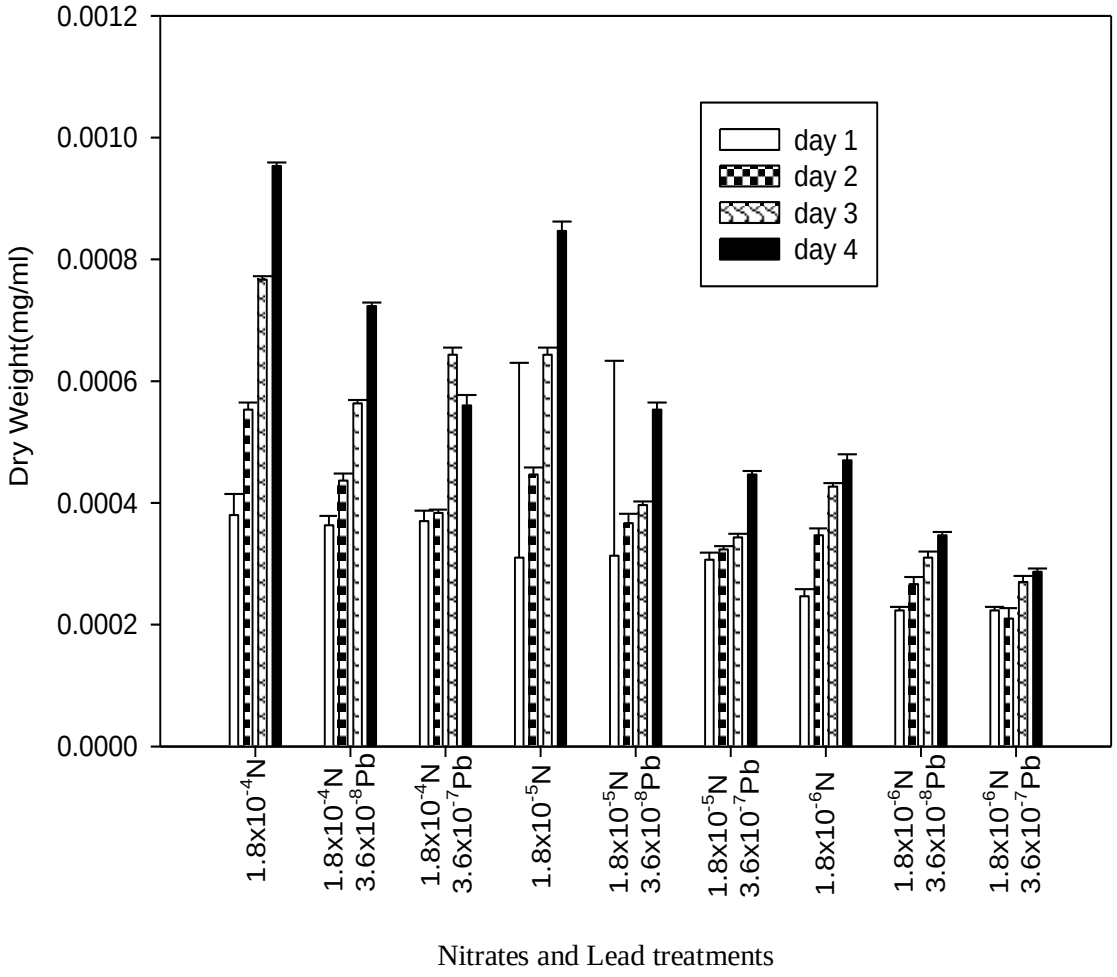


Figure 4.3: Dry weight of *Scenedesmus quadricauda* at varying Pb and N concentrations.

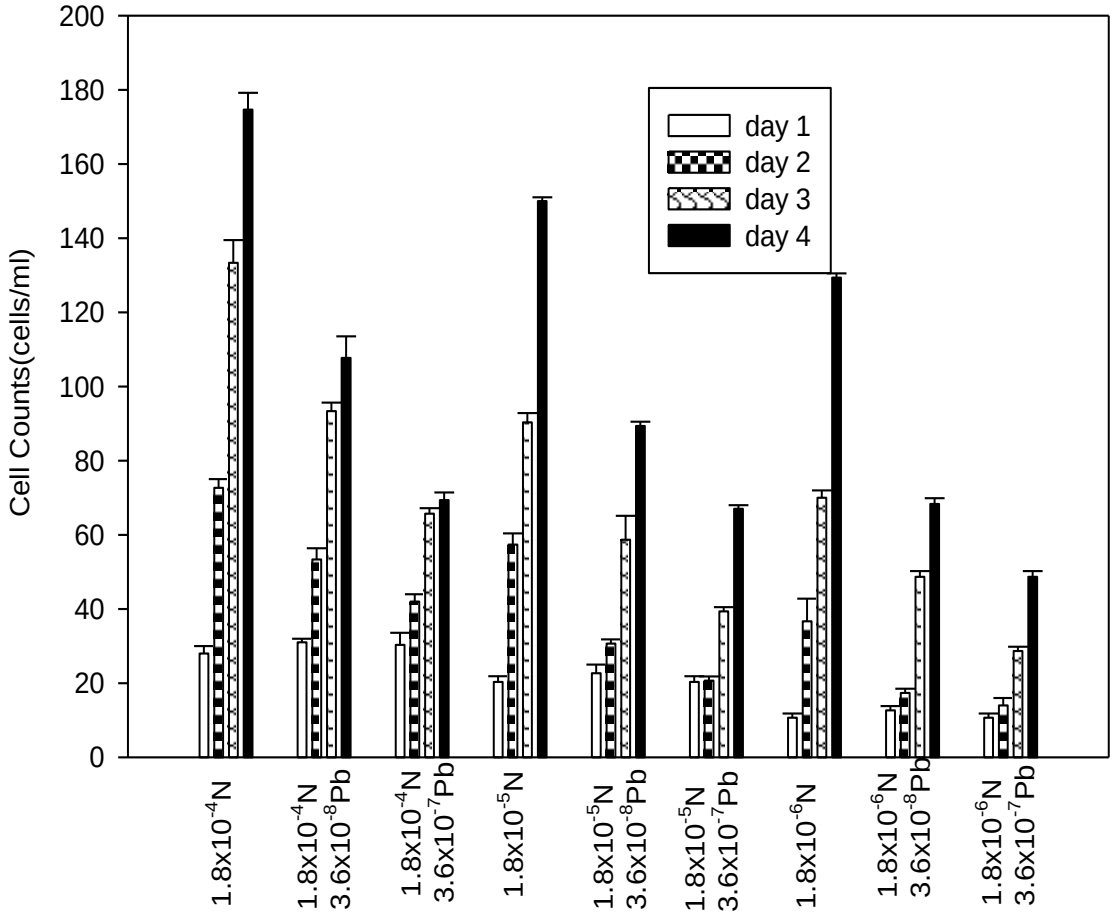
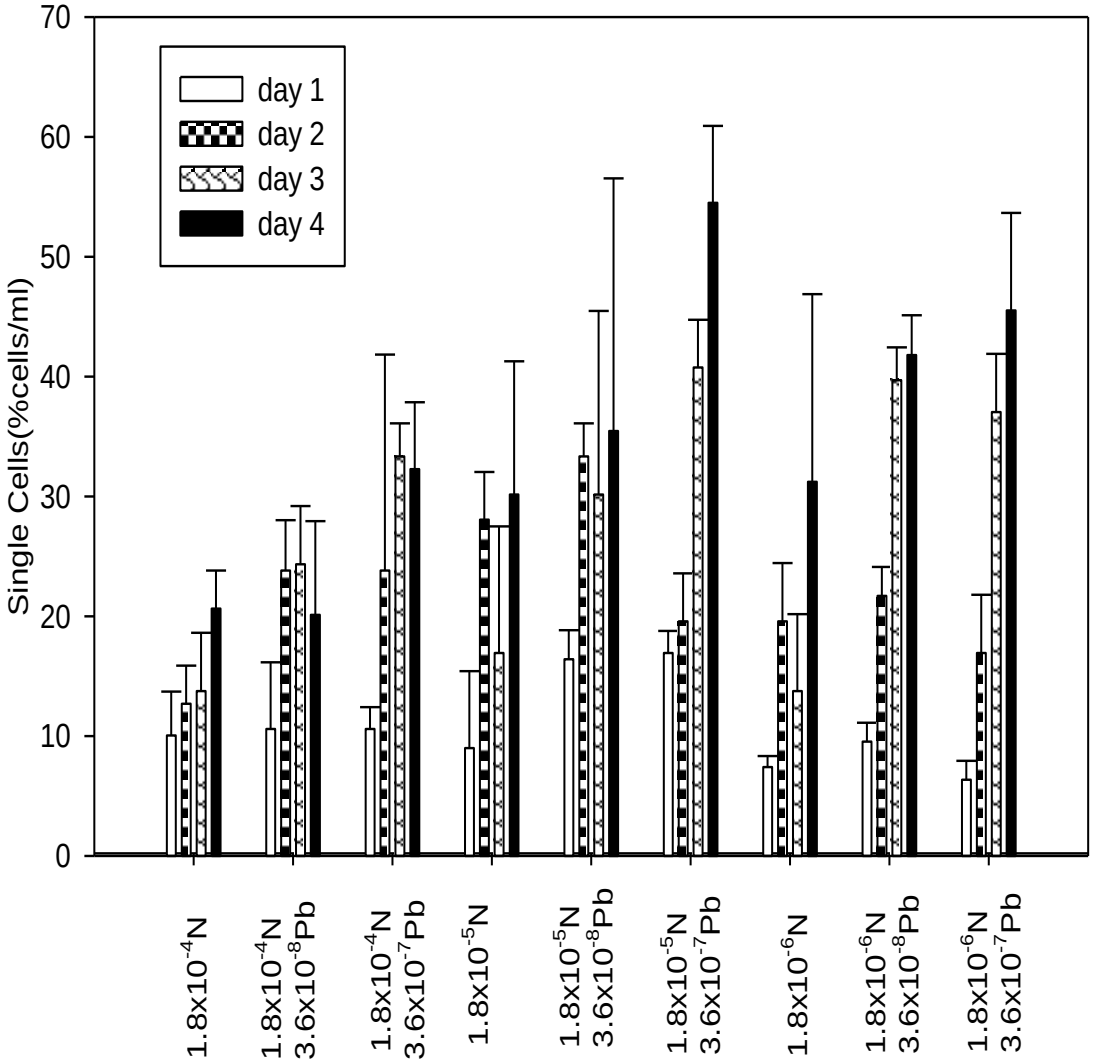


Figure 4.4: Cell counts (x10^4 cells per mL) of *Scenedesmus quadricauda* at varying Pb and N concentrations.

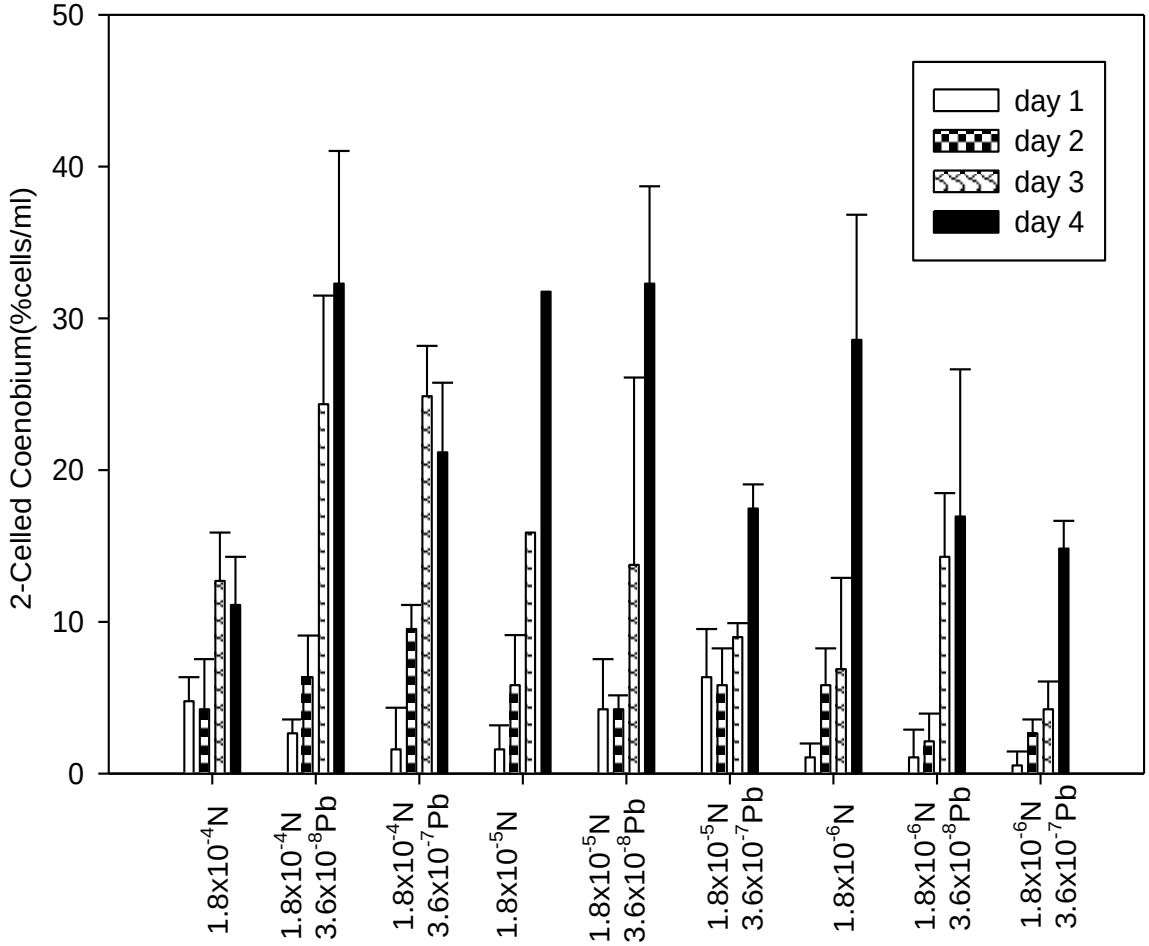
4.2The effect of Pb and NO_3 on the Phenotypic Plasticity of *S. quadricauda*

The number of single cells (Fig. 4.5) increased over the experimental period in all treatments. Although, there was a general significant increase in number of singles cells with increasing N/Pb stress. There was a reduction in the number of cells per coenobium as Pb concentration increased. There was a general increase in number of two cells with increasing N/Pb stress. There was a reduction in the number of cells per coenobium as Pb concentration increased. Furthermore, the production of three and four cells per coenobium showed that they decreased under N limitation and Pb stress as the experiment progressed. The production of three cells per coenobium (Fig.4.6) was only affected individually by N and Pb stress (Table 4.2).There was significant interaction between N limitation and Pb stress on the production of 4 cells per coenobium, the N treatments without Pb had higher number of four cells per coenobium compared to those with Pb addition (Fig.4.7).Time also had effect on the production of cneobium. The production of coenobium started on the first day in all N and Pb treatments (Fig.4.8).The production of coenobium increased on the second day, single cells had the highest production in all treatments, although four and three cell had the lowest production in culture medium with Pb compared to those without Pb and lso limited N concentrations(Fig.4.9).The production of coenobium increased on the third day, single cells had the highest production in all treatments with Pb addition and limited N concentrations, four and three cells production was high on third day in culture medium without Pb compared to cultue medium Pb, although the highest production of three and four cells was recorded the sample with the highest N concentrations (Fig.4.10).



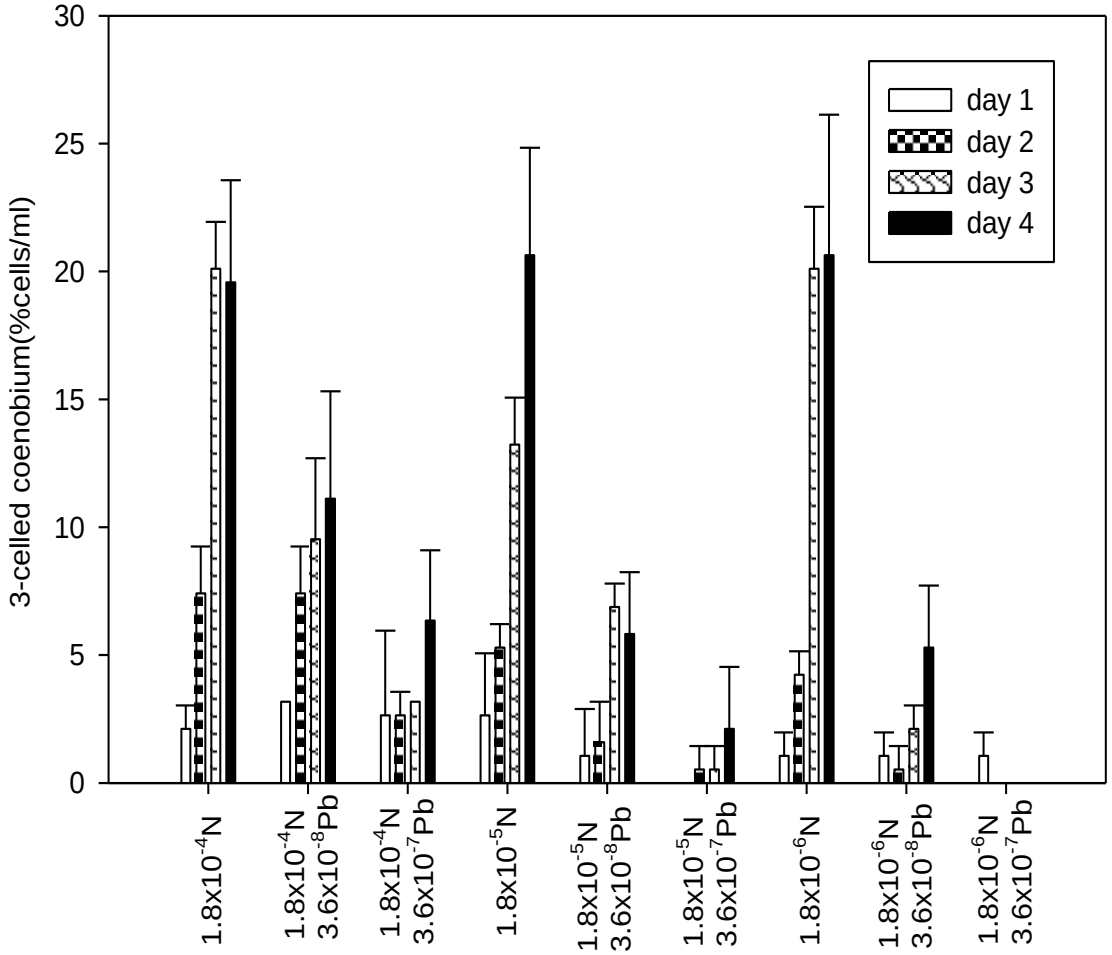
Nitrates and Lead treatment

Figure 4.5: Number ofsingle cells of *Scenedesmus quadricauda* at varying Pb and N concentrations



Nitrates and Lead treatments

Figure 4.6: Number oftwo-celled coenobia of *Scenedesmus quadricauda* at varying Pb and N concentrations



Nitrates and Lead treatments

Figure 4.7: Number ofthree-celled coenobia of *Scenedesmus quadricauda* at varying Pb and N concentrations.

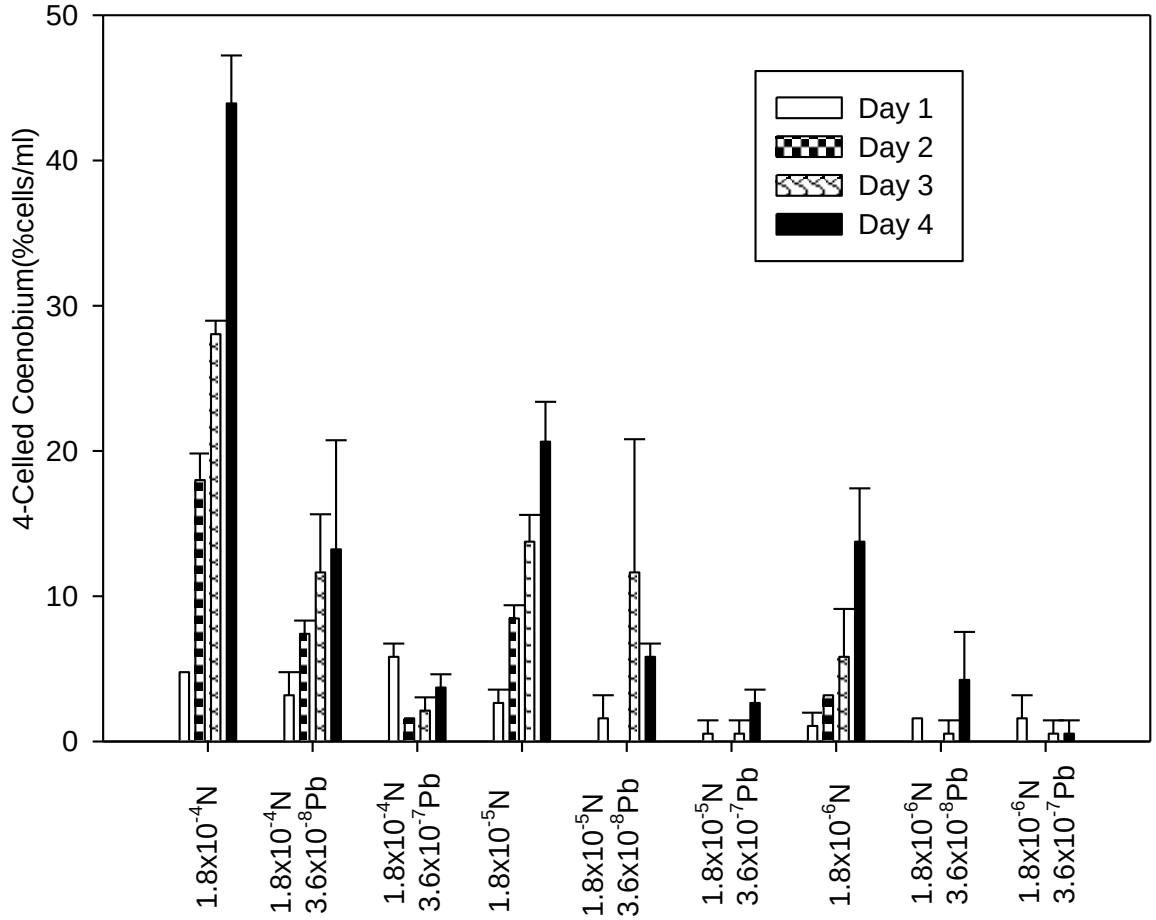


Figure 4.8: Number offour-celled coenobia of *Scenedesmus quadricauda* at varying Pb and N concentrations .

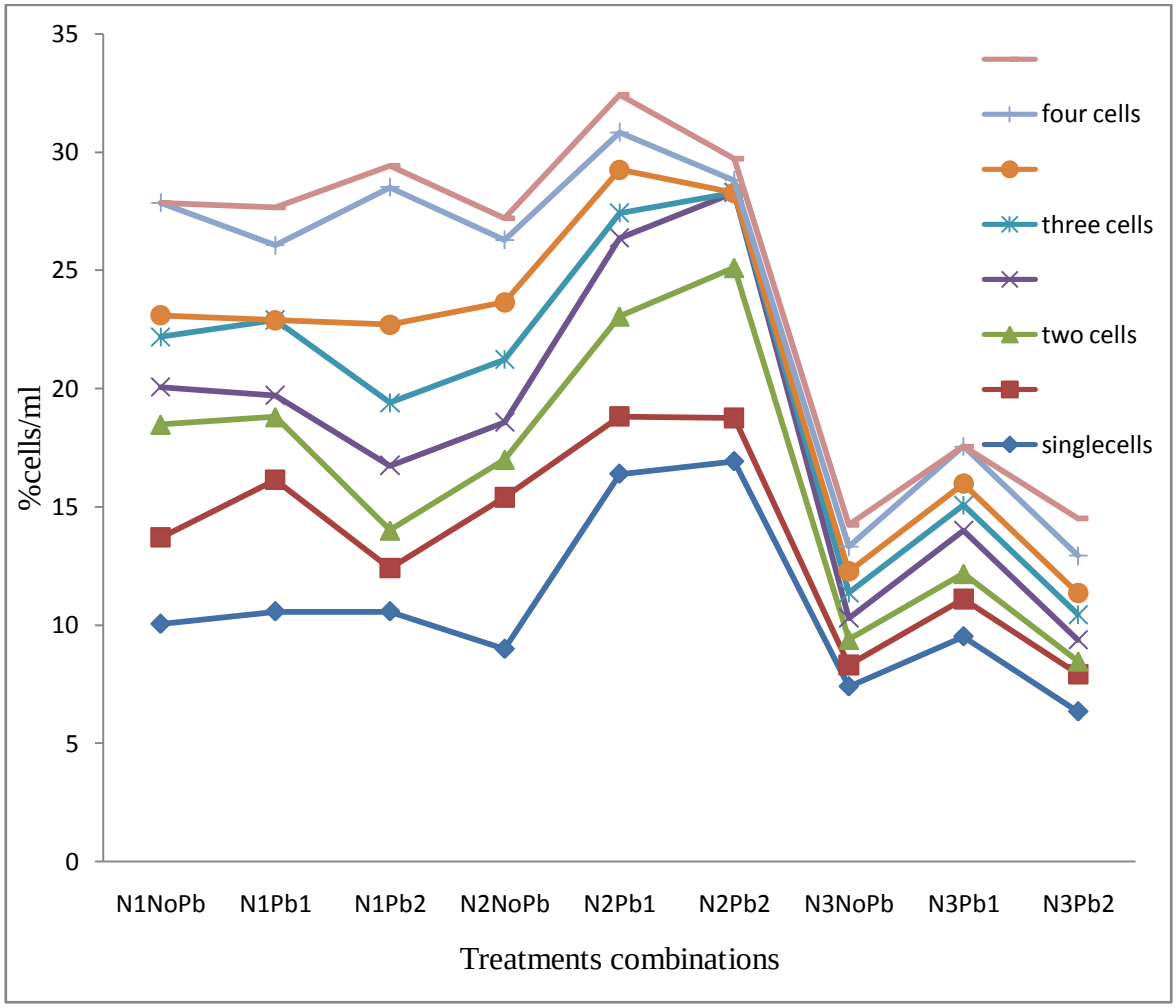


Figure 4.9: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and N concentrations on first day.

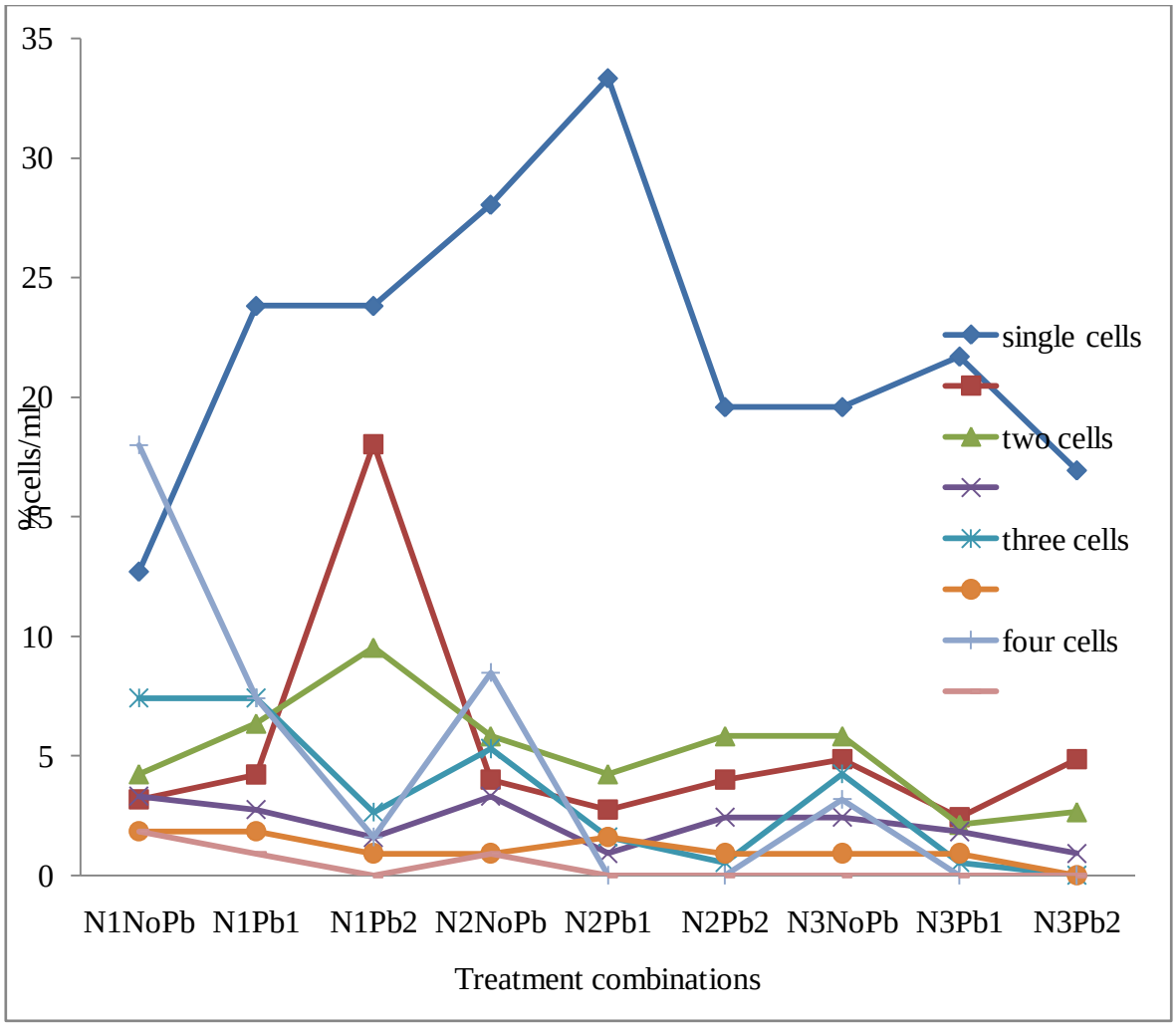


Figure 4.10: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and N concentrations on the second day.

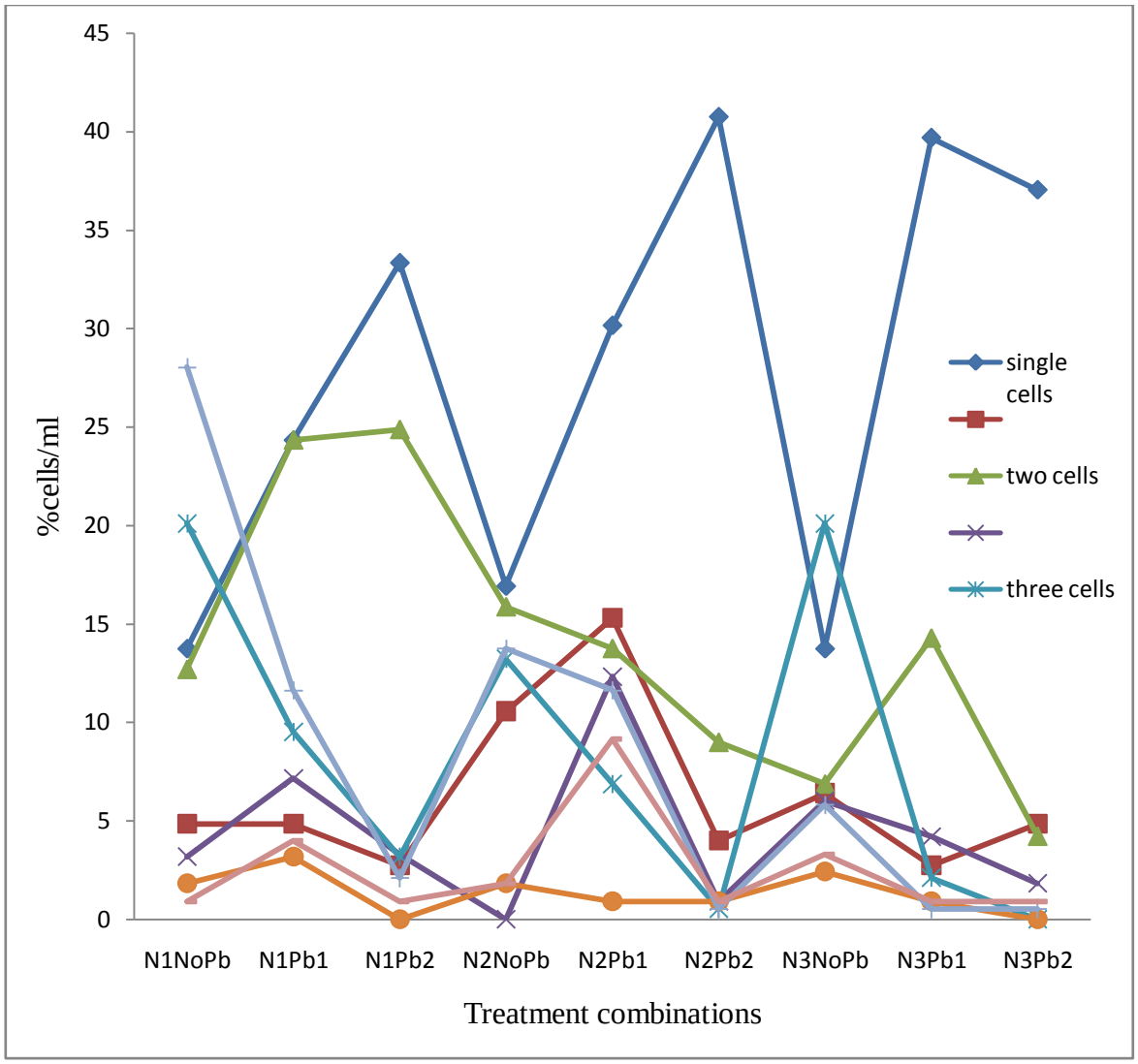


Figure 4.11: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and N concentrations on the third day.

The production of four and three cells production was high on fourth day in culture medium without Pb compared to cultue medium Pb, although the highest production of three and four cells was recorded the sample with the highest N concentrations (Fig.4.12).

4.3 Effect of Pb and NO_3on Total Protein Content and Antioxidant Enzymes of S. *quadricauda*

4.3.1 Effect on total protein content

Lowest protein production was obtained at lowest N concentration with the highest Pb concentration. The highest protein concentration was recorded in the control (10^{-4}M) without Pb addition. In general, the highest protein concentration was recorded in the highest N concentration compared to the limited N concentrations (10^{-5} and 10^{-6}) (Fig. 4.13).

4.3.2 Antioxidant enzymes

Superoxide dismutaseactivities increased throughout the experimental period (Fig.4.14). Inaddition, increasing N/Pb stress resulted in significantly (p <0.05) higher activities of SOD.Changes in catalase activity showed an initial increase in catalase activities whichbegan to decrease after 48 h. The lowest catalase activity was obtained in the control but as Nlimitation and Pb stress increased, CAT activity increased within the first 48 h of the experiment.Although, CAT activity decreased after 48 h, the activity of the enzyme was still higher underN/Pb stress conditions compared to the control throughout the experiment (Fig. 4.15). Peroxidase(POD) activity increased with decreasing N concentration in combination with Pb. Unlike whatwas obtained in CAT treatment, POD activity decreased mostly after 72 h.

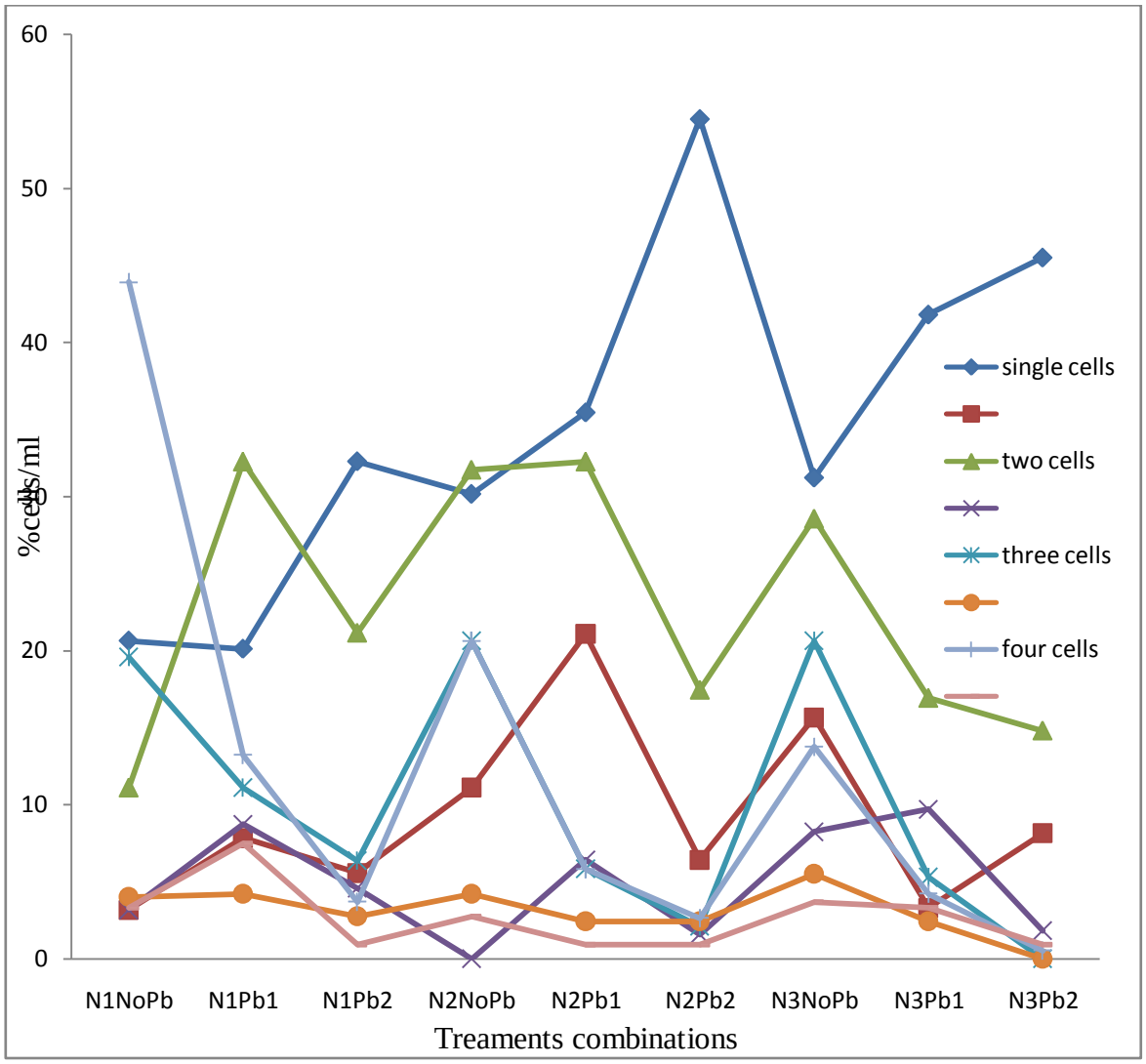


Figure 4.12: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and N concentrations on the fourth day.

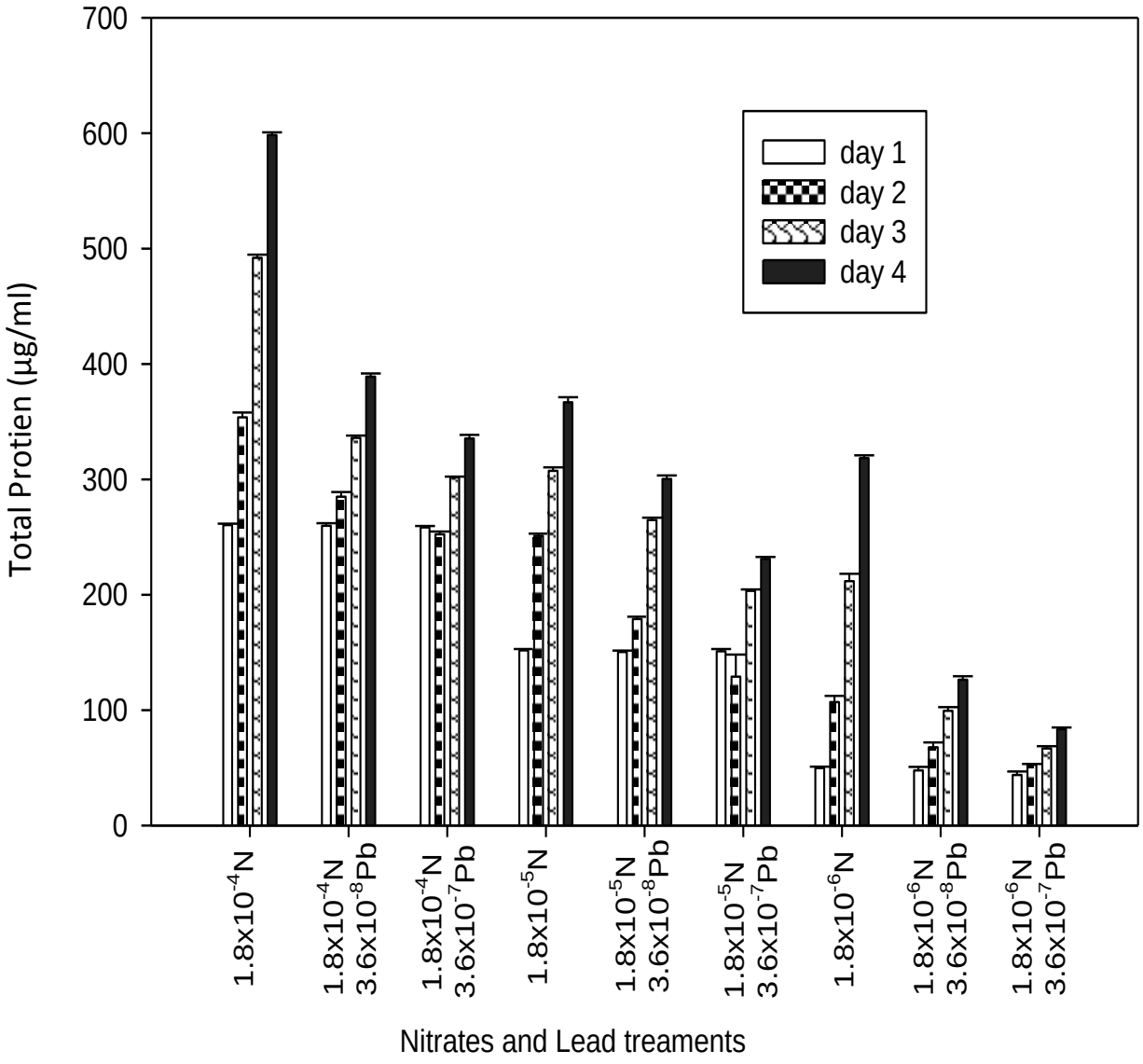


Figure 4.13: Total protein content of *Scenedesmus quadricauda* at varying N and Pb concentrations.

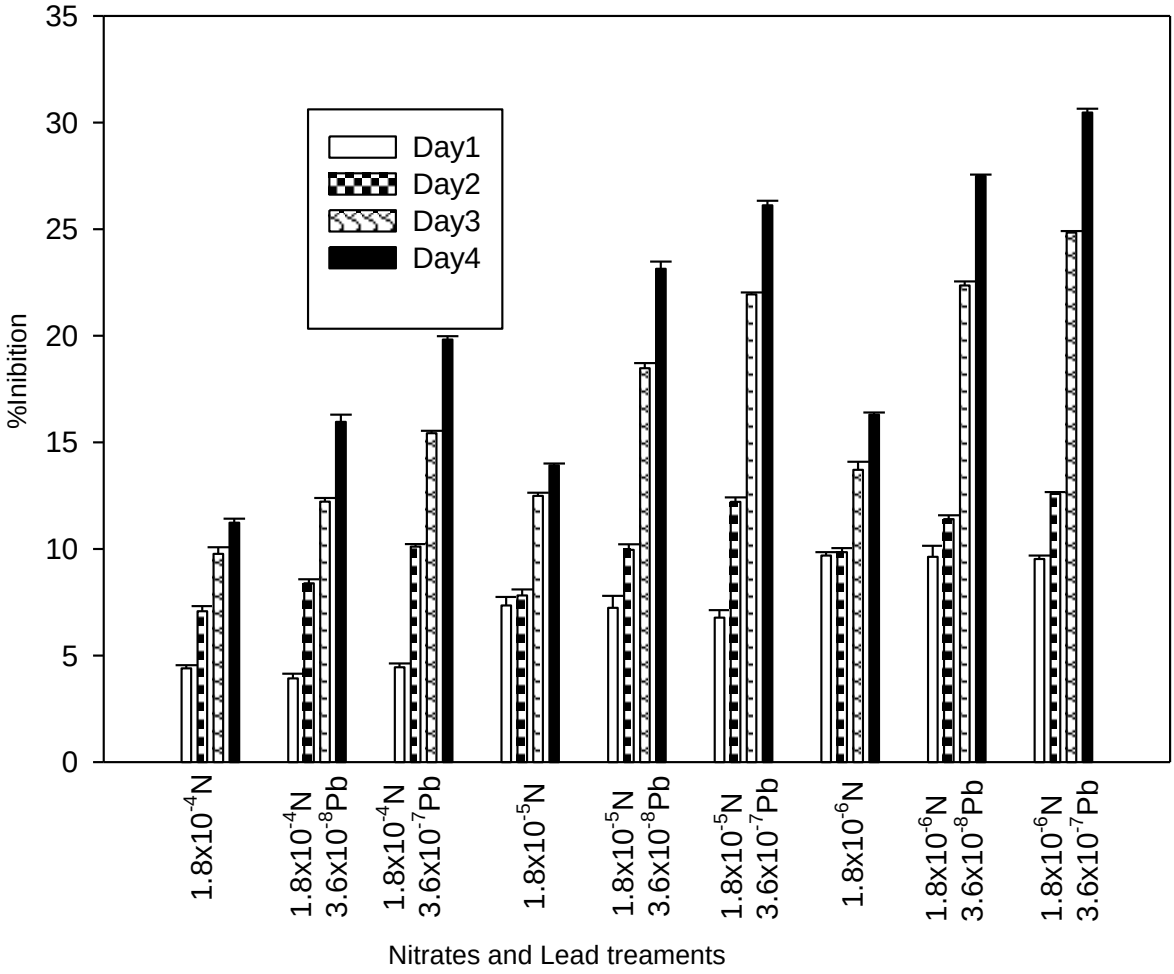
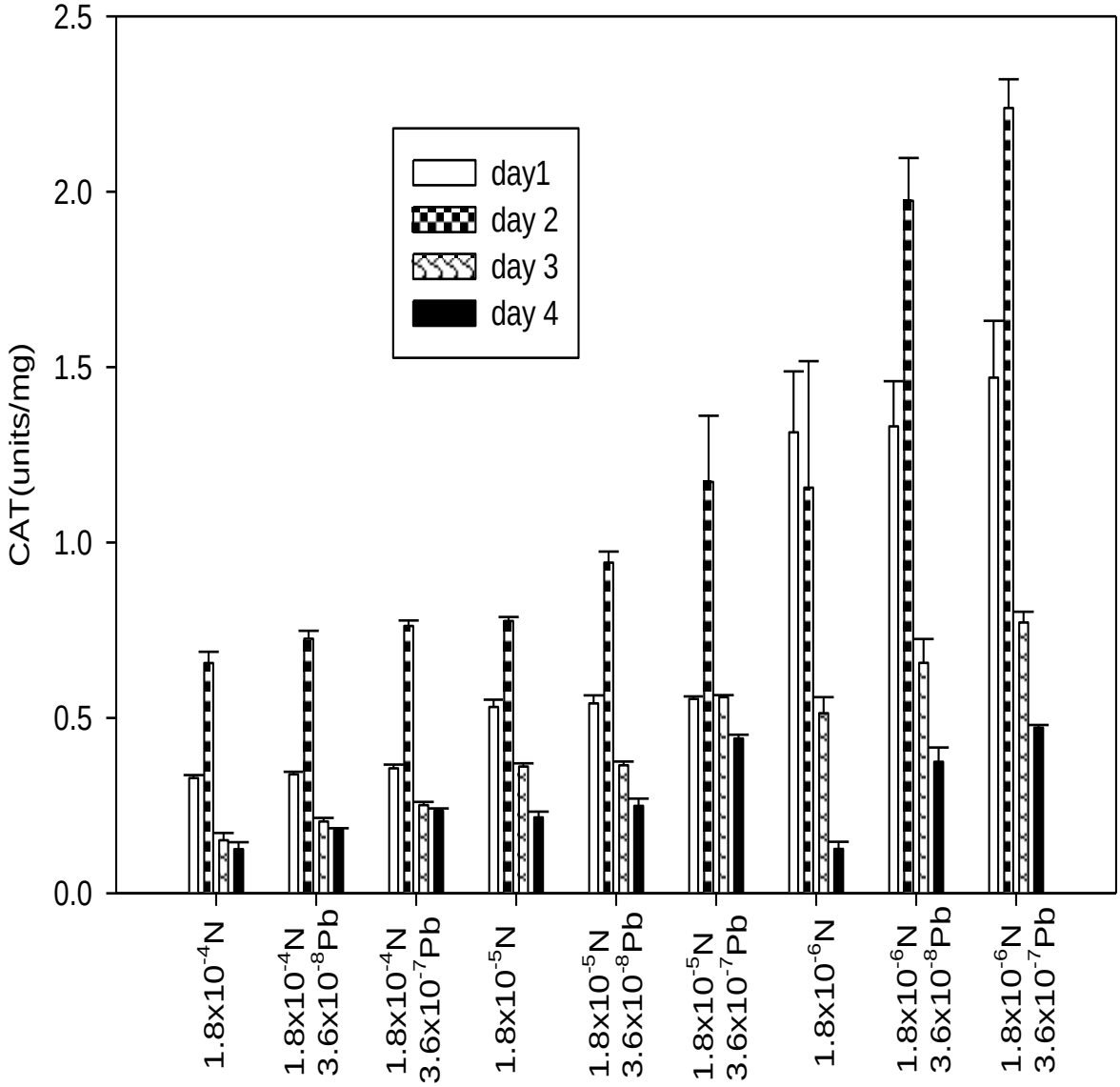


Figure 4.14: SOD activity (%inhibition) of *Scenedesmus quadricauda* at varying N and Pb concentrations



Figures 4.15: CAT activity (Specific activity) of *Scenedesmus quadricauda* at different N and Pb concentrations.

Perioxidase increased with increasing N/Pb stress, and each factor had significant interactive effect on thisenzyme (Fig.4.16).Glutathione-S-transferaseactivities increased throughout the experimental period (Fig.4.17). Inaddition, increasing N/Pb stress resulted in significantly (p <0.05) higher activities of GST. Chlorophyll *a*, dry weight, cells counts and total protein content decreased with limited N concentration and with Pb stress (Table 4.2). Contrary to the results in table below, the SACAT, SAPOD,SASOD and SAGST activity all increased under N limitation and addition of Pb, the presence of Pb stimulated high antioxidant activities (Tables 4.1 ANOVA P<0.05).Principal component analysis (PCA) showed that the first two components were responsible for 73% of the totalvariation (Fig. 4.18). SOD, POD, single cells, and the production of two cells per coenobium had a significant positive correlation with Pb concentration and presented a negative correlation withincreasing N concentration on the second PCA component. Cell counts, dry weightconcentration, production of three and four cells per coenobium, chlorophyll *a* and total proteins were positively associated with high N concentration. The limitation of N had a negative relationship with CAT activity, while with increasing Pb concentration the relationship was positive. Biomass production (cell counts, dry weight, and chlorophyll *a* production) was negatively associated with Pb.

4.4 The effect of Pb and P on Growth and Biomass Determination of S. *quadricauda*

Scenedesmus quadricauda growth rates (Table 4.2) decreased under P limitation and Pb addition in P-replete conditions and a combination of high (10^{-7}M), Pb and P limitation further inhibited the growth rate. Growth changes can also be seen in the figure 4.20

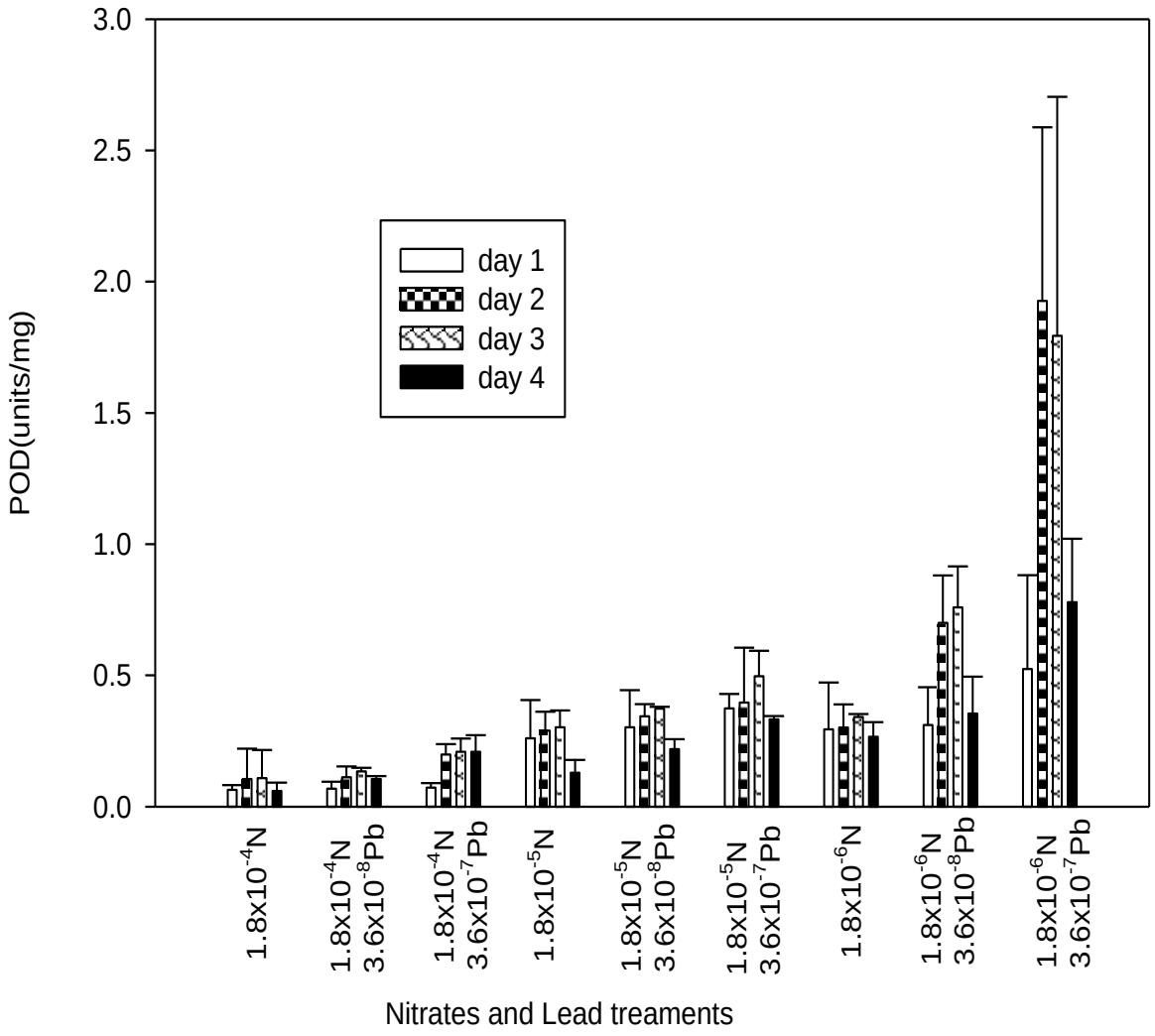


Figure 4.16: POD activity (Specific activity) of *Scenedesmus quadricauda* at varying N and Pb concentrations.

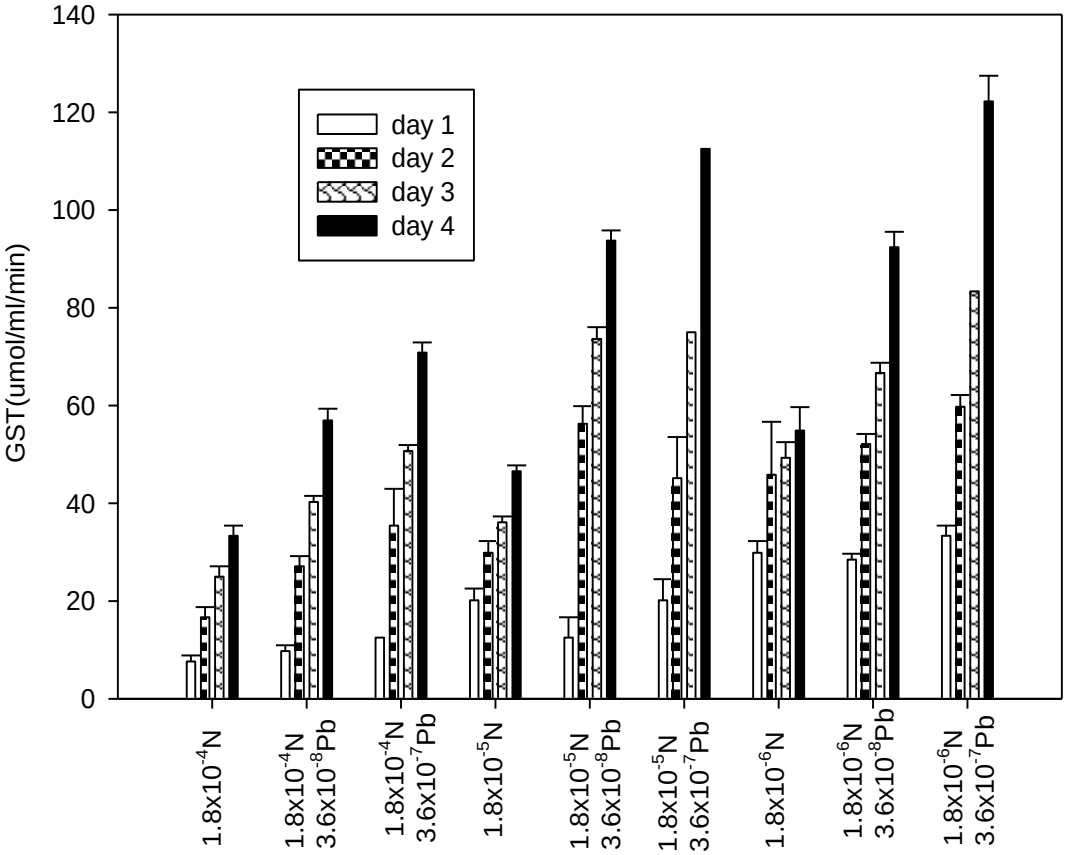


Figure 4.17: GST activity (Specific activity) of *Scenedesmus quadricauda* at varying N and Pb concentrations

Table 4.2:Analysis of variance results for the effect of Nitrogen (N) and Lead(Pb) on biomass production and antioxidant enzymes response of *Scenedesmus quaridicauda*.

Parameters	N	Pb	N/Pb
Chl *a*	30.176**(0)**	23.322**(0)**	1.102(0.359)
Dry weights	34.229**(0)**	16.111**(0.000)**	0.361(0.835)
Cell counts	8.1997**(0.000)**	13.6284**(0.000)**	0.123(0.973)
1 celled coenobium	3.5174**(0.033)**	6.4627**(0.002)**	0.2809(0.889)
2 celled coenobium	2.3072(0.104)	0.842(0.433)	1.3314(0.263)
3 celled coenobium	3.9665**(0.022)**	31.3462**(0)**	0.59(0.670)
4 celled coenobium	19.3924**(0)**	32.4649**(0)**	4.3861**(0.002)**
Protein	95.449**(0)**	22.915**(0)**	0.652(0.626)
SAGST	12.551**(0.000)**	11.8045**(0.000)**	0.3421(0.848)
SACAT	24.344**(0)**	3.2415**(0.043)**	0.7119(0.585)
SAPOD	34.9221**(0)**	17.5246**(0)**	8.7608**(0.000)**
SASOD	9.6308**(0.000)**	8.7117**(0.000)**	0.2667(0.899)

Values represent F values and those in parentheses represent P values. Note: values with $P<0.05$ are statistically significant and marked in bold. Abbreviations- SAGST=specific activity of Glutathione –S transferase; SACAT=specific activity of Catalase; SAPOD=specific activity of peroxidase, SASOD=specific activity superoxide dismutase.

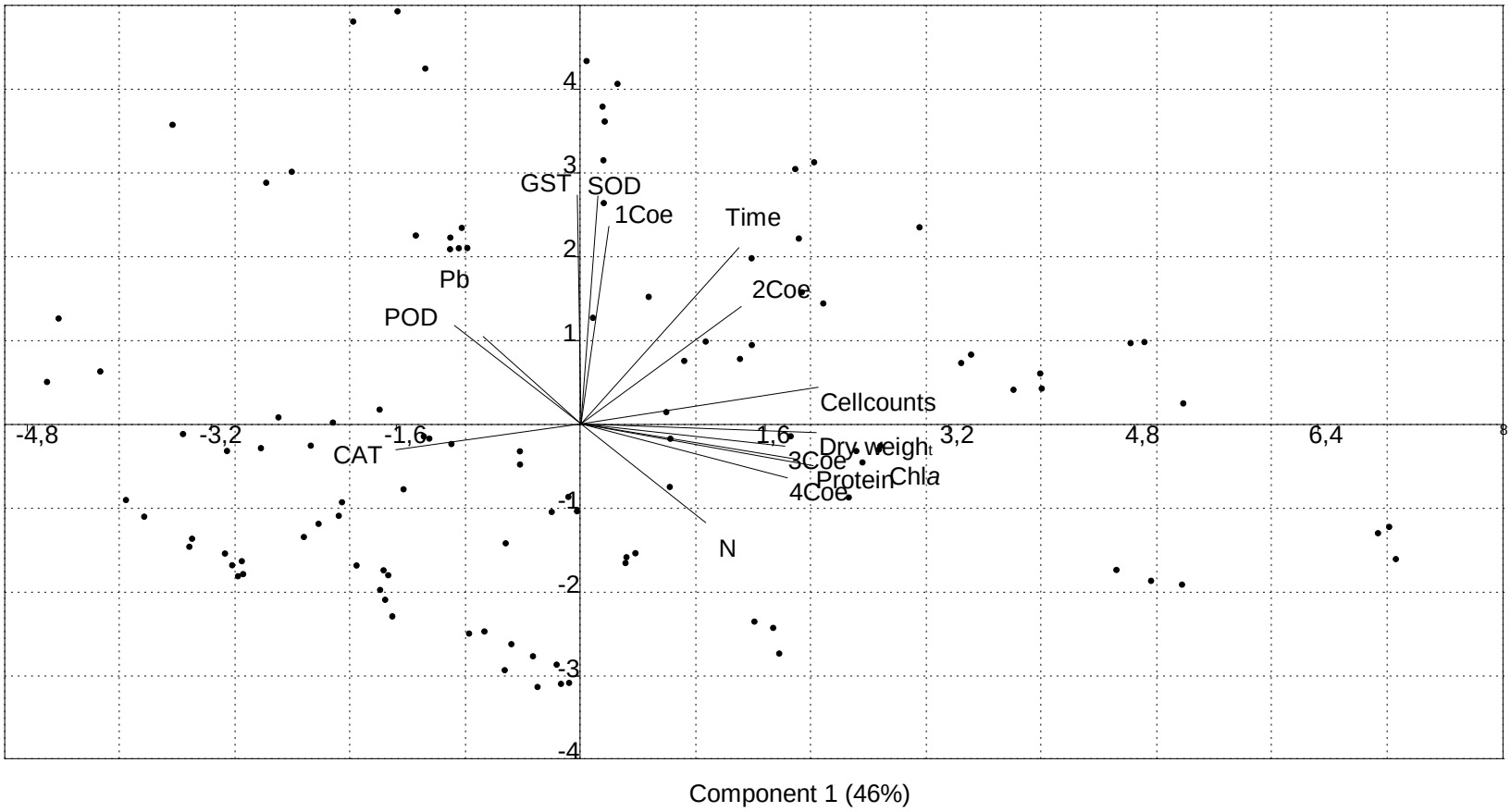


Figure 4.19: Principal component analysis (PCA chart) of Pb and N experiment

Table 4.3: Specific growth rates of *Scenedesmus quadricauda* as a function of different Lead(Pb) and Phosphates concentration determined after 96 h incubation.

	Pb(Mol L^{-1})		
Phosphorus(M)	NoPb Growth rate	3.6×10^{-8}(Pb1) Growth rate	3.6×10^{-7}(Pb2) Growth rate
1.2×10^{-5}	0.601±0.34	0.432±0.47	0.321±0.024
1.2×10^{-6}	0.549±0.013	0.269±0.016	0.256±0.018
1.2×10^{-7}	0.479±0.030	0.228±0.021	0.156±0.029

Values represent mean and standard deviation for n=3.

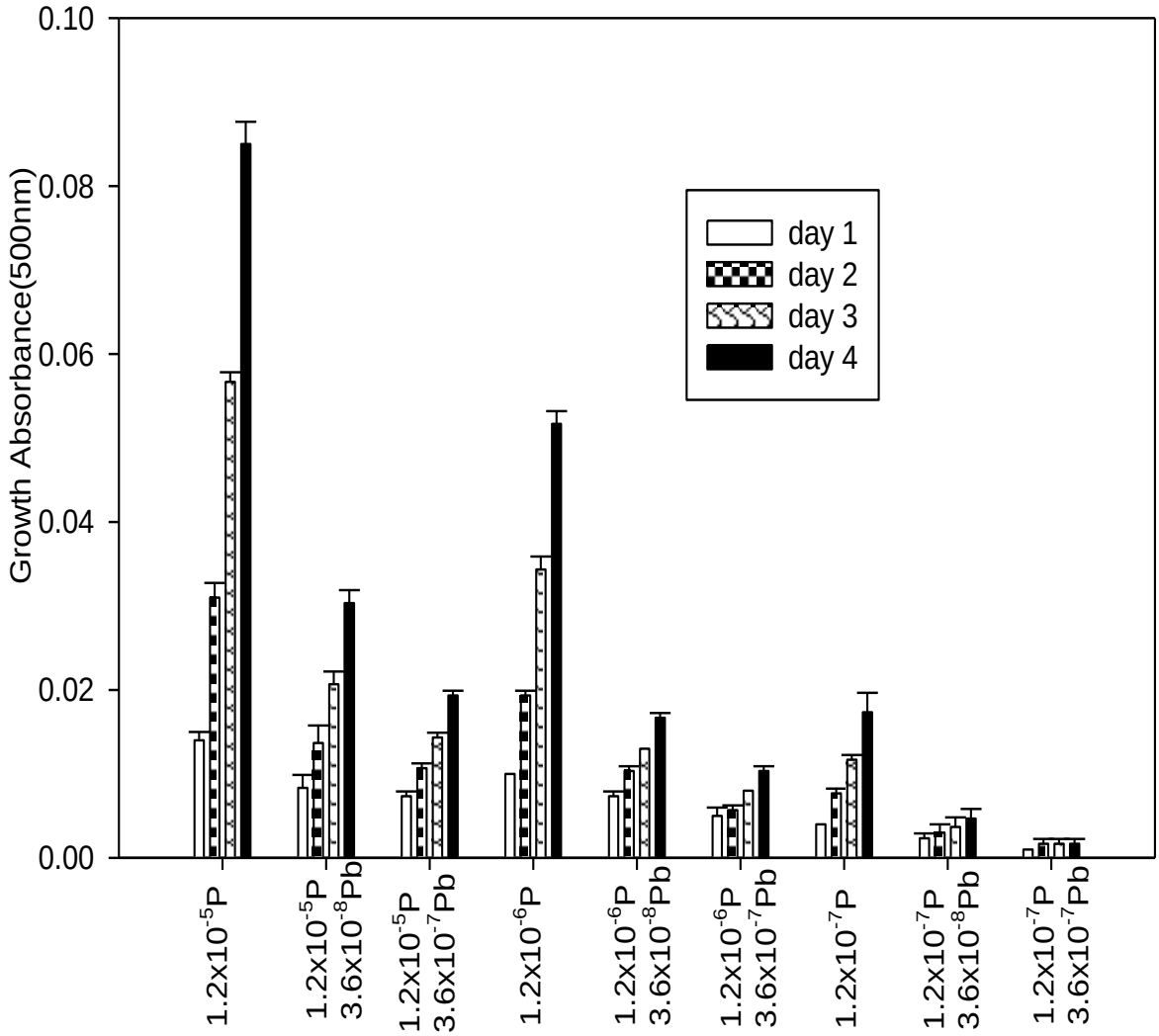


Figure 4.20: Growth absorbance at 550nm by *Scenedesmus quadricauda* at varying Pb and P concentrations.

Chlorophyll *a* concentrations reduced with replete P concentrations. The presence of Pb further inhibited the chlorophyll production, chlorophyll *a* started to decrease after the first day and the lowest production was seen in treatment with lowest P and highest Pb concentrations (Fig.4.21). The cell density was inhibited by P limitation and also addition of Pb (Fig.4.22). Dry weight per cell were inhibited by P limitation and also addition of Pb (Fig. 4.23).

4.5The effect of Pb and PO_4 on the Phenotypic Plasticity of *S. quadricauda*

The Phenotypic plasticity observed in *Scenedesmus quadricauda* showed that there was a reduction in the number of cells per coenobium at the highest Pb concentration. There were changes in the number of cells per coenobium compared to the control. The number of cells per coenobium diminishes as the Pb concentration increased. All the Pb concentrations observed, single cells were seen (Fig.4.24).At P limitation and highest Pb concentration, coenobium with decreased three cells (Fig4.26) and two cells (Fig.4.25) started to increase.At the control for all P concentrations, coenobium with four cells were dominant but there was a decrease with respect to P limitation and Pb addition (Fig.4.27).

Time also had effect on the production of cneobium. The production of coenobium started on the first day in all P and Pb experiment.There was general increase in the production of coenobium (Fig.4.28). The production of coenobiumcontinued after the first day in all P and Pb experiment.There was general increase in the production of coenobium, the addition of Pb inhibited the production of coenobium and the lowest production was recorded in the sample with lowest P and Pb concentration (Fig.4.29).

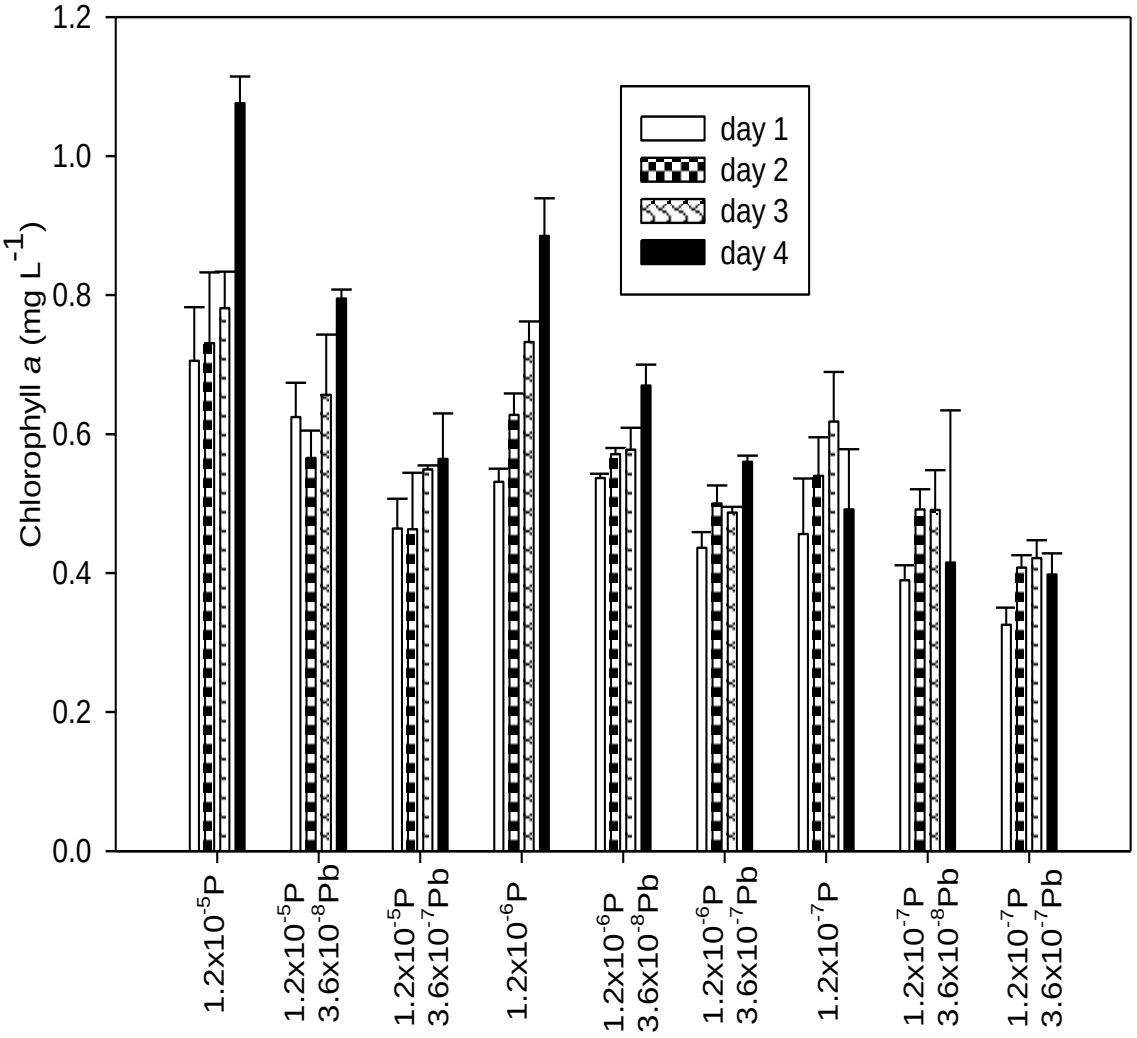


Figure 4.22: Clorophyll *a* content of *Scenedesmus quadricauda* at varying Pb and P concentrations.

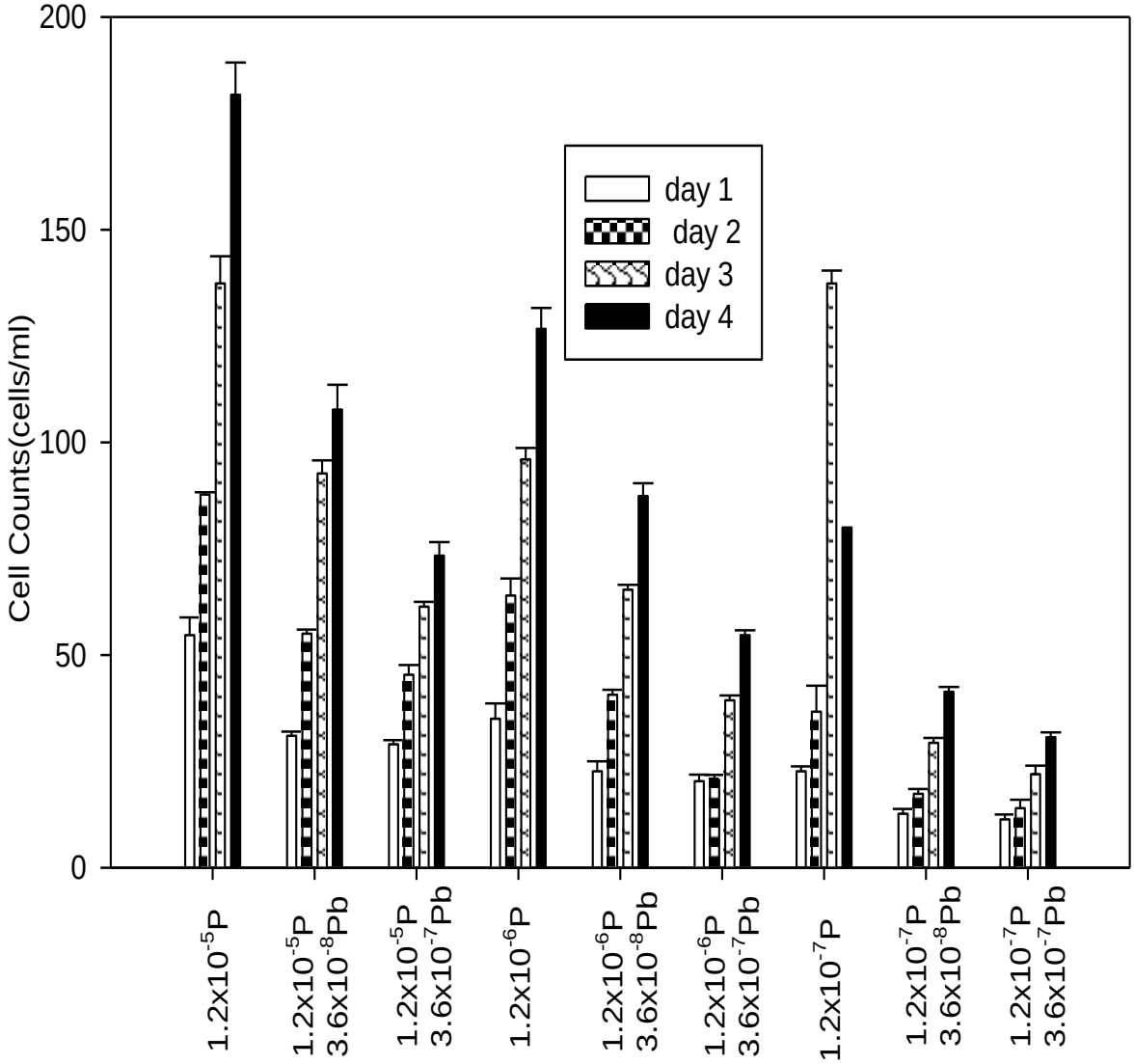


Figure 4.22: Cell Counts of *Scenedesmus quadricauda* at varying Pb and P concentrations.

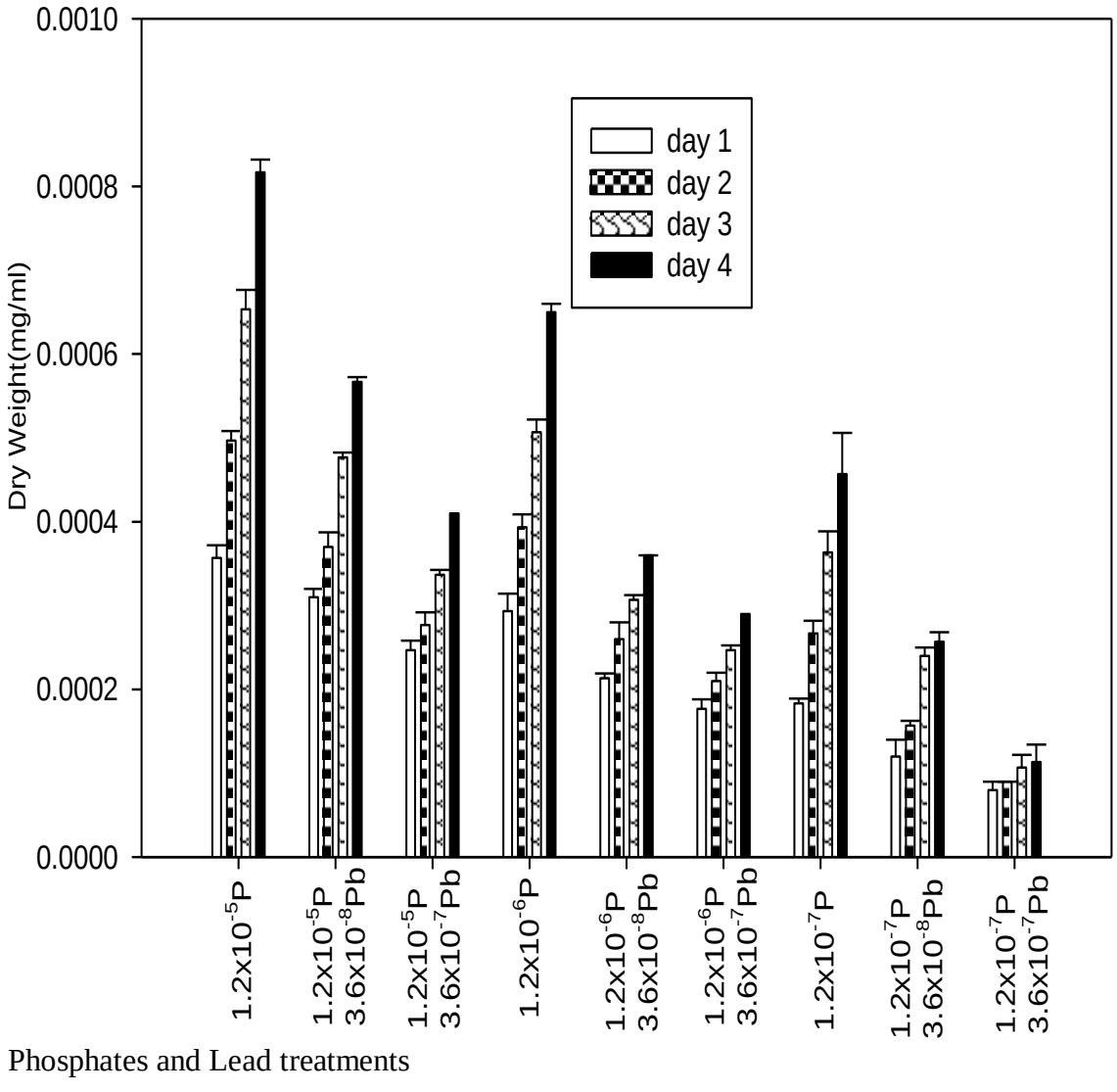
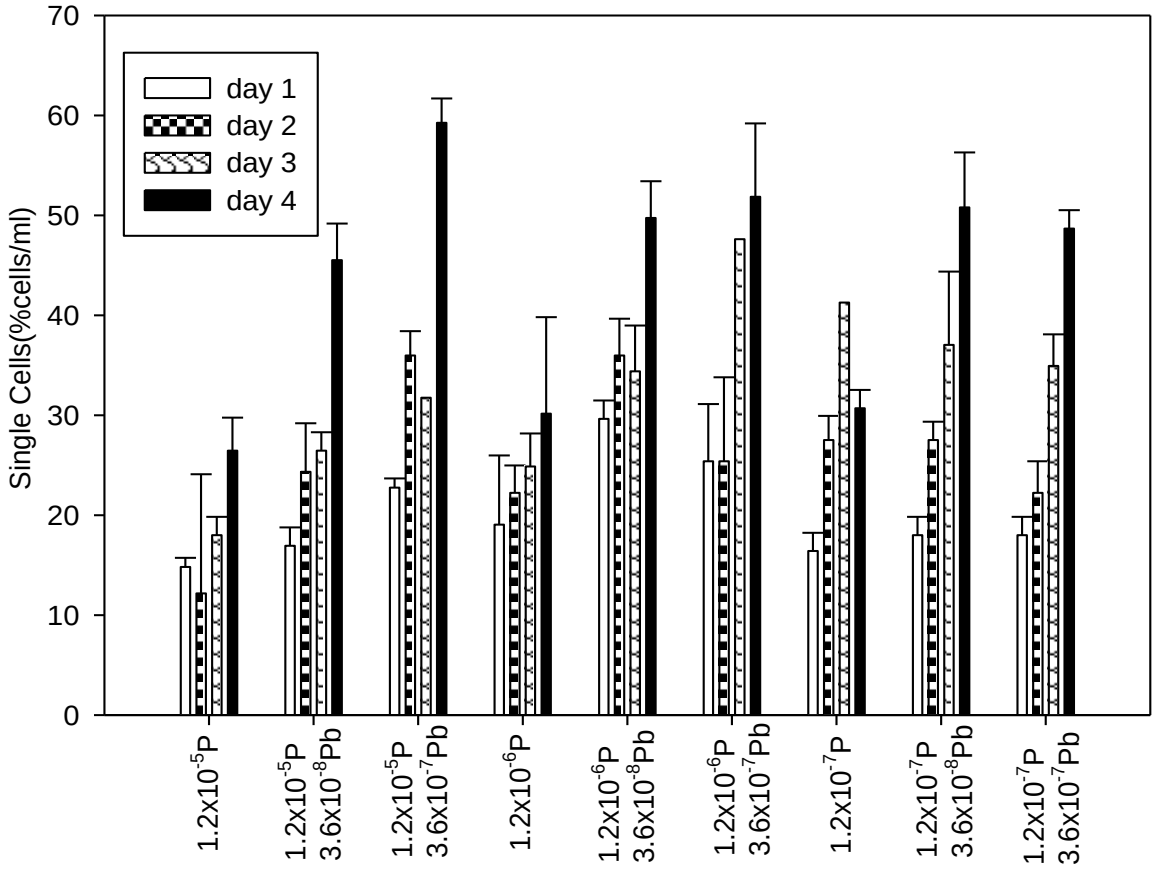


Figure 4.23: Dry weight of *Scenedesmus quadricauda* at varying Pb and P concentrations.



Phosphates and Lead experiments

Figure 4.24: Number ofsingle Cells Coenobia (Phenotypic plasticity) of *Scenedesmus quadricauda* at varying Pb and Pconcentrations.

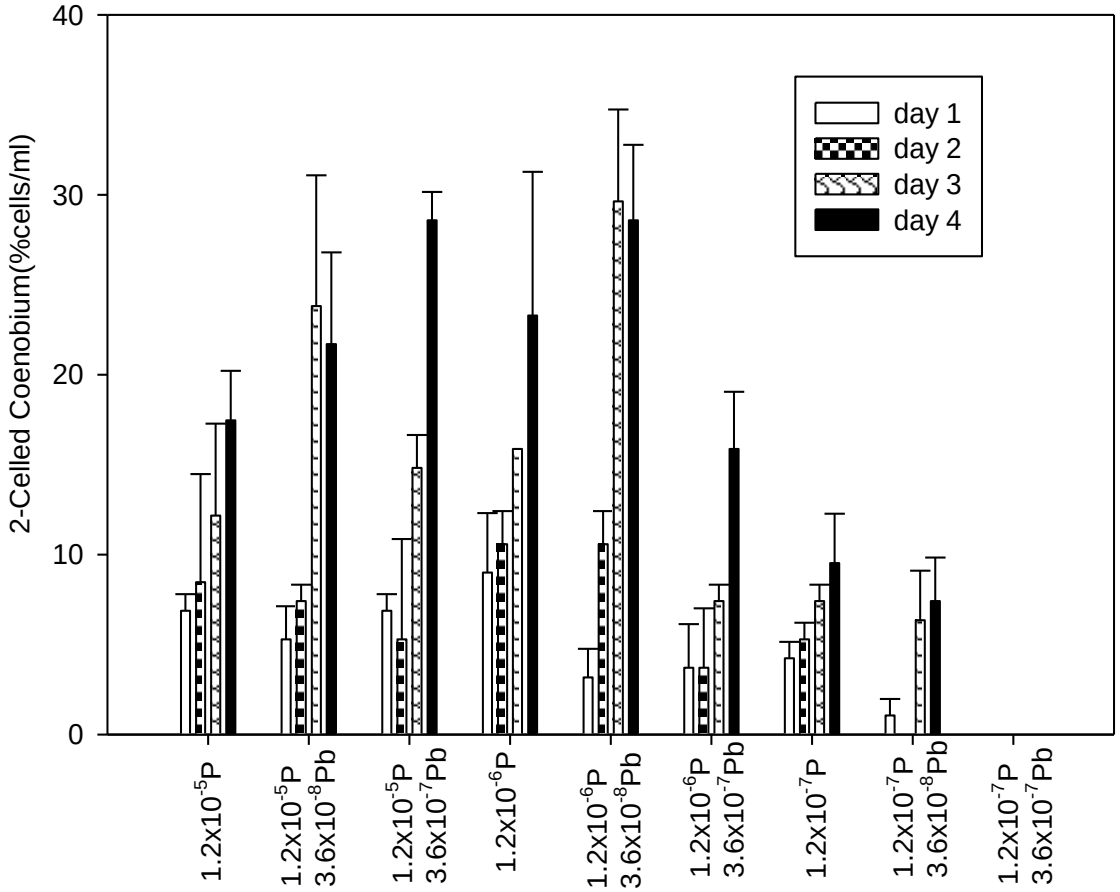


Figure 4.25: Number oftwo-celled Coenobia (Phenotypic plasticity) of *Scenedesmus quadricauda* at varying Pb and P concentrations.

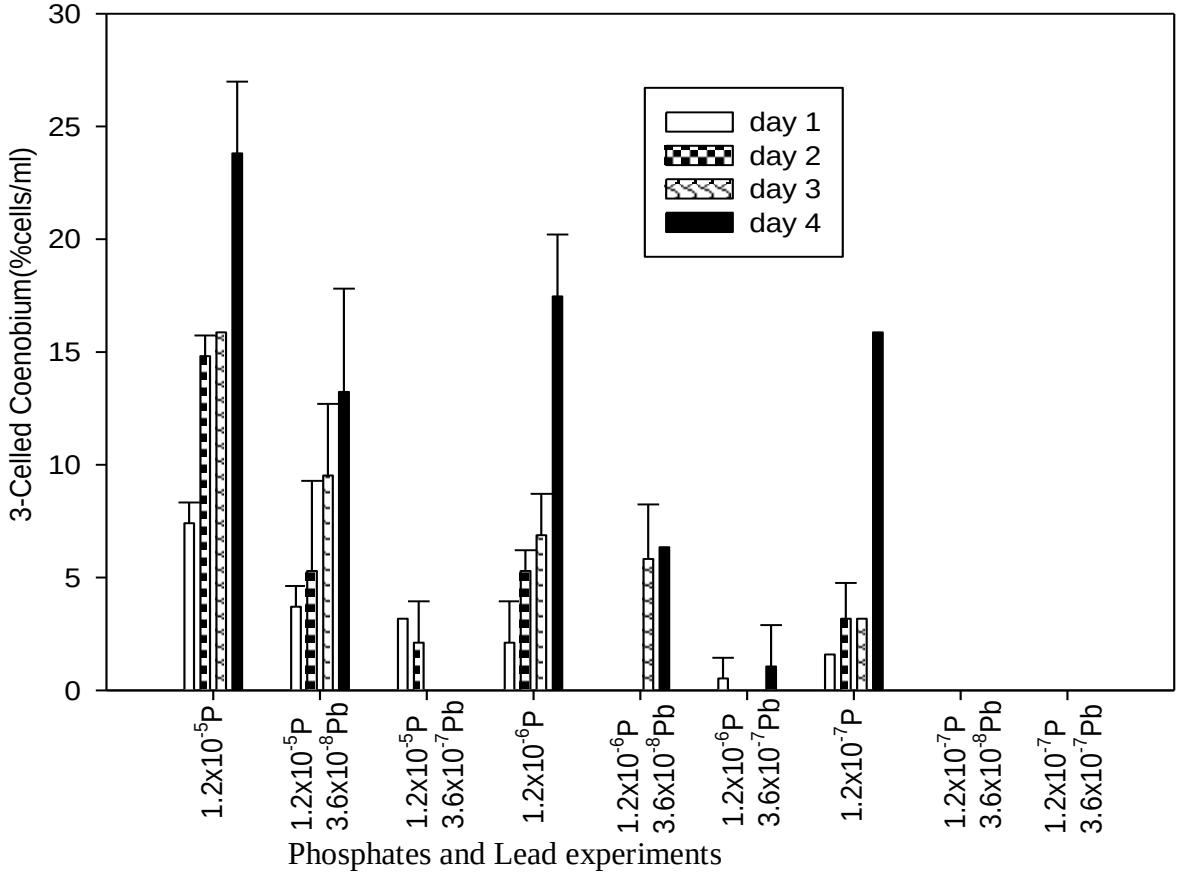


Figure.4.26: Number ofthree-celled Coenobia (Phenotypic plasticity) of *Scenedesmus quadricauda* at varying Pb and P concentrations.

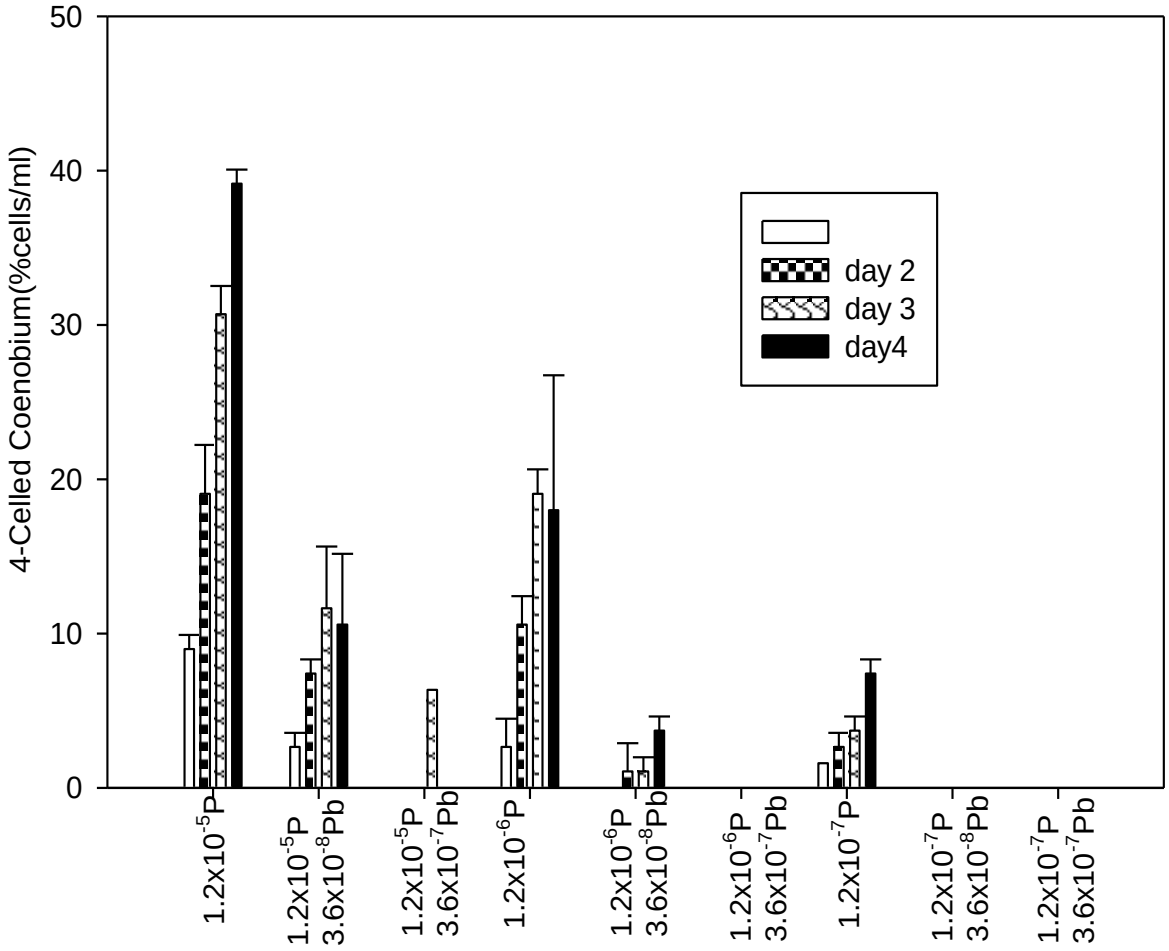


Figure 4.27: Number offour-Celled Coenobia (Phenotypic plasticity) of *Scenedesmus quadricauda* at varying Pb and P concentrations.

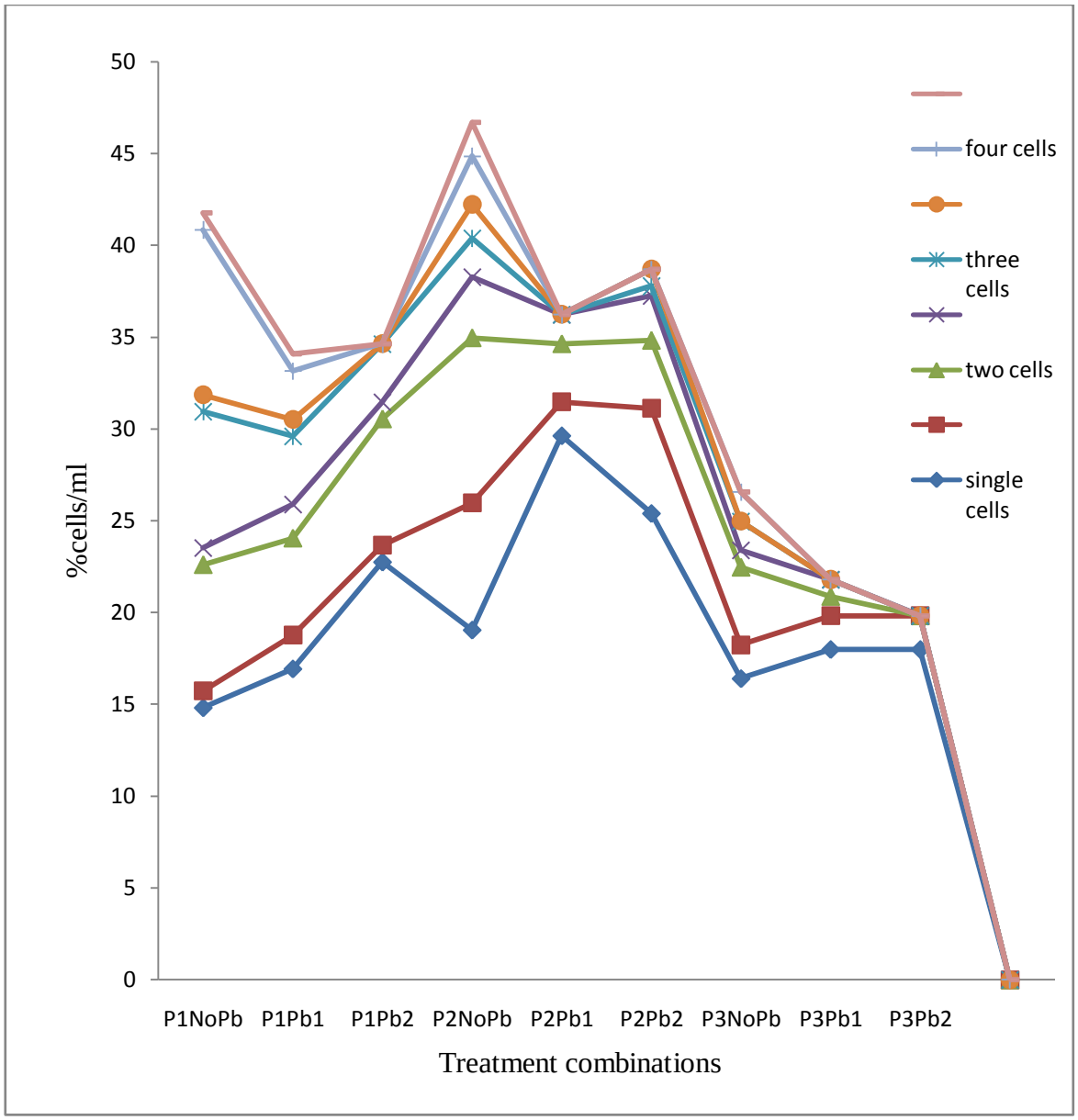


Figure 4.28: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and P concentrations on the first day.

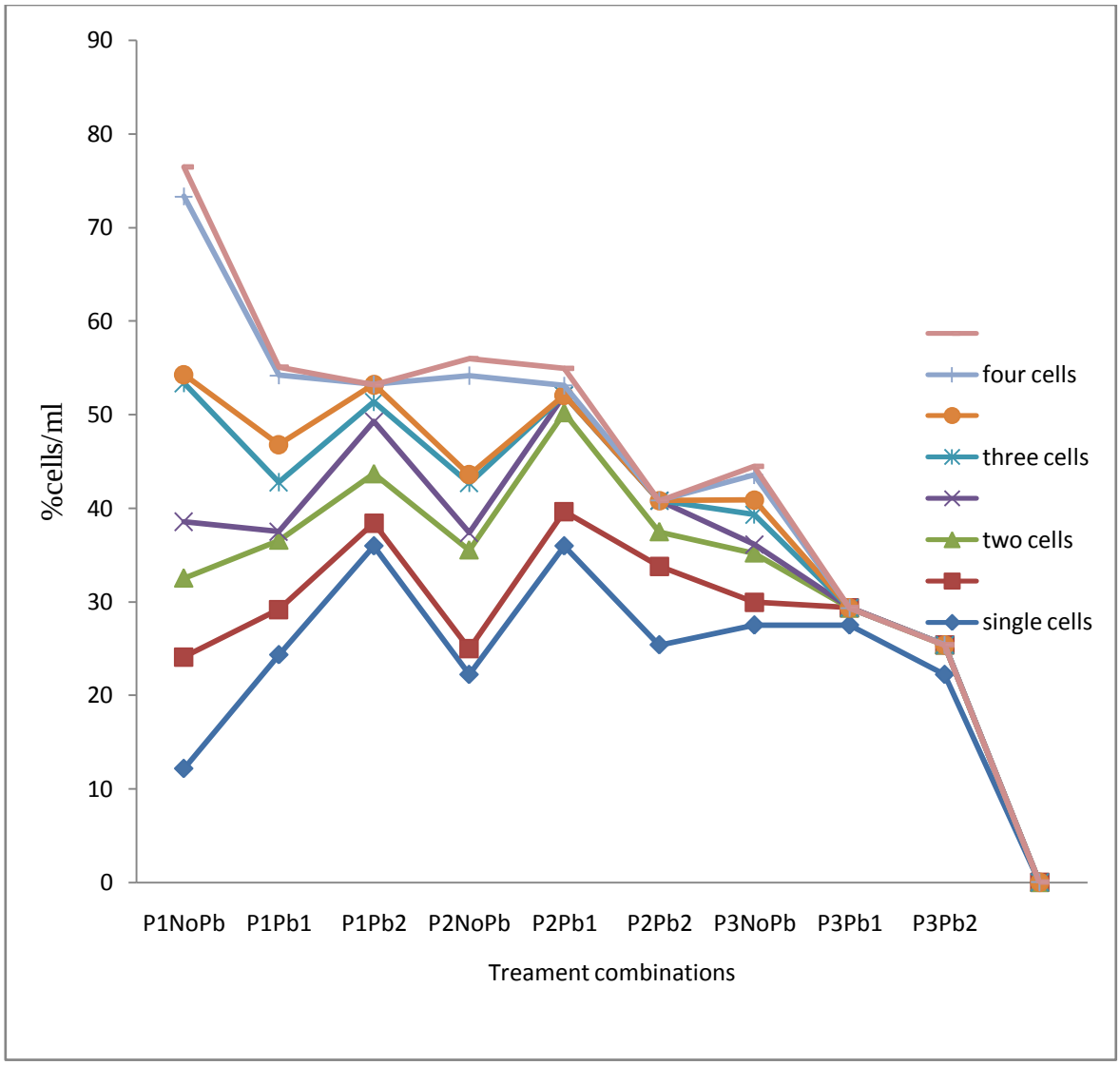


Figure 4.29: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and P concentrations on the second day.

There was general increase in the production of coenobium, limited P had effect on the production of coenobium as the experiment progressed, the third day recorded the highest production of three and four cells in controls compared to culture medium with Pb (Fig.4.30). There was general increase in the production of coenobium, limited concentration of P had effect on the production of coenobium as experiment progressed, the highest production of four cells coenobium was day recorded in culture medium with P concentration, P/Pb stress futher inhibited the production of coenobium after the four days experiments (Fig.4.31).

4.6 Effect of Pb and PO_4 on Total Protein Content and Antioxidant Enzymes of *S. quadricauda*

4.6.1 Effect on total protein content

Lowest protein production was obtained at limited P concentration with the highest Pb concentration. The highest protein concentration was recorded in the highest Pconcentration and No Pb addition compared to the limited P concentrations without Pb (Fig.4.32).

4.6.2Effect onAntioxidant Enzymes

Lead (Pb) stimulated the activity of antioxidant enzymes, increase in Superoxide dismutase activity was observed in limited P concentration with highest Pb concentration and also there was an increase in Superoxide dismutase activity in limited P concentration without Pb compared to the control without Pb (Fig.4.33).

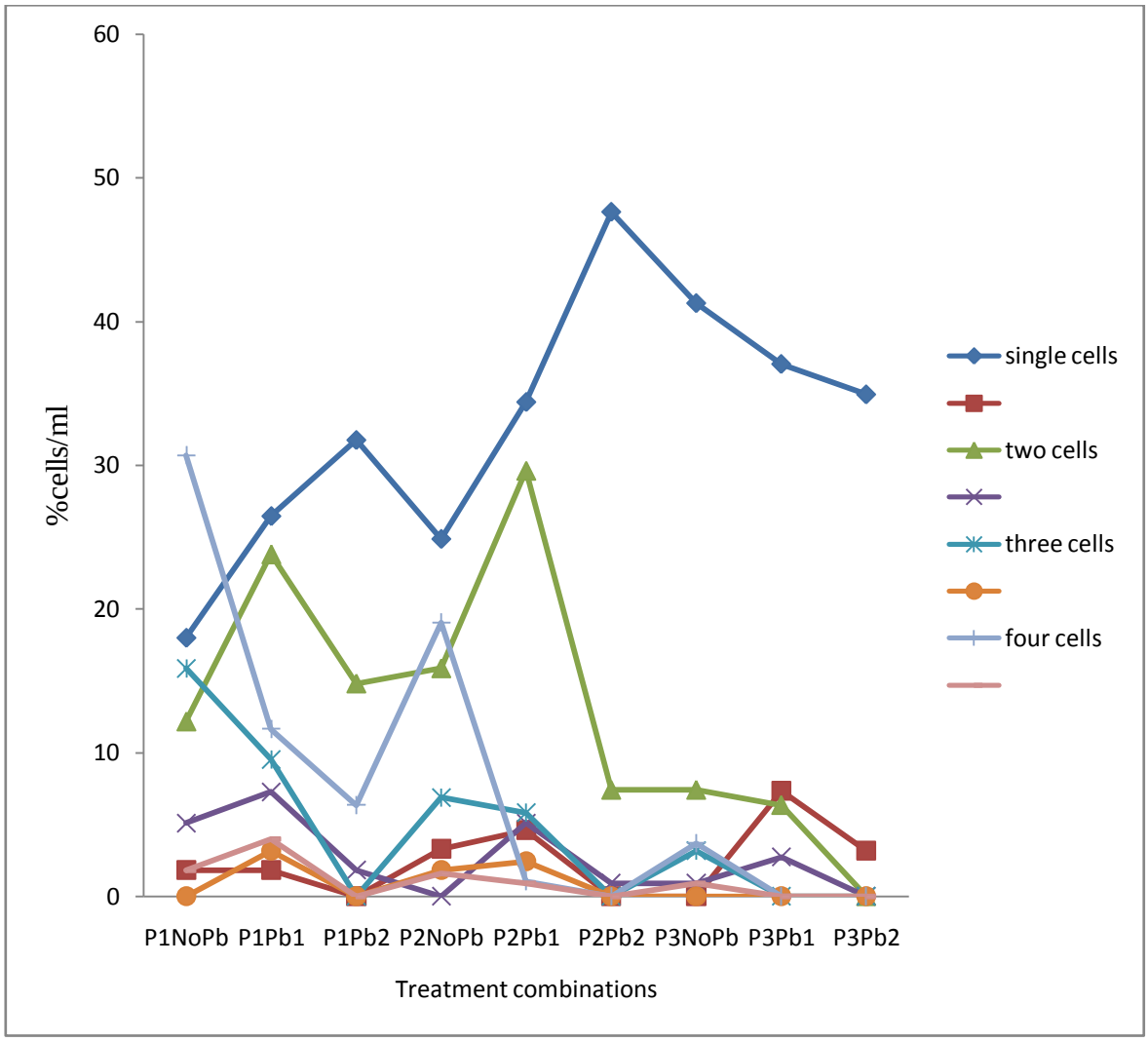


Figure 4.30: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and P concentrations on the third day.

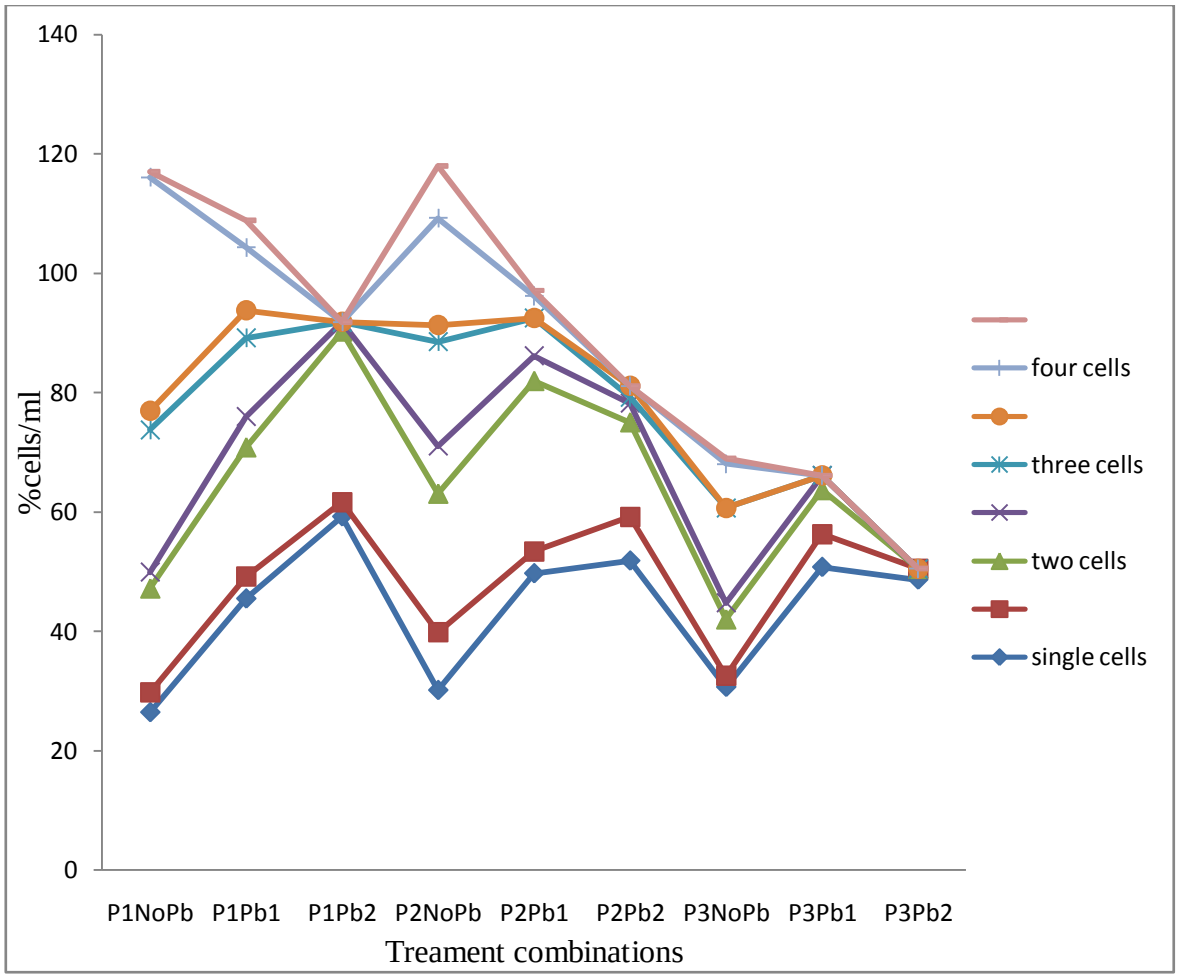


Figure 4.31: The production of coenobium of *Scenedesmus quadricauda* at varying Pb and P concentrations on the fourth day.

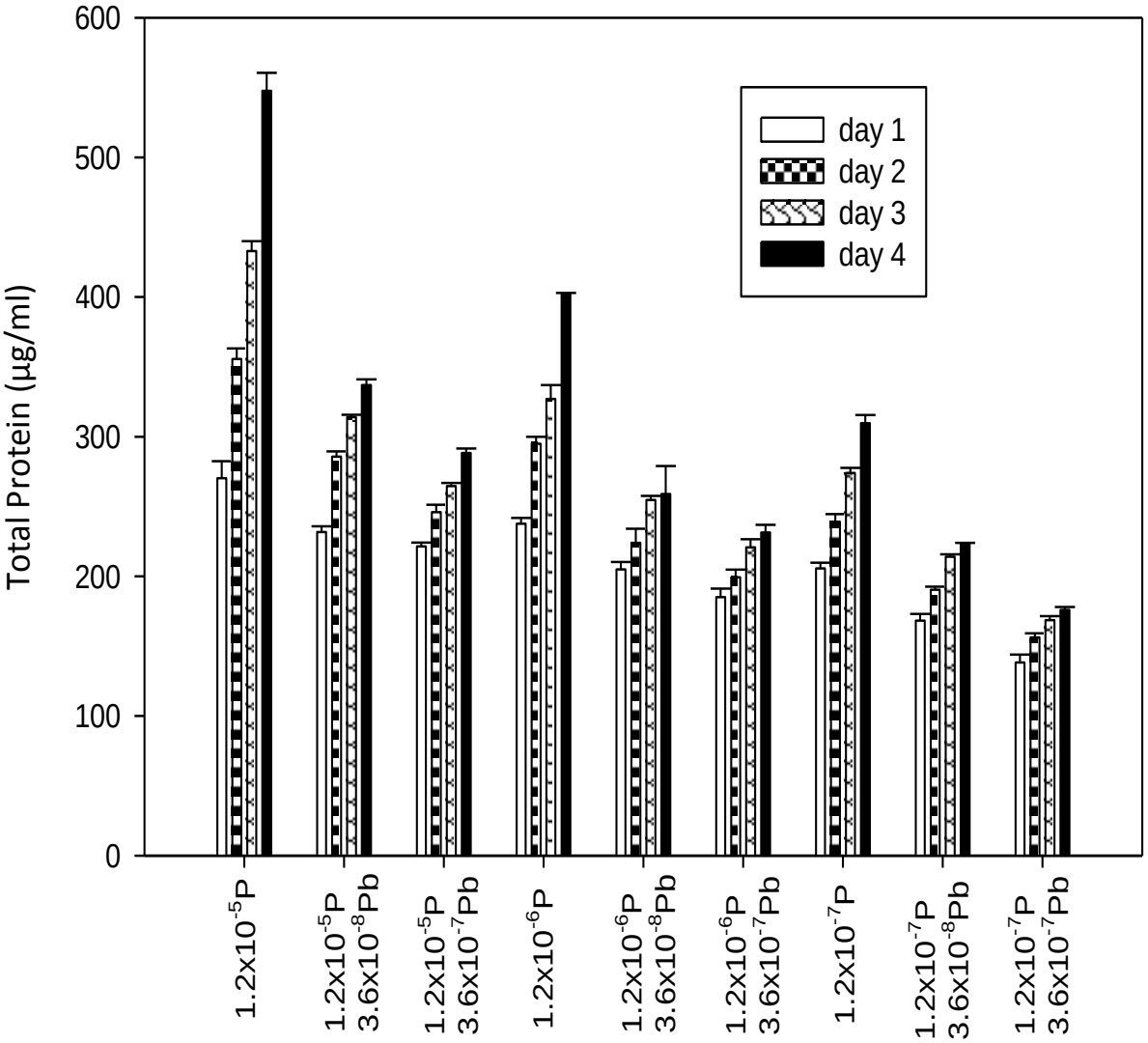


Figure 4.32: Total Protein content of *Scenedesmus quadricauda* at varying Pb and P concentrations.

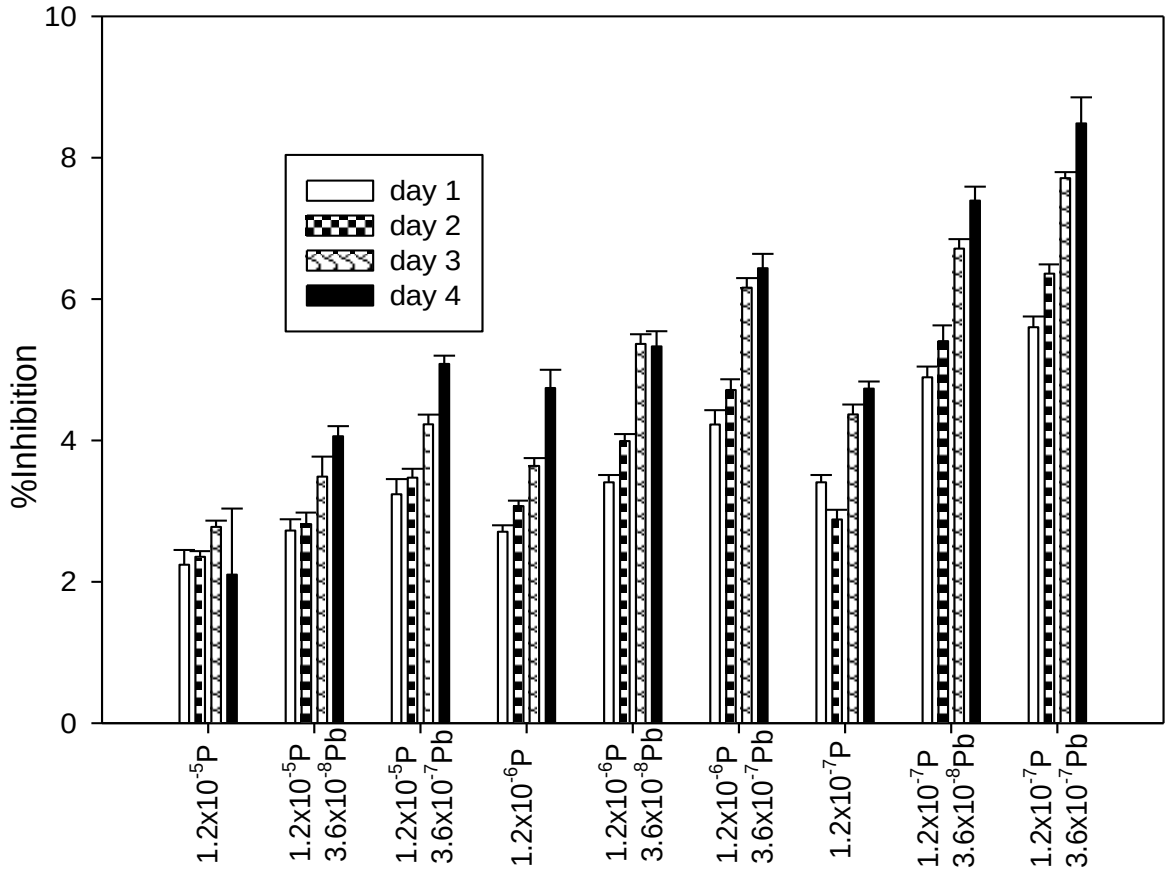


Figure 4.33: Superoxide dismutase (SOD) activity of *Scenedesmus quadricauda* at varying Pb and P concentrations**.**

Catalase activity increased at replete P concentration without Pb and further increase with the addition of Pb concentration compared to the control with and without Pb concentrations (Fig.4.34).Limited P concentration stimulated Peroxidase activity and the activity was futher increased with Pb addition. At limited P concentration with no Pb there was an increase in Peroxidase activity compared to the control without Pb (Fig.4.35).The content of GST increased at highest Pb concentration and lowest P concentration without Pb compared to the controls (Fig.4.36).The ANOVA table shows that chlorophyll *a* ,dry weight, cells counts and total protein content decreases with limited P concentration with Pb (Table 4.6). Contrary to the results obtained for biomass production, the antioxidant enzymes SACAT, SAPOD,SASOD and SAGST activity all increased under P replete condition and Pb stress (Tables 4.6 ANOVA $P<0.05$). Principal component analysis (PCA) showed that the first two components were responsible for 78% of total variation (Fig.4.36). Two celled coenobium, three celled coenobium, four celled coenobium and biomass production presented significant positive correlation with P whereas single cells present a negative correlation with P limitation. Phosphate limitation shows a negative correlation with CAT, POD, GST and SOD. Pb shows a positive correlation with total protein content and antioxidant enzymes.

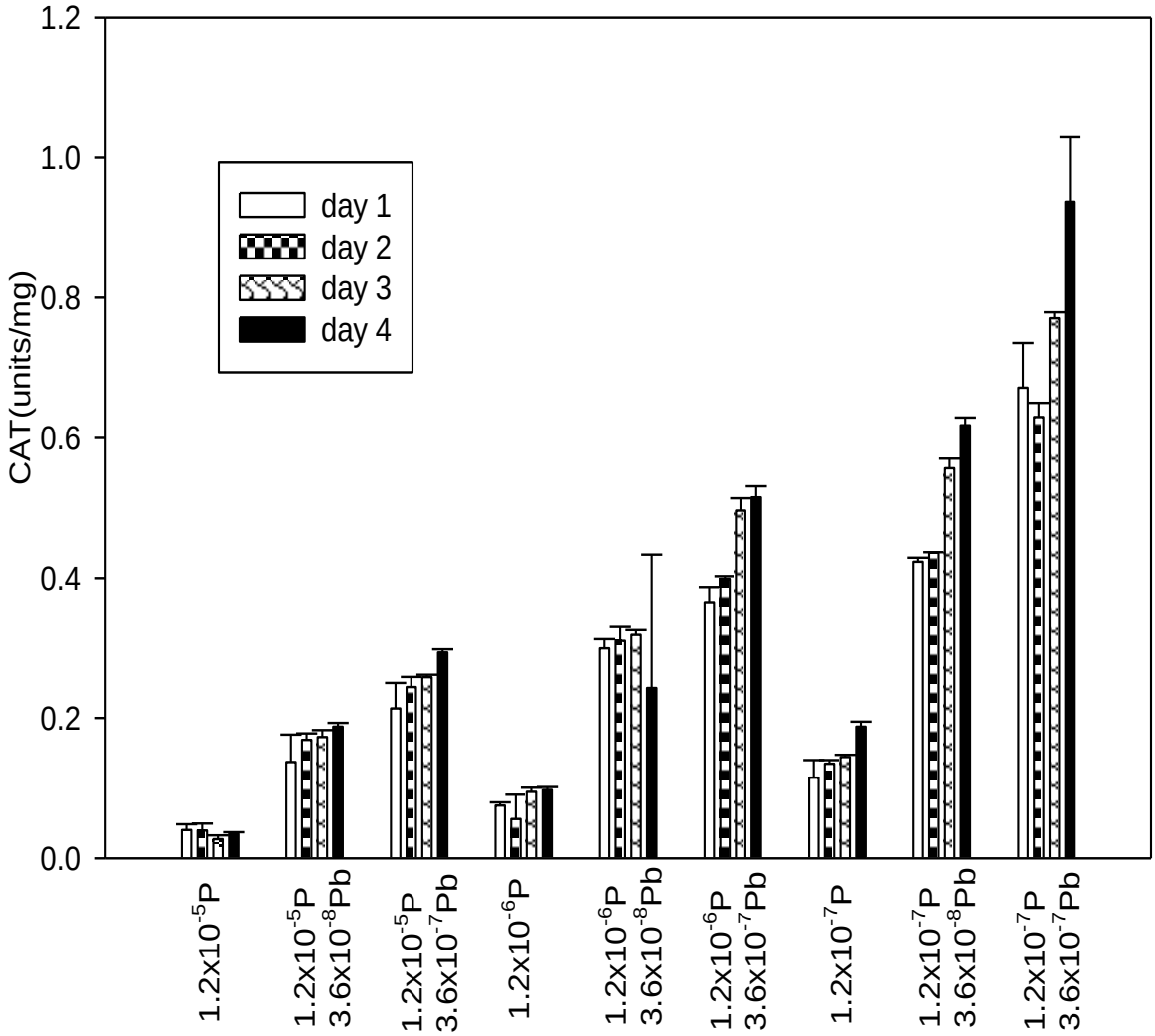


Figure 4.34: Catalase activity (CAT) activity of *Scenedesmus quadricauda* at varying Pb and P concentrations**.**

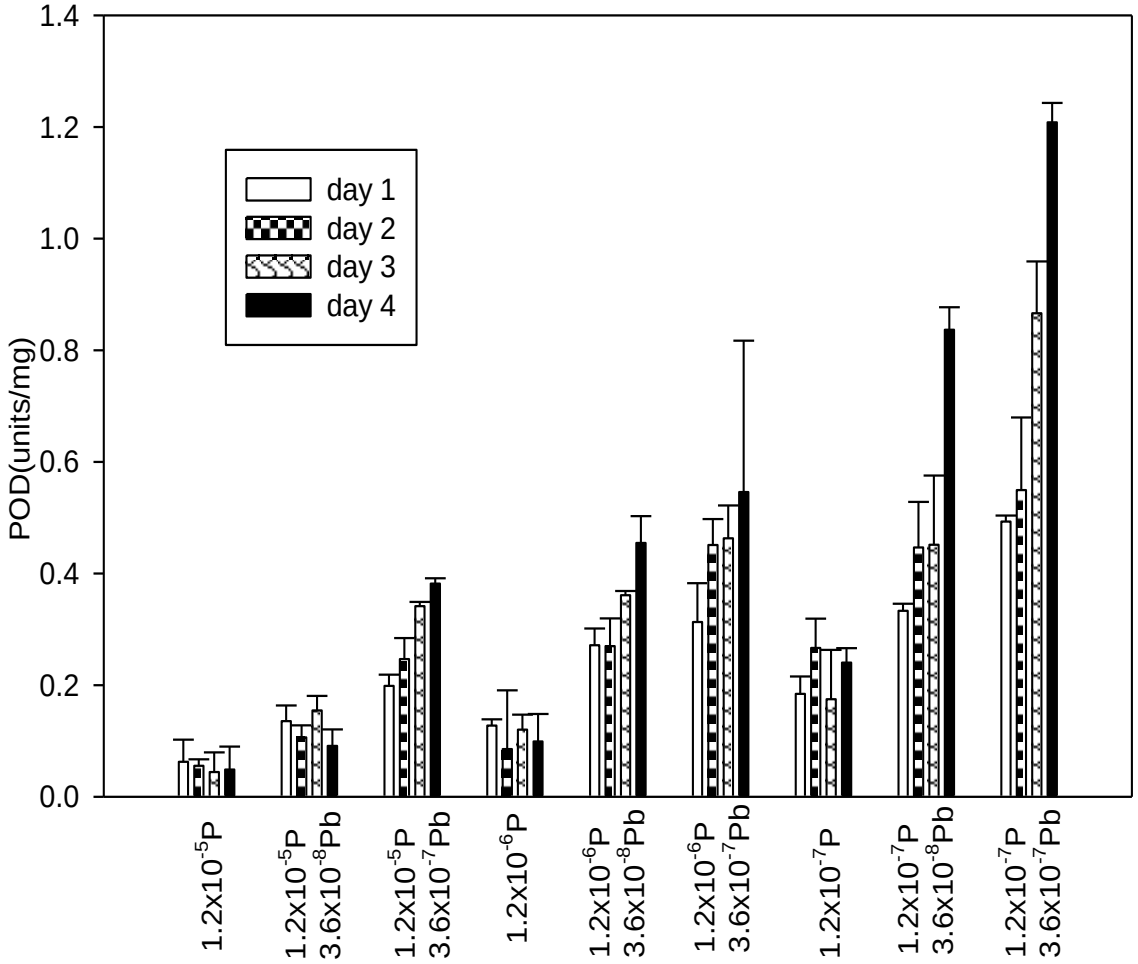


Figure 4.35: Peroxidase activity (POD) activity of *Scenedesmus quadricauda* at varying Pb and P concentrations**.**

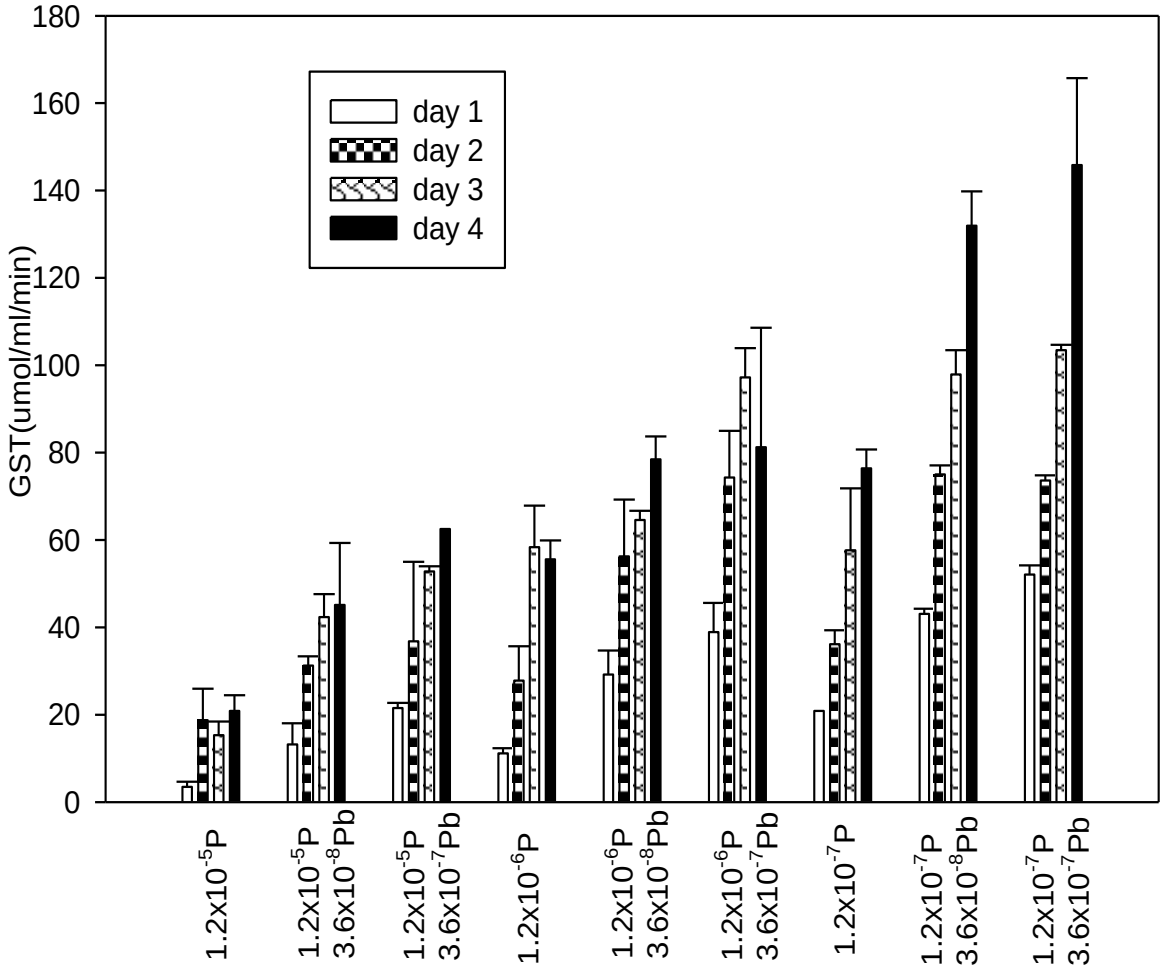


Figure 4.35: Glutathione S-transferase activity (GST) of*Scenedesmus quadricauda* at differentPb and P concentrations**.**

Table 4.4: Analysis of variance results for the effect of Phosphate (P) and Lead (Pb) on biomass production andantioxidant enzymes response of *Scenedesmus quaridicauda*.

Parameters	P	Pb	P/Pb
Chl *a*	40.546**(0)**	38.99**(0)**	2.882**(0.026)**
Dry weights	53.018**(0)**	53.097**(0)**	0.43(0.787)
Cell counts	27.6429**(0)**	27.1303**(0)**	1.1709(0.328)
1 celled coenobium	1.9539(0.147)	11.0308**(0.000)**	2.1984(0.075)
2 celled coenobium	22.4384**(0)**	4.449**(0.014)**	2.5605**(0.043)**
3 celled coenobium	24.0258**(0)**	44.3196**(0)**	2.9955**(0.022)**
4 celled coenobium	34.8152**(0)**	63.2335**(0)**	10.4248**(0.0)**
Protein	47.543**(0)**	55.293**(0)**	1.196(0.317)
SAGST	32.6191**(0)**	21.6866**(0)**	0.8172(0.517)
SACAT	204.493**(0)**	315.982**(0)**	26.086**(0)**
SAPOD	54.3928**(0)**	63.6612**(0)**	4.7291**(0.002)**
SASOD	71.958**(0)**	60.562**(0)**	3.461**(0.011)**

Values represent F values and those in parentheses represent P values. Note: values with $P<0.05$ are statistically significant and marked in bold. Abbreviations: SAGST=specific activity of Glutathione –S transferase; SACAT=specific activity of Catalase; SAPOD=specific activity of peroxidase, SASOD=specific activity superoxide dismutase.

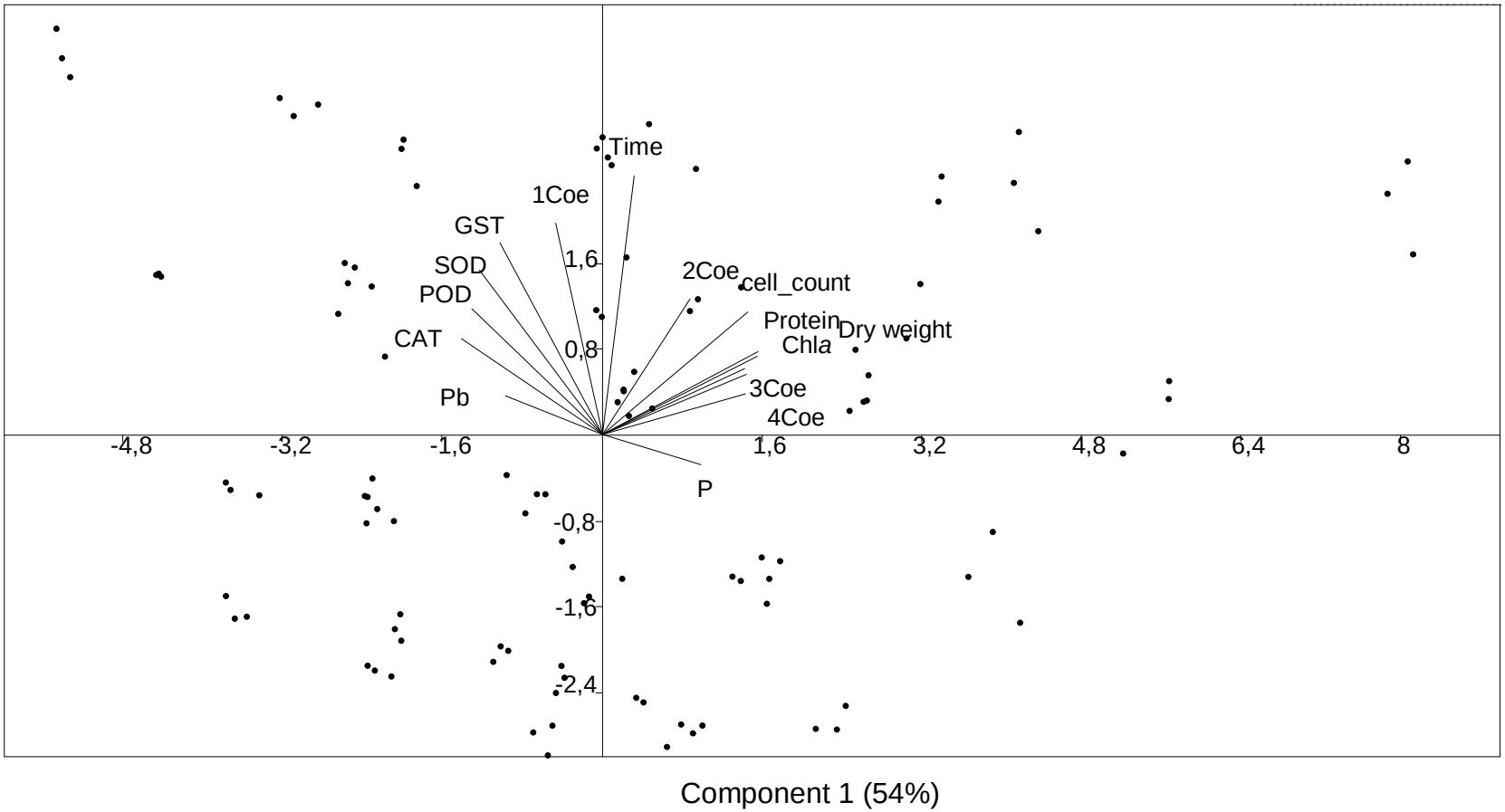


Figure 4.36: Principle component analysis (PCA chart) of Pb and P experiment

CHAPTER FIVE

5.0DISCUSSION

Growth and Biomass Production

It is well known that algal cells exposed to heavy metals may suffer serious physiologicalalterations (Trzcińska and Pawlik-Skowrońska 2013; Chia *et al.,* 2013). This agrees with the findings in thisinvestigation, where the growth and biomass production (cell number, dry weight production andchlorophyll *a*) of *S. quadricauda* decreased with exposure to ambient background concentrations of Pb. The decrease in chlorophyll *a*concentration observed in the present studywould have led to a decrease in photosynthesis and impairment of CO_2 assimilation. This in turn may have affected the growth rate and ability of themicroalga to increase its biomass over time. Furthermore, other studies have reported that soluble (free ion and labile) forms of metals like Pb can be implicated for the reduction in growth rates of microalgae including members of genus *Scenedesmus*(Mohammed and Markert 2006; Qian *et al.*, 2009; Bajguz 2011; Monterio *et al.*,2011). The positivecorrelation of growth rates and biomass with the concentrations of N and P without Pb addition supports the importance of these nutrients(P and N) for cell replication. These results arein accordance with other literature data on algal response to nutrient and metal stress (Hu and Zhou, 2010; Bhola*etal.,* 2011; Trzcińska and Pawlik-Skowrońska 2013; Chia *et al.,* 2013) that also reported decreased freshwater algaegrowth and biomass with decrease in N. the minimum requirement concentrations of N and P significantly reduced theinhibitory effect of Pb, which implies that the microalga is able to tolerate Pb when grownunder replete or high nutrient concentrations. Serra *et al.,*(2010)

showed that phytoplanktonspecies tend to be more sensitive to metal pollution when grown under limited nutrient (P)conditions. This means that under eutrophic conditions, the microalgae best adapted to these conditions will have better chances of tolerating heavy metal pollution.Other studies have also reported that soluble (free ion and labile) forms of metals canbe implicated for the reduction in growth rates of *Scenedesmus obliquus* (Yan and Pan, 2002; Costa and França,2003; Monteiro *et al.,* 2011). According to them,this can be attributed to the inhibition of normal cell division because of metal binding tosulfhydryl groups that are important in regulating such a metabolic process. Therefore, a directlink to basic physiological processes like cell division, membrane assembling, photosynthesisand respiration, which are necessary for the maintenance of the life of the cell, can beestablished. As more than 95% of Pb remained in solution as free and labile Pb, this would haveenhanced its effect on the physiology of *S. quadricauda.*

Phenotypic plasticity

Most ecotoxicological studies use biomass production and growth as the common endpoints.

However, morphological variations provide an alternative means of evaluating the effect of toxiccompounds to microalgae (Lombardi *et al.*, 2007). Coenobial structure was readily affected at N and P limitations/ Pb stress and exposure time. These results indicate that coenobium structure is a verysensitive parameter that promptly responds to nutrient and heavy metals variations in culturemedium. Thus, phenotypic plasticity in *S. quadricauda* represents the effects of Pb on its physiology, thereby serving as an important biomarker in monitoring and ecotoxicology-related investigations. A decrease in the number of cells per coenobium with increase in Cuconcentrations has also been shown by Peña-Castro *et*

al.,(2004) and Lombardi *et al.*, (2007) in *S.incrassatulus* and *S. acuminatus,* respectively and heavy-metal-contaminated textile effluents (Chia and Rilwan, 2014). Contrary to the present study, Mohammed and Markert(2006) observed that at 15 mg L^{-1} (6.3×10^{-5}) Pb concentrations, no morphological changes were observed in thismicroalga. The difference may have resulted from the combination of N, P limitation and Pb stress investigated in this study as well as the strain of *S.quadricauda*used.

Protein and antioxidant enzyme activities

The total protein content which is an important indicator of reversible and irreversible changes in metabolism is known to respond to a wide variety of stressors (Singh and Tewari 2003). Our present study shows that there was decrease in total protein content with respect to N and P limitation, which was further significantly decreased with the addition of Pb compared to control without Pb. Furthermore, the treatment with the highest N and P concentrations supported higher protein concentrations. This was similar to the findings of Chia *et al. (*2013*)* who found a decrease in protein content with N/P limitation and Cd addition in *C vulgaris*cultures.

The significantly strong positive correlation of protein productionwith N and P concentration can be explained by the fundamentalrole N plays in protein metabolism, as it is a keyingredient for protein synthesis (Illman *et al.*, 2000). Proteinsynthesis was negatively affected by the presence of Pb in themedium having more than 50 % total protein concentrationreduction under some exposure conditions. It is possible thatchlorophylls and proteins and even chloroplast proteins representedan emergency source of nitrogen and sulfur to ensurecell growth under N, P limitation and Pb exposure (Carfagna*et al.*, 2013). This reduction in protein content can also be

related to the reduction in chlorophyll content observed under the different growth conditions investigated in this study,which may have resulted in the shortage of carbon skeletonneeded for protein synthesis due to low photosynthetic rate(Carfagna*et al.*,2013). Several studies have demonstrated that oxygen radicals are generated during algalmetabolism, especially in algae exposed to pollutant metals (Pinto *et al.,* 2003; Zhang *et al.*,2013). When this occurs, an important palliative measure is the induction of antioxidants toscavenge the free radicals for maintenance of normal growth. This supports the transient upregulationof antioxidant enzymes families like SOD, POD, CAT and GST after the exposure of*S. quadricauda* to Pb/N and Pb/P stress in this study. The increase in the production of these enzymes bythe microalga means that they act together to reduce the impact of Pb toxicity. A number of studies further support our findings that when faced with environmental stresses, these enzymesare accumulated in various microalgae (Wang *et al.*, 2004; Jamers*et al.*, 2006; Knauert and Knauer, 2008; Szivak *etal.*, 2009). For example, the exposure of *S. vacuolatus* to Curesulted in the increased production and activities of CAT and POD (Sabatini *et al.*, 2009).

Catalase and Perioxidase are two major systems for the enzymatic removal of hydrogen peroxide and superoxideradicals in plant cells (Torres *et al.*, 2008). Their increase activity provides a potent means of controlling andreducing peroxidative damage of cells exposed to Pb and other heavy metal stresses. Thus, it canbe observed from this study that the interactive effects of Pb/N conditions were mostly additiveresulting in higher enzyme activities with increasing severity of the treatment conditions, *i.e*.high Pb/low N and P compared to only N and P treatments without Pb. However, our results show that the measurement of CAT and POD activity at the end of experimental periods may not always

reflect the changes in the enzyme activity as this will mask some effects of the treatments on the activity of the enzymes. The reduction in CAT and POD activities may have been as a result of their inactivation by Pb due to its binding with their essential thiol groups or replacing their catalytic metal ions (Pinto *et al.,* 2003; Szivak*et al.*, 2009;Yadav, 2010;Zhang *et al.*,2013). The mechanism of defense of this microalga to Pb toxicity can be directly associated with the activities of these enzymes as they showed a significant positive association with increasing Pb concentrations.

The increased activity of SOD is due to the pivotal role it plays as the first line of defense against ROS, dismutating superoxide to H_2O_2 and O_2. Similar increase in SOD activity has been reported in *Nannochloropsis oculata* (Lee and Shin, 2003) and *Gracilaria tenuistipitata* (Collen *et al.*, 2003). SOD and GST activities increased throughout the experimental period, demonstrating that both enzymes may have played a more active role in the regulation of oxidative stress in*S. quadricauda.* The increased SOD activityrecorded in this study suggests that superoxide (O_2^-) levelswere increased under Pb exposure and N, P limitation.Superoxide reacts with sensitive and critical cellular moleculartargets, for example, the reaction with NO radical and transformationinto toxic peroxynitrite (Miller, 2012). The magnitude of the Pb stress was aggravated by N and P limitationwhich in turn determined the level of oxidative stress asobserved by the activity of SOD. Although not determined in the present study, the compartmentalization of differentforms of SOD throughout microalgal cells makes them counteractheavy metal pollution effectively (Miller, 2012).Therefore, SOD outcompetes the damaging reactions of thesuperoxide and, in the process, protects the cell from superoxidetoxicity.

The increased GST activity observed in*S. quadricauda* can be tied to its determinant function in thecellular detoxification processes. With increasing concentrationof Pb in the experiments, the increased GST may implythat the enzyme may have been involved in the catalysis of theconjugation of glutathione (GSH) via a sulfhydryl group to themetal to detoxify it or reduce its toxicity *to S. quadricauda*.This detoxification process using GSH is achieved in twoways: first is to bind both the substrate at the enzyme'shydrophobic H-site and GSH at the adjacent, hydrophilic G-site,which together form the active site of the enzyme; andsubsequently, to activate the thiol group of the GSH, enablingthe nucleophilic to attach upon the substrate (Salinas andWong 1999). By this action, GST would prevent thismicroalga or any other plant under similar conditions fromoxidative injury by using GSH to reduce organic hydroperoxides(Vestena *et al.,* 2011), which are the oxidative degradationbyproducts of membrane lipids (Chia *et al.,* 2013) and/ornucleic acids. Thus, the upregulation of GST activity wouldhave helped in combating the xenobiotic agent, in this case Pb,and its toxicity associated endobiotic compounds (ROS).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 **Conclusion**

In conclusion, low concentrations of N, P and the presence Pb at low concentrations could inhibit the growth of *S. quadricauda.* High concentrations of Pb^{2+} led to ROS production and oxidative stress in *S. quadricauda which* was evidenced by changes in the activities of SOD, CAT, POD GST. In addition, exposure to high concentrations of Pb^{2+} resulted in decrease of chlorophyll *a* content, growth rates, dry weight, cell counts, total protein content and changes in the phenotypic plasticity. Nitrates in the range from 10^{-5} to 10^{-6}M and P ranging from 10^{-6} to 10^{-7}M affects the physiology and biochemical composition of *S. qauadriacauda,* this also shows an additive interactive damaging effect on the microalga when combined with Pb exposure even at low concentrations(10^{-8}M).

Increasing levels of heavy metals in the environment may have varying physiological and biochemical effects depending on the concentration of important nutrients like phosphates and nitrates

6.2 Recommendation

Microalgae are some of the most important organisms in our ecosystem because they are the main primary producers at the base of aquatic food webs. Microalgae are characterized by rapid growth rates and ubiquitous distribution throughout natural environments, yet they show a critical sensitivity to environmental variations (e. g. nutrient levels and presence of pollutants); this is why the use of microalgae as test organisms is gaining

importance. Furthermore, microalgal toxicity tests are quick and inexpensive, and can be effectively used to assess those toxic substances that are found in concentrationstoo low for conclusive detection via highertrophic organisms. Finally,there is a likely application of microalgae in determiningtoxicity of metal ions in situ because the microalgalspecies used already exist in a contaminated environment,so any change in the levels of the toxic metals will bedirectly reflected upon in the size of its population.It is obvious that microalgae form the basis of food chain in an aquatic ecosystem and any alteration in the physiology of microalgae will direct or indirectly affect other aquatic life that depends on it for survival. So the indiscriminate pollution of the aquatic system with substances that are harmful to system should be stopped. Government and authorized regulatory bodies should put down strict rules and regulations that will check industries and individuals waste disposal.

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Raw data for phosphate experiments

Time (days)	P conc.	Pb conc	cell count	Chl a(ug mL-1)	Dry wght/ml	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SSOD	SAGST	SACAT	SAPOD
1	P1	NPb	50	0.4227	3.60E-04	10	4	4	5	258	2.241195	4.166667	0.037145	0.046981
1	P1	NPb	56	0.469824	3.40E-04	9	4	5	6	282	2.449414	4.166667	0.033983	0.107458
1	P1	NPb	58	0.377016	3.70E-04	9	5	5	6	271	2.032086	2.083333	0.049508	0.033546
1	P1	Pb1	31	0.408552	3.00E-04	10	2	3	2	235	2.760085	18.75	0.154965	0.103159
1	P1	Pb1	32	0.362508	3.10E-04	10	4	2	2	227	2.863203	10.41667	0.092878	0.153518
1	P1	Pb1	30	0.352956	3.20E-04	12	4	2	1	233	2.553191	10.41667	0.164521	0.149564
1	P1	Pb2	28	0.24852	2.40E-04	14	4	2	0	223	3.068783	22.91667	0.171898	0.203832
1	P1	Pb2	29	0.294204	2.40E-04	15	4	2	0	223	3.171247	20.83333	0.232063	0.176654
1	P1	Pb2	30	0.292404	2.60E-04	14	5	2	0	218	3.477345	20.83333	0.237385	0.215457
1	P2	NPb	32	0.327816	3.10E-04	7	4	2	3	241	2.760085	10.41667	0.071577	0.125739
1	P2	NPb	39	0.306192	2.70E-04	15	5	2	1	239	2.760085	12.5	0.080195	0.13947
1	P2	NPb	34	0.3225	3.00E-04	14	8	0	1	233	2.604997	10.41667	0.074034	0.11705
1	P2	Pb1	24	0.317904	2.10E-04	18	3	0	0	211	3.407881	31.25	0.290679	0.251328
1	P2	Pb1	24	0.32358	2.20E-04	20	2	0	0	203	3.510638	22.91667	0.292693	0.306016
1	P2	Pb1	20	0.32466	2.10E-04	18	1	0	0	201	3.304904	33.33333	0.314677	0.256294
1	P2	Pb2	19	0.24744	1.70E-04	15	4	0	0	180	4.223865	41.66667	0.340741	0.319865
1	P2	Pb2	20	0.263388	1.70E-04	13	2	1	0	192	4.425711	31.25	0.37934	0.378788
1	P2	Pb2	22	0.27438	1.90E-04	20	1	0	0	183	4.021164	43.75	0.377049	0.240106

Time (days)	P conc.	Pb conc	cell count	Chl a(ug mL-1)	Dry wght/ml	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SSOD	SAGST	SACAT	SAPOD
1	P3	NPb	22	0.328536	1.90E-04	11	2	1	1	202	3.407881	20.83333	0.142327	0.20252
1	P3	NPb	22	0.253476	1.80E-04	9	3	1	1	205	3.304904	20.83333	0.093496	0.14782
1	P3	NPb	24	0.238968	1.80E-04	11	3	1	1	210	3.510638	20.83333	0.109524	0.20202
1	P3	Pb1	12	0.24852	1.40E-04	10	1	0	0	170	4.726891	41.66667	0.422794	0.347594
1	P3	Pb1	12	0.229056	1.20E-04	12	0	0	0	172	4.926625	43.75	0.417878	0.325934
1	P3	Pb1	14	0.22374	1.00E-04	12	1	0	0	163	5.026178	43.75	0.429192	0.325339
1	P3	Pb2	12	0.178056	9.00E-05	12	0	0	0	144	5.4716	52.08333	0.598958	0.484007
1	P3	Pb2	10	0.204276	7.00E-05	10	0	0	0	138	5.570016	50	0.694444	0.505051
1	P3	Pb2	12	0.203556	8.00E-05	12	0	0	0	133	5.766234	54.16667	0.720551	0.489861
2	P1	NPb	87	0.3699	4.90E-04	8	7	9	12	347	2.370917	22.91667	0.035903	0.043664
2	P1	NPb	88	0.487848	5.10E-04	0	1	10	14	359	2.267932	22.91667	0.05072	0.067528
2	P1	NPb	88	0.457392	4.90E-04	15	8	9	10	361	2.422328	10.41667	0.031856	0.054562
2	P1	Pb1	56	0.324936	3.50E-04	12	5	6	4	290	2.62743	31.25	0.16523	0.104493
2	P1	Pb1	55	0.326016	3.80E-04	18	4	3	5	284	2.8826	29.16667	0.161972	0.085361
2	P1	Pb1	54	0.366744	3.80E-04	16	5	1	5	283	2.933473	33.33333	0.179476	0.128493
2	P1	Pb2	44	0.227256	2.60E-04	21	7	2	0	252	3.33855	16.66667	0.228175	0.2405
2	P1	Pb2	48	0.32466	2.80E-04	23	0	2	0	243	3.489583	41.66667	0.256344	0.211997
2	P1	Pb2	44	0.281412	2.90E-04	24	3	0	0	243	3.59001	52.08333	0.248457	0.286819
2	P2	NPb	60	0.37242	4.10E-04	15	6	3	6	300	3.136435	18.75	0.070278	0.040404

Time (days)	P conc.	Pb conc	cell count	Chl a(ug mL-1)	Dry wght/ml	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SSOD	SAGST	SACAT	SAPOD
2	P2	NPb	64	0.39684	3.80E-04	15	8	3	6	290	2.984293	33.33333	0.016523	0.010449
2	P2	NPb	68	0.360708	3.90E-04	12	6	4	8	295	3.085774	31.25	0.081215	0.205444
2	P2	Pb1	40	0.337008	2.80E-04	20	6	0	2	226	3.890041	66.66667	0.296829	0.268168
2	P2	Pb1	42	0.34764	2.40E-04	24	8	0	0	233	4.089027	41.66667	0.30025	0.221095
2	P2	Pb1	40	0.343044	2.60E-04	24	6	0	0	213	3.989637	60.41667	0.332942	0.320102
2	P2	Pb2	20	0.283212	2.10E-04	14	3	0	0	193	4.876797	83.33333	0.397237	0.455331
2	P2	Pb2	22	0.313668	2.00E-04	22	0	0	0	203	4.582904	77.08333	0.396552	0.403045
2	P2	Pb2	20	0.303396	2.20E-04	12	4	0	0	202	4.68107	62.5	0.403259	0.49505
2	P3	NPb	30	0.352236	2.50E-04	17	3	1	1	233	2.984293	35.41667	0.13573	0.260112
2	P3	NPb	38	0.287088	2.70E-04	16	4	2	2	240	2.933473	33.33333	0.139757	0.32197
2	P3	NPb	42	0.332412	2.80E-04	19	3	3	2	244	2.729659	39.58333	0.129611	0.217337
2	P3	Pb1	18	0.293484	1.60E-04	18	0	0	0	193	5.652911	75	0.43696	0.353274
2	P3	Pb1	16	0.278256	1.60E-04	16	0	0	0	189	5.224304	72.91667	0.436067	0.481
2	P3	Pb1	18	0.312948	1.50E-04	18	0	0	0	189	5.331821	77.08333	0.436067	0.505051
2	P3	Pb2	16	0.2478	9.00E-05	16	0	0	0	158	6.235955	72.91667	0.60654	0.412351
2	P3	Pb2	14	0.232572	9.00E-05	14	0	0	0	153	6.34119	72.91667	0.645153	0.564468
2	P3	Pb2	12	0.253476	9.00E-05	12	0	0	0	158	6.498599	75	0.636867	0.67127
3	P1	NPb	130	0.434688	6.40E-04	10	4	10	18	440	2.795574	12.5	0.026136	0.030992
3	P1	NPb	140	0.49632	6.40E-04	12	9	10	20	426	2.852154	14.58333	0.022496	0.017783

	Time (days)	P conc.	Pb conc	cell count	Chl a(ug mL-1)	Dry wght/ml	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SSOD	SAGST	SACAT	SAPOD
3	P1	NPb	142	0.475416	6.80E-04	12	10	10	20	433	2.682216	18.75	0.033199	0.083981	
3	P1	Pb1	92	0.353952	4.80E-04	18	11	4	10	313	3.190255	41.66667	0.171459	0.164585	
3	P1	Pb1	96	0.452796	4.70E-04	16	20	8	5	313	3.526012	47.91667	0.183706	0.174267	
3	P1	Pb1	90	0.374856	4.80E-04	16	14	6	7	316	3.748558	37.5	0.163766	0.124664	
3	P1	Pb2	62	0.333492	3.30E-04	20	10	2	4	263	4.245554	52.08333	0.25507	0.345662	
3	P1	Pb2	62	0.327456	3.40E-04	20	10	2	4	264	4.355301	52.08333	0.257734	0.332874	
3	P1	Pb2	60	0.327456	3.40E-04	20	8	2	4	267	4.08046	54.16667	0.262016	0.346158	
3	P2	NPb	94	0.4209	5.20E-04	14	10	5	11	317	3.748558	47.91667	0.09674	0.090813	
3	P2	NPb	95	0.456312	4.90E-04	15	10	5	12	327	3.526012	60.41667	0.08792	0.143638	
3	P2	NPb	99	0.441084	5.10E-04	18	10	3	13	337	3.637413	66.66667	0.09953	0.125888	
3	P2	Pb1	66	0.330612	3.10E-04	20	20	2	0	253	5.217391	62.5	0.325758	0.365313	
3	P2	Pb1	66	0.341244	3.10E-04	20	15	4	1	258	5.491329	66.66667	0.312016	0.352361	
3	P2	Pb1	64	0.367464	3.00E-04	25	21	5	1	253	5.381944	64.58333	0.318182	0.365313	
3	P2	Pb2	38	0.29736	2.50E-04	30	4	0	0	214	6.034483	89.58333	0.492601	0.509771	
3	P2	Pb2	40	0.287448	2.40E-04	30	5	0	0	223	6.303725	100	0.481315	0.482403	
3	P2	Pb2	40	0.292044	2.50E-04	30	5	0	0	225	6.142365	102.0833	0.51537	0.397306	
3	P3	NPb	58	0.418824	3.40E-04	26	4	2	2	271	4.217926	62.5	0.141451	0.072683	
3	P3	NPb	60	0.336648	3.60E-04	26	5	2	2	273	4.385965	41.66667	0.147436	0.2331	
3	P3	NPb	64	0.357192	3.90E-04	26	5	2	3	278	4.497664	68.75	0.144784	0.218007	

Time (days)	P conc.	Pb conc	cell count	Chl a(ug mL-1)	Dry wght/ml	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SSOD	SAGST	SACAT	SAPOD
3	P3	Pb1	30	0.266904	2.30E-04	26	2	0	0	213	6.837607	93.75	0.544405	0.533504
3	P3	Pb1	28	0.283212	2.40E-04	18	5	0	0	216	6.571429	104.1667	0.554591	0.308642
3	P3	Pb1	30	0.333132	2.50E-04	26	5	0	0	213	6.731318	95.83333	0.571401	0.512164
3	P3	Pb2	24	0.237528	9.00E-05	24	0	0	0	172	7.608696	104.1667	0.780039	0.792812
3	P3	Pb2	22	0.253116	1.10E-04	22	0	0	0	167	7.759088	104.1667	0.768962	0.834694
3	P3	Pb2	20	0.268344	1.20E-04	20	0	0	0	167	7.759088	102.0833	0.763224	0.970786
4	P1	NPb	180	0.625536	8.20E-04	15	10	15	25	533	1.023018	16.66667	0.03596	0.022741
4	P1	NPb	175	0.67086	8.00E-04	16	13	13	24	557	2.617011	22.91667	0.03269	0.027202
4	P1	NPb	190	0.640404	8.30E-04	19	10	17	25	553	2.665996	22.91667	0.036392	0.095896
4	P1	Pb1	101	0.475416	5.70E-04	26	10	5	10	333	4.191617	29.16667	0.187062	0.06825
4	P1	Pb1	110	0.4701	5.60E-04	30	15	10	5	337	3.903904	50	0.193373	0.080928
4	P1	Pb1	112	0.485328	5.70E-04	30	16	10	5	341	4.076739	56.25	0.182674	0.124411
4	P1	Pb2	77	0.29556	4.10E-04	39	19	0	0	286	5.04451	62.5	0.294872	0.370841
4	P1	Pb2	72	0.34728	4.10E-04	36	18	0	0	292	4.988124	62.5	0.288813	0.389166
4	P1	Pb2	71	0.37278	4.10E-04	37	17	0	0	287	5.21327	62.5	0.297184	0.385387
4	P2	NPb	130	0.49632	6.50E-04	12	14	10	15	402	4.761905	54.16667	0.095357	0.060305
4	P2	NPb	129	0.535968	6.40E-04	23	10	10	14	402	4.477612	60.41667	0.095357	0.082919
4	P2	NPb	121	0.560748	6.60E-04	22	20	13	5	403	4.988124	52.08333	0.102254	0.154147
4	P2	Pb1	88	0.42198	3.60E-04	30	19	4	2	268	5.230126	72.91667	0.025031	0.457938
4	P2	Pb1	90	0.39648	3.60E-04	30	20	4	2	273	5.575821	83.33333	0.326465	0.40515
4	P2	Pb1	84	0.386928	3.60E-04	34	15	4	3	236	5.180534	79.16667	0.377648	0.50077

Time (days)	P conc.	Pb conc	cell count	Chl a(ug mL-1)	Dry wght/ml	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SSOD	SAGST	SACAT	SAPOD
4	P2	Pb2	56	0.342324	2.90E-04	30	10	2	0	225	6.21118	85.41667	0.532407	0.720539
4	P2	Pb2	54	0.332772	2.90E-04	38	8	0	0	234	6.597938	106.25	0.51193	0.2331
4	P2	Pb2	54	0.332772	2.90E-04	30	12	0	0	235	6.501548	52.08333	0.501596	0.68343
4	P3	NPb	80	0.400716	4.00E-04	20	5	10	5	303	4.731861	72.91667	0.183443	0.265027
4	P3	NPb	80	0.170856	4.80E-04	20	5	10	5	313	4.631579	75	0.195953	0.242037
4	P3	NPb	80	0.175452	4.90E-04	18	8	10	4	313	4.831933	81.25	0.183706	0.212993
4	P3	Pb1	42	0.250596	2.70E-04	36	3	0	0	223	7.172131	137.5	0.605942	0.815328
4	P3	Pb1	40	0.281772	2.50E-04	30	5	0	0	224	7.456588	135.4167	0.62035	0.811688
4	P3	Pb1	42	0.352236	2.50E-04	30	6	0	0	223	7.55102	122.9167	0.627429	0.883272
4	P3	Pb2	30	0.22158	9.00E-05	30	0	0	0	178	8.11359	147.9167	0.861423	1.191692
4	P3	Pb2	32	0.237168	1.20E-04	32	0	0	0	174	8.484848	164.5833	0.908764	1.184256
4	P3	Pb2	30	0.257712	1.30E-04	30	0	0	0	176	8.853119	125	1.040009	1.248278

ANOVA table for phosphate experiments

	Degr. of - Freedom	cell count - SS	cell count - MS	cell count - F	cell count - p	Chl a(ug mL-1) - SS	Chl a(ug mL-1) - MS	Chl a(ug mL-1) - F	Chl a(ug mL-1) - p	Dry wght/ml - SS	Dry wght/ml – MS	Dry wght/ml - F	Dry wght/ml - p
Intercept	1	336005.3	336005	444.79	0	12.65027	12.6503	3445.2	0	0.000011	1.1E-05	1155.18	0
P conc.	2	41764.7	20882	27.643	0	0.29776	0.14888	40.546	0	0.000001	1E-06	53.018	0
Pb conc	2	40990.2	20495	27.13	0	0.28633	0.14317	38.99	0	0.000001	1E-06	53.097	0
P conc.*Pb conc	4	3538.2	884.5	1.1709	0.3283	0.04233	0.01058	2.882	0.0264	0	0	0.43	0.786867
Error	99	74787.7	755.4			0.36352	0.00367			0.000001	0		
Total	107	161080.7				0.98994				0.000003			

	Degr. of - Freedom	1Coe – SS	1Coe - MS	1Coe – F	1Coe - p	2Coe - SS	2Coe - MS	2Coe - F	2Coe - p	3Coe - SS	3Coe - MS	3Coe - F	3Coe - p
Intercept	1	40290.7	40290.7	811.497	0	4319.3	4319.3	207.61	0	972	972	143.982	0
P conc.	2	194.02	97.01	1.9539	0.14715	933.69	466.84	22.438	0	324.389	162.194	24.0258	0
Pb conc	2	1095.35	547.68	11.0308	4.7E-05	185.13	92.565	4.449	0.014	598.389	299.194	44.3196	0
P conc.*Pb conc	4	436.59	109.15	2.1984	0.07463	213.09	53.273	2.5605	0.043	80.889	20.2222	2.9955	0.0222
Error	99	4915.33	49.65			2059.8	20.806			668.333	6.7508		
Total	107	6641.3				3391.7				1672			

	Degr. of - Freedom	4Coe - SS	4Coe - MS	4Coe - F	4Coe - p	Protein (ug/mL) - SS	Protein (ug/mL) - MS	Protein (ug/mL) - F	Protein (ug/mL) - p	SSOD - SS	SSOD - MS	SSOD - F	SSOD – p
Intercept	1	1430.08	1430.1	132	0	7207500	7207500	3073.631	0	2141.5	2142	2857.89	0
P conc.	2	754.389	377.19	34.815	0	222972	111486	47.543	0	107.84	53.92	71.958	0
Pb conc	2	1370.17	685.08	63.234	0	259318	129659	55.293	0	90.763	45.38	60.562	0
P conc.*Pb conc	4	451.778	112.94	10.425	0	11217	2804	1.196	0.31742	10.373	2.593	3.461	0.010864
Error	99	1072.58	10.834			232150	2345			74.185	0.749		
Total	107	3648.92				725658				283.16			

	Degr. of – Freedom	SAGST - SS	SAGST - MS	SAGST - F	SAGST – p	SACAT - SS	SACAT - MS	SACAT - F	SACAT - p	SAPOD - SS	SAPOD - MS	SAPOD – F	SAPOD - p
Intercept	1	317100.7	317100.7	544.3178	0	9.568111	9.568111	2121.411	0	10.97754	10.9775	539.58	0
P conc.	2	38005.5	19002.7	32.6191	0	1.844633	0.922316	204.493	0	2.21321	1.1066	54.393	0
Pb conc	2	25267.7	12633.9	21.6866	0	2.850321	1.425161	315.982	0	2.59033	1.29516	63.661	0
P conc.*Pb conc	4	1904.3	476.1	0.8172	0.51716	0.470615	0.117654	26.086	0	0.38412	0.09603	4.7201	0.001579
Error	99	57674	582.6			0.446516	0.00451			2.01412	0.02034		
Total	107	122851.5				5.612084				7.20177			

Time (days)	N conc.	Pb conc	Chl a	dry weight	cell counts	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SOD	SAGST	SACAT	SAPOD
1	1.80E-04	0	0.428376	7.20E-03	30	5	4	2	3	184.0625	4.233577	6.25	0.520656	0.065854
1	1.80E-04	0	0.439008	7.20E-03	26	9	3	1	3	182.8125	4.512373	8.333333	0.503248	0.091168
1	1.80E-04	0	0.428376	8.40E-03	28	5	2	1	3	184.0625	4.442826	8.333333	0.489417	0.115244
1	1.80E-04	3.80E-08	0.388728	7.00E-03	31	10	2	2	2	181.5625	3.882784	10.41667	0.45393	0.100141
1	1.80E-04	3.80E-08	0.367464	7.20E-03	32	3	2	2	4	184.0625	3.741746	10.41667	0.463384	0.131708
1	1.80E-04	3.80E-08	0.285288	7.60E-03	30	7	1	2	3	184.0625	4.163623	8.333333	0.479004	0.057622
1	1.80E-04	3.80E-07	0.352236	7.00E-03	29	8	0	1	3	181.5625	4.233577	12.5	0.490878	0.116831
1	1.80E-04	3.80E-07	0.34692	7.60E-03	28	6	3	0	4	182.8125	4.512373	12.5	0.477037	0.116032
1	1.80E-04	3.80E-07	0.342324	7.60E-03	34	6	0	4	4	182.8125	4.581818	12.5	0.471795	0.074592
1	1.80E-05	0	0.338448	6.40E-03	20	5	1	2	1	115.3125	6.911142	18.75	0.681481	0.420465
1	1.80E-05	0	0.338448	6.40E-03	19	2	0	3	2	116.5625	7.43338	18.75	0.70706	0.441953
1	1.80E-05	0	0.338088	5.80E-03	22	10	2	0	2	115.3125	7.692308	22.91667	0.739657	0.394186
1	1.80E-05	3.80E-08	0.276096	6.00E-03	24	12	2	0	2	115.3125	6.647808	16.66667	0.66486	0.420465
1	1.80E-05	3.80E-08	0.338448	6.40E-03	24	10	5	0	1	115.3125	7.303371	8.333333	0.714724	0.433604
1	1.80E-05	3.80E-08	0.328176	6.40E-03	20	9	1	2	0	114.0625	7.756813	12.5	0.705753	0.199253
1	1.80E-05	3.80E-07	0.281412	6.00E-03	19	10	2	0	1	116.5625	6.382979	18.75	0.731725	0.571939
1	1.80E-05	3.80E-07	0.353676	6.00E-03	20	12	4	0	0	114.0625	6.911142	25	0.714155	0.464923
1	1.80E-05	3.80E-07	0.256272	6.40E-03	22	10	6	0	0	115.3125	7.042254	16.66667	0.731346	0.433604
1	1.80E-06	0	0.293844	4.80E-03	10	4	1	0	1	50.3125	9.52381	31.25	1.257143	0.180689
1	1.80E-06	0	0.212028	5.20E-03	12	5	0	1	1	52.8125	9.830508	31.25	1.088757	0.258203
1	1.80E-06	0	0.288528	4.80E-03	10	5	1	1	0	49.0625	9.708079	27.08333	1.347771	0.432349

Time (days)	N conc.	Pb conc	Chl a	dry weight	cell counts	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SOD	SAGST	SACAT	SAPOD
1	1.80E-06	3.80E-08	0.304116	4.40E-03	12	5	0	1	1	52.8125	9.090909	29.16667	1.306509	0.344271
1	1.80E-06	3.80E-08	0.319344	4.60E-03	12	6	2	0	1	51.5625	9.646739	27.08333	1.375354	0.411387
1	1.80E-06	3.80E-08	0.167424	4.40E-03	14	7	0	1	1	51.5625	10.13514	29.16667	1.133737	0.088154
1	1.80E-06	3.80E-07	0.2988	4.40E-03	12	4	0	0	2	47.8125	9.400545	31.25	1.423094	0.126758
1	1.80E-06	3.80E-07	0.263388	4.60E-03	10	3	0	1	1	46.5625	9.462219	33.33333	1.358389	0.748424
1	1.80E-06	3.80E-07	0.134448	4.40E-03	10	5	1	1	0	50.3125	9.708079	35.41667	1.2	0.542067
2	1.80E-04	0	0.47334	1.08E-02	70	8	5	4	10	239.0625	6.816585	16.66667	1.010196	0.348584
2	1.80E-04	0	0.458112	1.12E-02	74	10	2	4	12	242.8125	7.142857	18.75	0.943286	0.05616
2	1.80E-04	0	0.442524	1.12E-02	74	6	1	6	12	244.0625	7.272727	14.58333	0.922749	0.055872
2	1.80E-04	3.80E-08	0.388008	8.60E-03	56	12	5	4	4	199.0625	8.551724	27.08333	1.063946	0.228343
2	1.80E-04	3.80E-08	0.39792	9.00E-03	50	17	2	4	5	196.5625	8.171745	29.16667	1.048225	0.123332
2	1.80E-04	3.80E-08	0.330972	8.60E-03	54	16	5	6	5	201.5625	8.425414	25	1.003204	0.12779
2	1.80E-04	3.80E-07	0.37278	7.60E-03	40	2	5	2	1	180.3125	9.979633	37.5	1.089544	0.218476
2	1.80E-04	3.80E-07	0.388008	7.80E-03	42	23	6	1	1	177.8125	10.22343	27.08333	1.088694	0.298237
2	1.80E-04	3.80E-07	0.357192	7.60E-03	44	20	7	2	1	177.8125	10.10169	41.66667	1.050967	0.3238
2	1.80E-05	0	0.368184	8.80E-03	60	15	6	3	6	177.8125	7.592191	31.25	1.088694	0.059647
2	1.80E-05	0	0.378096	9.20E-03	54	18	2	3	5	179.0625	7.725632	27.08333	1.086446	0.101539
2	1.80E-05	0	0.377736	8.80E-03	58	20	3	4	5	176.5625	8.123652	31.25	1.112684	0.077233
2	1.80E-05	3.80E-08	0.281052	7.00E-03	30	23	2	1	0	132.8125	10.18974	60.41667	1.313255	0.536185

Time (days)	N conc.	Pb conc	Chl a	dry weight	cell counts	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SOD	SAGST	SACAT	SAPOD
2	1.80E-05	3.80E-08	0.353316	7.40E-03	32	20	3	2	0	131.5625	9.681979	54.16667	1.267458	0.426114
2	1.80E-05	3.80E-08	0.37386	7.60E-03	30	20	3	0	0	134.0625	10	54.16667	1.229526	0.42947
2	1.80E-05	3.80E-07	0.276096	6.60E-03	20	12	4	0	0	107.8125	12.22527	35.41667	1.404444	0.337286
2	1.80E-05	3.80E-07	0.328176	6.40E-03	22	15	2	1	0	109.0625	11.98347	50	1.344413	0.208388
2	1.80E-05	3.80E-07	0.317904	6.40E-03	20	10	5	0	0	87.8125	12.40576	50	1.691578	0.776448
2	1.80E-06	0	0.260868	6.80E-03	30	9	2	3	2	89.0625	9.89011	39.58333	1.700117	0.238171
2	1.80E-06	0	0.363948	6.80E-03	38	13	4	2	2	90.3125	10.01372	39.58333	1.644752	0.134214
2	1.80E-06	0	0.348	7.20E-03	42	15	5	3	2	84.0625	9.641873	58.33333	0.889219	0.108145
2	1.80E-06	3.80E-08	0.34872	5.60E-03	18	14	2	0	0	65.3125	11.35135	54.16667	2.054226	0.533565
2	1.80E-06	3.80E-08	0.368184	5.20E-03	16	12	2	0	0	60.3125	11.5903	50	2.208636	0.854137
2	1.80E-06	3.80E-08	0.19644	5.20E-03	18	15	0	1	0	64.0625	11.23139	52.08333	2.06439	0.851441
2	1.80E-06	3.80E-07	0.328176	4.60E-03	16	14	1	0	0	54.0625	12.47498	62.5	2.268979	2.606411
2	1.80E-06	3.80E-07	0.32394	4.00E-03	14	10	2	0	0	53.4375	12.6498	58.33333	2.187914	1.701223
2	1.80E-06	3.80E-07	0.1185	4.00E-03	12	8	2	0	0	54.0625	12.59161	58.33333	2.109441	1.345244
3	1.80E-04	0	0.557232	1.52E-02	128	12	6	12	17	326.5625	9.478022	22.91667	0.390303	0.046397
3	1.80E-04	0	0.567144	1.54E-02	132	8	8	12	18	329.0625	9.726027	27.08333	0.375689	0.018418
3	1.80E-04	0	0.556512	1.54E-02	140	6	10	14	18	329.6875	10.0955	25	0.363349	0.013787
3	1.80E-04	3.80E-08	0.457392	1.12E-02	92	18	11	4	10	229.6875	12.01602	39.58333	0.312925	0.171511
3	1.80E-04	3.80E-08	0.452436	1.14E-02	92	16	15	6	7	232.1875	12.25033	41.66667	0.284791	0.202292

Time (days)	N conc.	Pb conc	Chl a	dry weight	cell counts	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SOD	SAGST	SACAT	SAPOD
3	1.80E-04	3.80E-08	0.390084	1.12E-02	96	12	20	8	5	230.9375	12.36702	39.58333	0.294632	0.209948
3	1.80E-04	3.80E-07	0.417744	1.26E-02	67	20	15	2	2	209.6875	15.29563	50	0.251366	0.151741
3	1.80E-04	3.80E-07	0.428016	1.30E-02	66	20	18	2	1	209.0625	15.51282	50	0.206278	0.231916
3	1.80E-04	3.80E-07	0.334848	1.30E-02	64	23	14	2	1	209.6875	15.45863	52.08333	0.196523	0.231224
3	1.80E-05	0	0.482532	1.26E-02	90	3	10	9	10	210.9375	12.34568	37.5	0.813235	0.308866
3	1.80E-05	0	0.4974	1.30E-02	93	14	10	9	8	213.4375	12.64525	35.41667	0.808199	0.369138
3	1.80E-05	0	0.390444	1.30E-02	88	15	10	7	8	214.6875	12.46575	35.41667	0.79903	0.254069
3	1.80E-05	3.80E-08	0.280332	8.00E-03	66	8	15	4	14	187.8125	18.23417	70.83333	0.525568	0.467907
3	1.80E-05	3.80E-08	0.392964	7.80E-03	56	23	11	5	4	185.3125	18.4949	75	0.527487	0.1717
3	1.80E-05	3.80E-08	0.383412	8.00E-03	54	26	0	4	4	185.9375	18.70229	75	0.499944	0.097785
3	1.80E-05	3.80E-07	0.265464	6.80E-03	38	23	6	1	0	147.1875	22.02563	75	0.481812	0.823522
3	1.80E-05	3.80E-07	0.34836	6.80E-03	40	26	5	0	1	148.4375	21.83486	75	0.503579	0.663477
3	1.80E-05	3.80E-07	0.363588	7.00E-03	40	28	6	0	0	148.4375	21.93036	75	0.503579	0.561404
3	1.80E-06	0	0.403236	8.60E-03	68	5	0	13	6	150.3125	13.35593	45.83333	1.10298	0.0504
3	1.80E-06	0	0.377736	8.40E-03	70	8	6	14	2	157.8125	13.64865	52.08333	1.026271	0.048005
3	1.80E-06	0	0.383052	8.60E-03	72	13	7	11	3	151.5625	14.1129	50	1.068591	0.129959
3	1.80E-06	3.80E-08	0.368544	6.40E-03	50	23	12	1	0	81.5625	22.35723	66.66667	0.869476	0.037153
3	1.80E-06	3.80E-08	0.39828	6.00E-03	49	26	7	2	0	85.3125	22.54545	64.58333	0.775092	0.515041
3	1.80E-06	3.80E-08	0.3225	6.20E-03	47	26	8	1	1	82.1875	22.16809	68.75	0.71128	0.737412
3	1.80E-06	3.80E-07	0.327816	5.20E-03	30	26	2	0	1	61.5625	24.91187	83.33333	0.513706	1.476696
3	1.80E-06	3.80E-07	0.225456	5.60E-03	28	24	2	0	0	62.1875	24.77928	83.33333	0.601005	1.218212

Time (days)	N conc.	Pb conc	Chl a	dry weight	cell counts	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SOD	SAGST	SACAT	SAPOD
3	1.80E-06	3.80E-07	0.19464	5.40E-03	28	20	4	0	0	64.0625	24.82353	83.33333	0.523577	0.898744
4	1.80E-04	0	0.681132	1.90E-02	170	11	5	15	26	394.0625	11.00671	33.33333	0.36479	0.061519
4	1.80E-04	0	0.609588	1.92E-02	179	15	7	10	30	396.5625	11.36364	31.25	0.357657	0.064952
4	1.80E-04	0	0.650676	1.90E-02	175	13	9	12	27	394.0625	11.30435	35.41667	0.342902	0.146108
4	1.80E-04	3.80E-08	0.516864	1.44E-02	101	7	26	10	3	262.1875	15.96958	54.16667	0.270481	0.161809
4	1.80E-04	3.80E-08	0.531732	1.46E-02	110	15	20	5	10	264.6875	16.28788	58.33333	0.260685	0.166005
4	1.80E-04	3.80E-08	0.459468	1.44E-02	112	16	15	6	12	265.3125	15.59516	58.33333	0.270907	0.13706
4	1.80E-04	3.80E-07	0.57498	1.10E-02	67	20	10	5	3	232.8125	19.63636	70.83333	0.214049	0.611755
4	1.80E-04	3.80E-07	0.452796	1.16E-02	70	17	15	5	2	229.0625	19.92754	68.75	0.175716	0.436562
4	1.80E-04	3.80E-07	0.353952	1.10E-02	71	24	15	2	2	229.6875	19.87915	72.91667	0.158549	0.474954
4	1.80E-05	0	0.591564	1.66E-02	150	24	20	10	14	247.1875	13.85424	47.91667	0.662958	0.165498
4	1.80E-05	0	0.606072	1.70E-02	149	11	20	14	14	250.3125	14.02359	45.83333	0.643196	0.133167
4	1.80E-05	0	0.4683	1.72E-02	151	22	20	15	11	252.8125	13.85424	45.83333	0.633045	0.269693
4	1.80E-05	3.80E-08	0.340164	1.08E-02	88	7	25	5	4	206.5625	23.09496	91.66667	0.389713	0.359419
4	1.80E-05	3.80E-08	0.437928	1.12E-02	90	30	18	4	3	210.3125	23.49854	95.83333	0.355423	0.331397
4	1.80E-05	3.80E-08	0.407112	1.12E-02	90	30	18	2	4	209.0625	22.82353	93.75	0.330045	0.253658
4	1.80E-05	3.80E-07	0.336648	9.00E-03	67	30	10	3	2	164.6875	25.87571	112.5	0.32587	0.478408
4	1.80E-05	3.80E-07	0.39792	8.80E-03	68	35	11	1	2	166.5625	26.1677	112.5	0.299187	0.445733
4	1.80E-05	3.80E-07	0.339804	9.00E-03	66	38	12	0	1	164.6875	26.29213	112.5	0.279317	0.469208
4	1.80E-06	0	0.452796	9.40E-03	128	13	15	15	10	219.0625	16.03535	52.08333	0.695578	0.17983

Time (days)	N conc.	Pb conc	Chl a	dry weight	cell counts	1Coe	2Coe	3Coe	4Coe	Protein (ug/mL)	SOD	SAGST	SACAT	SAPOD
4	1.80E-06	0	0.468024	9.60E-03	130	15	15	15	10	221.5625	16.29956	52.08333	0.679079	0.034192
4	1.80E-06	0	0.427656	9.20E-03	130	31	24	9	6	219.0625	16.56211	60.41667	0.673704	0.048416
4	1.80E-06	3.80E-08	0.37314	7.00E-03	67	24	4	5	5	100.3125	27.48092	91.66667	0.4108	0.332295
4	1.80E-06	3.80E-08	0.37314	6.80E-03	68	27	12	3	2	97.8125	27.40175	89.58333	0.382109	0.356278
4	1.80E-06	3.80E-08	0.3735	7.00E-03	70	28	16	2	1	101.5625	27.55991	95.83333	0.330256	0.65641
4	1.80E-06	3.80E-07	0.276096	5.80E-03	47	23	10	0	1	72.1875	30.2935	122.9167	0.119481	0.629673
4	1.80E-06	3.80E-07	0.265824	5.60E-03	50	30	10	0	0	74.0625	30.43933	127.0833	0.168214	1.186549
4	1.80E-06	3.80E-07	0.122016	5.80E-03	49	33	8	0	0	72.8125	30.65693	116.6667	0.144778	0.853167

ANOVA table for nitrate experiments.

	Degr. of - Freedom	Chl a(ug mL-1) - SS	Chl a(ug mL-1) - MS	Chl a(ug mL-1) - F	Chl a(ug mL-1) - p	dry weigt/ml - SS	dry weigt/ml - MS	dry weigt/ml - F	dry weigt/ml - p	cell counts - SS	cell counts - MS	cell counts – F	cell counts - p
Intercept	1	15.02728	15.02728	2697.943	0	0.00002	0.00002	1178.404	0	354893.3	354893	280.6237	0
N conc.	2	0.33615	0.16808	30.176	0	0.000001	0.000001	34.229	0	20739.6	10369.8	8.1997	0.000507
Pb conc	2	0.2598	0.1299	23.322	0	0.000001	0	16.111	0.000001	34470.6	17235.3	13.6284	0.000006
N conc.*Pb conc	4	0.02456	0.00614	1.102	0.359881	0	0	0.361	0.835515	622.3	155.6	0.123	0.973913
Error	99	0.55142	0.00557			0.000002	0			125201.2	1264.7		
Total	107	1.17193				0.000003				181033.7			

	Degr. of - Freedom	1Coe - SS	1Coe - MS	1Coe – F	1Coe - p	2Coe - SS	2Coe - MS	2Coe - F	2Coe - p	3Coe - SS	3Coe - MS	3Coe - F	3Coe - p
Intercept	1	24330.01	24330.01	377.717	0	5362.231	5362.231	131.681	0	1481.481	1481.481	128.2612	0
N conc.	2	453.13	226.56	3.5174	0.03344	187.907	93.954	2.3072	0.10487	91.63	45.815	3.9665	0.022025
Pb conc	2	832.57	416.29	6.4627	0.002301	68.574	34.287	0.842	0.43391	724.13	362.065	31.3462	0
N conc.*Pb conc	4	72.37	18.09	0.2809	0.889737	216.87	54.218	1.3314	0.26361	27.259	6.815	0.59	0.670645
Error	99	6376.92	64.41			4031.417	40.721			1143.5	11.551		
Total	107	7734.99				4504.769				1986.519			

	Degr. of - Freedom	4Coe - SS	4Coe - MS	4Coe – F	4Coe - p	Protein (ug/mL) - SS	Protein (ug/mL) - MS	Protein (ug/mL) - F	Protein (ug/mL) - p	SAGST - SS	SAGST - MS	SAGST - F	SAGST - p
Intercept	1	2002.083	2002.083	119.0249	0	5441880	5441880	1024.83	0	248168.4	248168.4	427.4884	0
N conc.	2	652.389	326.194	19.3924	0	1013677	506838	95.449	0	14577.1	7288.6	12.5551	0.000014
Pb conc	2	1092.167	546.083	32.4649	0	243361	121680	22.915	0	13705.7	6852.9	11.8045	0.000025
N conc.*Pb conc	4	295.111	73.778	4.3861	0.002627	13850	3463	0.652	0.626759	794.4	198.6	0.3421	0.848919
Error	99	1665.25	16.821			525693	5310			57472.1	580.5		
Total	107	3704.917				1796581				86549.4			

	Degr. of - Freedom	SACAT - SS	SACAT - MS	SACAT – F	SACAT - p	SAPOD - SS	SAPOD - MS	SAPOD - F	SAPOD - p	SSOD - SS	SSOD - MS	SSOD - F	SSOD - p
Intercept	1	45.70927	45.70927	258.3231	0	15.42396	15.42396	175.2667	0	19837.38	19837.38	535.9524	0
N conc.	2	8.61531	4.30765	24.3444	0	6.14649	3.07324	34.9221	0	712.94	356.47	9.6308	0.000151
Pb conc	2	1.14716	0.57358	3.2415	0.043291	3.08443	1.54222	17.5246	0	644.9	322.45	8.7117	0.000327
N conc.*Pb conc	4	0.50389	0.12597	0.7119	0.585688	3.0839	0.77098	8.7608	0.000004	39.48	9.87	0.2667	0.898738
Error	99	17.51767	0.17695			8.71228	0.088			3664.32	37.01		
Total	107	27.78402				21.0271				5061.64			