THE EFFECT OF ATRAZINE IN COMBINATION WITH CADMIUM AND COPPER ON THE MICROALGA *SCENEDESMUS QUADRICAUDA* (TURPIN)

BY

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NIGERIA.

NOVEMBER, 2014

DECLARATION

I declare that the work in this thesis titled "The effect of Atrazine in combination with Cadmium and Copper on the microalgae *Scenedesmus quadricauda*" was performed by me in the Department of Biological Sciences under the supervision of Dr. W.S Japhet and Dr. M.A Chia. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma in any other institution

Sadiya Yunusa GALADIMA _____ _____

Signature Date

CERTIFICATION

This thesis titled "THE EFFECT OF ATRAZINE IN COMBINATION WITH CADMIUM AND COPPER ON THE MICROALGA *Scenedesmus quadricauda*" by Sadiya Galadima YUNUSA meets the regulations governing the award of the degree of Masters in Botany of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to God Almighty and my beloved Husband Mr. Samuel Simon Awala, to my lovely Parents Arch. and Mrs. Dembo Yunusa Galadima for their love support and encouragement.

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ABSTRACT

The interactive effects of cadmium (Cd) copper (Cu) and atrazine on *Scenedesmus quadricauda* was investigated in this study. Individually, the metals at 1-2 $mg.L^{-1}$ (Cd and Cu) and herbicide at 0.005-0.5 $mg.L^{-1}$(atrazine) significantly decreased algal growth, dry weight production and chlorophyll content, while antioxidant enzyme activities increased under the same conditions. The toxicity of both the metals and atrazine increased additively when the microalgae were exposed to them simultaneously. These changes in biomass production, chlorophyll content and antioxidant enzyme activities were observed to be significant with two-way ANOVA ($p<0.05$). Principal components analysis (PCA) results showed a significant positive correlation of atrazine and the metals with increasing protein content and antioxidant enzyme activities. In addition, a significant negative correlation was observed between growth, biomass production and chlorophyll content of *S. quadricauda* and the combination of metal and herbicide treatment. These results demonstrate that the combination of these metals and atrazine is toxic to this micro alga as a result of both additive and synergistic interactive toxic effects they have on it. This may have cascading effects to other levels of the aquatic food web, if other microalgae are also unable to withstand the levels investigated in this study. Considering that the concentrations tested in this study are within the maximum acceptable limits in water, it is really a cause for concern due to risk aquatic resources may face from the combination of these metals and atrazine.

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CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND INFORMATION

1.1 The Microalga *Scenedesmus quadricauda*

Freshwater microalgae serve as indicator species for monitoring changes in the physico-chemistry of aquatic ecosystems, especially in cases of organic and inorganic pollution (Torres *et al*., 2008). Algae are crucial for the ecological balance of aquatic ecosystems as they occupy the base of the food chain and are the most abundant plant forms in water bodies (Torres *et al*., 2008).

Microalgae are a group of fast growing unicellular or simple multicellular microorganism which have the ability to fix carbon dioxide while capturing solar energy with efficiency 10 to 50 times greater than that of terrestrial plants and higher biomass production compared to energy crops (Wang *et al.,* 2008). Algae are important primary producers and food source for the primary consumers; as such they are essential components of aquatic eco- systems (He *et al*., 2012). The use of microalgae in heavy metal toxicity testing is well established today due to their ubiquity in aquatic ecosystems, where they influence and/or are influenced by most aquatic processes (El-Naggar and El-Sheekh, 1998).

Scenedesmus is a genus of microalgae that belongs to chlorophyceae family. They are colonial and non-motile. *Scenedesmus quadricauda* is a fresh water microalgae having coenobium with varying number of cells (usually 2-8 cells), arranged in one or two rows. The coenobium has terminal cells with spines. The cells may be oval or lunar and have

one wall plastid with one starch centre. *Scenedesmus quadricauda* is a potent indigenous microalga for biomass production and carbon dioxide sequestration. The microalga also has potential as a valuable biological assay material in toxicity testing (Ma *et al*., 2006). *Scenedesmus quadricauda* is an autotrophic fresh water microalgae, used as food for other small aquatic organisms.

1.2 Cadmium, Copper and Atrazine

Cadmium is a toxic heavy metal and is considered non essential for living organisms. It is used in a wide variety of industrial processes. It is used in the production of battery, paint, as a colouring agent, stabilizer and alloy mixtures (Branco *et al*., 2010). Human activities have been increasing the cadmium levels in soils and waters, affecting many organisms in the primary trophic levels such as microalgae. Sources of contamination of cadmium in water include corrosion of galvanized pipes, erosion of natural deposits, discharge from metal refineries, runoff from waste batteries and paints (Branco *et al*., 2010). The MCL (maximum concentration level) for cadmium is 0.005mg/L (U-S-EPA, 1996). Cadmium is not essential to most plants except marine diatoms (Chia *et al*. 2013).

Cadmium toxicity is linked to the formation of reactive oxygen species (ROS), leading to oxidative stress (Morelli and Scarano, 2004). These authors reported that, above normal concentrations, reactive oxygen species (ROS) are potentially toxic and can react with lipids, proteins, photosynthetic pigments and nucleic acids, leading to lipid peroxidation, decrease in chlorophyll and accessory pigments content, membrane damage, inactivation of enzymes, DNA alterations and oxidation of proteins. These cellular changes affect cell growth, viability and reproduction. Metals inhibit the activity of enzymes at cellular levels by reacting with their sulphydryl groups. The study of algal-metal interactions is of

particular importance, principally because algae are the base of the food chain (Battah, 2010).

Copper is an essential trace element for algae, it participates in photosynthetic electron transport, but is toxic at higher concentrations (Faith and Zaki,2005). Copper (Cu) is considered as a micronutrient for plants and plays important role in CO_2 assimilation and ATP synthesis. Cu is also an essential component of various proteins like plastocyanin of photosynthetic system and cytochromeoxidase of respiratory electron transport chain (Demirevska-kepova *et al*., 2004). Enhanced industrial and mining activities have contributed to the increasing occurrence of Cu in ecosystems. Cu is also added to soils from different human activities including mining and smelting of Cu-containing ores. Mining activities generate a large amount of waste rocks and tailings, which get deposited at the surface.

Atrazine is a selective pre and post emergence herbicide widely used on agricultural crops such as corn, sorghum, sugarcane and in non-cropped areas such as wheat fallow. It is a highly toxic herbicide that is frequently detected in surface water due to its intensive application. Algae are among the aquatic organisms most susceptible to atrazine pollution in water (Qian *et al*., 2007). Average rates of 1 to 2.5 pounds/acre are usually applied by ground boom application, but higher concentrations may be used under non selective conditions. Atrazine or its metabolites are frequently detected at concentrations above the maximum contamination level. United States Environmental Protection Agency (USEPA) has established a maximum concentration level (MCL) for atrazine at 3µg/L (USEPA, 1996). Lack of degradability is one reason that atrazine is commonly observed in surface

waters and well drinking water supplies. This long residence time in surface water indicate that it may have the opportunity to enter the food chain (USEPA, 1996).

1.3 Antioxidant Enzymes

Plants including microalgae possess several antioxidation defence systems and enzymes like Catalase (CAT), Superoxide dismutase (SOD), Glutathione Peroxidase (GPX), Ascorbate Peroxidase (APX), Glutathione Reductase (GR) and Glutathione-S-Transferase (GST) to scavenge toxic reactive oxygen species and to protect themselves from the oxidant stress (Seth *et al*., 2007). Diverse array of pollutants stimulate a variety of toxicity mechanisms such as oxidative damage to membrane lipids, DNA, Proteins and changes to antioxidant enzymes (Valanidis *et al*., 2006). Elevated activities of antioxidant enzymes may help to alleviate the oxidative damage caused by ROS (Qian *et al*., 2007). Glutathione –s –Transferase is an enzyme associated with atrazine detoxification in plants by detoxifying ROS through Ascorbate-glutathione cycle (Yadav, 2010). SOD converts superoxide radical into hydrogen peroxide and molecular oxygen, while the Catalase and Peroxidase convert hydrogen peroxide into water. In these two ways, two toxic species, superoxide radical and hydrogen peroxide are converted to the harmless product water (Weydert and Cullen, 2010). Various methods to identify oxidized amino acids in blood proteins as biomarkers of free radical damage, especially for metal-catalyzed oxidations, have been developed recently (Valavanidis *et al.*, 2006).

According to Weydert and Cullen (2010), there are three SOD enzymes that are highly compartmentalized, Manganese-containing Superoxide dismutase (MnSOD) is localized in the mitochondria, Copper and Zinc-containing Superoxide dismutase (CuZnSOD) is located in the cytoplasm, nucleus and extracellular SOD (ECSOD). Catalase is found in

peroxisomes and cytoplasm. Biological systems have developed during their evolution adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cellular components from oxidative damage (Valavanidis *et al*., 2006).

The individual toxicity of heavy metals and herbicides has been assessed, but they can interact and potentially increase their toxicity on microalgae. Correspondingly, the combined effect of herbicides and metals could be described qualitatively as antagonistic, non-reaction or synergistic. Generally algae are sensitive to pollutants; these pollutants may affect species composition of the algal community (Walsh, 1999). The toxicity of heavy metal (cadmium) in conjunction with atrazine herbicide was inhibited up to 100ppm of metal concentration in bean plant (Azmat *et al*., 2006). Combination of Butachlor and Thiobencarb at concentrations higher than 5 and 3 ppm, respectively, significantly decreased total soluble protein in *Nostoc muscorum* (Dowidar *et al*., 2010). Mathad *et al*. (2006) confirmed that *Helichrysum stuhmannii* and *Scenedesmus quadricauda* exposed to the various concentration of the bi- metallic combinations of chromium- iron, revealed no marked morphological variations at the lower metal concentration but, exhibited distinct abnormalities such as chlorosis, enlargement or reduction of cells, fragmentation and shrinkage of protoplasm or chloroplast, loss of cellular contents and cell lyses at the higher metal concentrations. Walsh (1999) stated that copper ions (2 ppm) inhibited toxicity of the herbicide Paraquat (1, 1-dimethyl- 4,4'-bipyridinum ion, 2 ppm) on *C. pyrenoidosa*, whereas cyanide ion (16 ppm) enhanced herbicide activity.

1.4 Statement of Research Problem

Environmental pollution by organic compounds and metals became extensive as mining and industrial activities increased in the 19th century and have intensified since then (Torres *et al*., 2008). And nowadays, environmental problems are multiple and complex, especially those arising from the identification and assessment of the toxicity of chemical substances in the aquatic ecosystem (Ma *et al*., 2006).

Adverse effects of herbicides such as oxidative stress, inhibition of Hill reaction of photosynthesis, reduction of electron transport through photosystem II leading to photoinhibition, reduce utilization of carbon dioxide, decrease in growth or biomass production, alteration of ultra-structures of the cellular organelles and change in community structure on non-target plants are of particular concern because of the annual, wide spread and increasingly worldwide use of these chemicals (Van der Brink and Ter Braak, 1999). Atrazine functions by binding to the plastoquinone-binding protein in photo system II, algal death results from starvation and oxidative damage caused by breakdown in the electron transport process (He *et al*., 2012)

There is little information about the joint effect of herbicides and heavy metals on aquatic organisms. As a food source, microalgae may facilitate the uptake of contaminants into higher organisms, increasing the possibility of toxicity (Okay *et al*., 2000). Therefore, stress on stress effect of both metals and herbicide on microalgae can cascade into the food chain. The stresses are numerous and often plant or location specific. They include increased UV-B radiation, water, high salinity, metal toxicity, herbicides, fungicides, air pollutants, light, temperature, topography and hypoxia that is restricted oxygen supply in waterlogged and compacted soil (Ali and Alqurainy, 2000). They further emphasize that

stress depend on tissue and/or species, on membrane properties, on endogenous antioxidant content and on the ability to induce the response in the antioxidant system.

Low concentrations of trace metal for long periods of time can lead to metabolism modification, elimination of the algae species that are unable to adapt themselves to the new conditions which will eventually affect the biodiversity of the environment (Tripath *et al*, 2006). Cadmium enters algal cells either by means of active transport or by endocytosis through chelating proteins and affects various physiological and biochemical processes of the algae. The toxicity primarily results from their binding to the sulphydryl group in proteins or disrupting protein structure or displacing essential elements (Arunakumara and Xuecheng, 2007). Heavy metal irons could interrupt routine metabolic processes by competing for the Protein binding sites, active Enzymes and various biological reactive groups, causing poor or no growth (Arunakumara and Xuecheng, 2007).

These pollutants stimulate a variety of toxicity mechanisms, such as oxidative damage to membrane lipids, DNA, Proteins, Carbohydrates and changes to Antioxidant Enzymes. Oxygen free radicals are essential in the physiological control of cell function in biological systems and are continuously produced in living cells but, during these metabolic processes, a small proportion (2–3%) of free radicals may escape from the protective shield of Antioxidant mechanisms, causing oxidative damage to cellular components. The amount of oxidized proteins and lipids in the algal cells indicates the severity of the metal stress on the microalgae.

Heavy metal ions can cause plasma membrane depolarization and acidification of the cytoplasm. In fact, membrane injury is one important effect of heavy metal ions that may

lead to the disruption of cellular homeostasis (Cardozo *et al*., 2002) however, study on the biochemical composition changes and antioxidant enzymes responses will bring in the effect of metals on microalgae (Tripathi*et al*, 2006).Cadmium (Cd) may displace or substitute for essential trace metals and interfere with proper functioning of enzymes, associated cofactors and disorganizes chloroplast causing the damage of photosynthetic pigments. Metals trigger changes in the transcript levels of numerous genes encoding proteins and induce the synthesis of several proteins, including metallothionein in algae (Torres *et al*., 2008).Heavy metals can interfere in the photosynthetic activity by increased photoinhibition from excess of light (Heckathorn *et al*., 2004).Herbicides like S-metalochlor within the K15 3 mode of action are currently thought to inhibit very long chain fatty acid synthesis (Husted *et al*., 1996)

1.5 Justification of The Study

Having an insight on the factors responsible for differential toxicity and physiological response of algae upon exposure to these chemicals will improve our ability to predict the impact of aquatic contaminants on freshwater ecosystem. The combined effect of Cadmium and atrazine on the physiological responses of *Scenedesmus quadricauda* is a subject of intensive investigation, as most studies focus on the individual toxicity of herbicides or heavy metals on microalgae. Investigation on toxicity of herbicide and heavy metals at environmentally relevant concentrations to fresh water green algae will help to provide a scientific basis to assess the ecological risk of both pollutant groups in aquatic ecosystem accurately (He *et al*., 2012).

Understanding of the regulatory mechanisms of metal and herbicide tolerance, and the components involved in the mechanism will be helpful in metal removing processes from aquatic ecosystem (Li *et al*., 2006). The potential of oxygen free radicals and other reactive oxygen species to damage tissues and cellular components called oxidative stress, in biological systems has become a topic of significant interest for environmental toxicology studies (Valavanidis *et al*., 2006). The balance between environmental pollutants and Antioxidant defences (enzymatic and Non-enzymatic) in biological systems can be used to assess toxic effects under stressful environmental conditions, especially oxidative damage induced by different classes of chemical pollutants. Therefore, the role of these Antioxidant systems and their sensitivity can be of great importance in environmental toxicology studies (Valavanidis *et al*., 2006).

Characterization of mutants and transgenic plants with altered expression of antioxidant is a potentially powerful approach to understanding the functioning of the antioxidant system and its role in protecting plants against stress, and significant progress is now being made in this area (Ali and Aqurainy, 2000).The knowledge of the effect of surface water contamination by heavy metals and herbicides on non-target organisms is imperative for freshwater algal communities given their role as the primary producers and the base position they occupy in most lotic and lentic environment food chains. Information on the impact of heavy metals and herbicides on the aquatic ecosystem and biodiversity are needed to assist in the provision of policies with appropriate regulations of the quantity of chemicals released into the aquatic environments, which result in their pollution.

The study will assess the level of contamination of aquatic life by these herbicide and heavy metals and thus emphasize the need to tackle the issue of environmental pollution as a result of these chemicals.

1.6 Aim of The Study

To assess the joint effects of herbicide (atrazine) and heavy metals (cadmium) and (copper), on the growth, biomass production, biochemical composition and physiological response of *Scenedesmus quadricauda*.

1.7 Objectives of the Study

1. To determine the interactive effect of cadmium and atrazine on the growth and biomass of *Scenedesmus quadricauda*,
2. To evaluate the interactive effect of copper and atrazine on the growth and biomass production of *Scenedesmus quadricauda,*
3. To determine the interactive effect of cadmium and atrazine on the antioxidant responses of *Scenedesmus quadricauda.*
4. To determine the interactive effect of copper and atrazine on the antioxidant responses of *Scenedesmus quadricauda.*

1.8 Research Hypotheses

1. There is no significant interactive effect of cadmium and atrazine on the biomass of *Scenedesmus quadricauda*,

2. There is no significant effect of copper and atrazine on the growth and biomass production of *Scenedesmus quadricauda,*
3. There is no significant interactive effect of cadmium and atrazine on the antioxidant enzyme of *Scenedesmus quadricauda*.
4. There is no significant interactive effect of copper and atrazine on the antioxidant enzyme of *Scenedesmus quadricauda*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Microalgae

Algae are the most vulnerable non-target species. As an important primary producer and food source for the primary consumers, microalgae are essential components of the aquatic ecosystem. Agricultural and industrial chemicals are entering the aquatic environment at an increasing rate, and most are taken up into tissues of aquatic organisms. As a result there are growing public concerns of the wide range of herbicides introduced into the environment from surface runoff and leaching (He *et al*., 2012). Ma *et al*. (2006) indicated that investigations using different algal species as test organisms have shown that algae vary greatly in their responses to herbicides. Aquatic organisms are more sensitive to the toxic effects of herbicides compared to terrestrial organisms, and in this respect they may provide experimental data for evaluation of oxidative stress, mutagenicity and other adverse effects (Lackner, 1998).

Scenedesmus quadricauda have attracted considerable attention for their capacity to eliminate heavy metals; however these chemicals have adverse effects like growth inhibition, limited photosynthesis and respiration on this important group of algae. Algae show immense ability to grow in the presence of varying heavy metal concentrations as a

result of a variety of tolerance mechanisms (El-Gamal, 2008). Since natural pools of water are normally polluted by a mixture of substances, these metals may exert their toxicity simultaneously.

2.2 Herbicides

Herbicides are the principal means of weed control but the introduction of these compounds into aquatic environment through runoff has severe consequences for non-target plants. Herbicides are important chemicals used all over the world; their concentrations at any given location vary from year to year and season to season depending upon rainfall and other associated factors. Atrazine concentrations have been reported to remain elevated almost year round in some lakes and reservoirs (USEPA, 1996). Selective toxicity of herbicides or other aquatic contaminants could alter species composition, decrease diversity, interfere with the normal succession pattern and alter food web as a whole. Results obtained from an investigation of the Yang River and Guanting Reservoir in China indicated that large portions of water bodies were contaminated with atrazine and its concentrations ranging from 0.22to 26mgL^{-1} (Ren *et al*., 2002). Therefore, a possible toxicity of atrazine to microalgae actually exists in waters of China.

2.3 Oxidative Stress

The damage caused by reactive oxygen species (ROS) is known as oxidative stress. Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox sensitive signalling pathways (Wu *et al*., 2008).Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a

biological system. The inability to readily detoxify the reactive intermediates or to repair the resulting damage, will cause disturbance in the normal redox state of cells through the production of peroxides and free radicals that damage all components of the cell, including Proteins, Lipids and DNA.

Reactive oxygen species are produced in many aerobic cellular metabolic processes; they include but are not limited to species such as superoxide and hydrogen peroxide which react with various intracellular targets including Lipids, Proteins and DNA. The biological effects of ROS on these intracellular targets are dependent on their concentration and increase level of these species present during oxidative stress (Weydert and Cullen, 2010). Formation of ROS is a normal process in photosynthesis, but can become a problem under stress conditions when production is increased (Asada, 1999).

Increased levels of ROS are cytotoxic while lower levels are necessary for the regulation of several key physiological mechanisms. Increased levels of ROS can result in ROS – induced damage such as cell death, mutations, chromosomal aberrations and carcinogenesis (Weydert and Cullen, 2010).All forms of life maintain a reducing environment within their cells. This reducing environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. To cope with metal stress, algae possess mechanisms to protect themselves from metal injury and poisoning, these mechanisms include extra cellular and intracellular sequestration of metal ions (Tripathi and Gaur, 2006). Freshwater algae form the base of aquatic food webs and changes in algal community structure and composition may have cascading effects on other component of the community due to changes in food availability and important community level interactions (Lockert *et al*., 2006).

2.4 Toxicity of Atrazine, Copper and Cadmium

2.4.1 Atrazine

According to He *et al*. (2012), atrazine acts by inhibiting photosynthesis (photo system II). Inhibitory effect of atrazine to freshwater algae should therefore be considered environmentally significant. British and Belgian biologists found that concentration of atrazine above 3ppb reduced chlorophyll levels in algae in experimental streams and concentrations above 11ppb reduced the amount of algae (Herbicide factsheet, 2001).

German scientists reported about 25% reduction in photosynthesis in freshwater green algae exposed to 12ppb of atrazine (Herbicide factsheet, 2001). Numerous studies have indicated that atrazine inhibits growth, photosynthesis of freshwater algae and algal responses to atrazine vary widely depending upon concentrations used, duration of exposure and algal species tested (Tang *et al*., 1997). They elaborated that at low concentrations atrazine stimulated both growth and chlorophyll content of green alga.

Exposure to Glufosinate increased malondialdehyde content in *Chlorella vulgaris* suggesting that there was some oxidative damage (Qian *et al*., 2008). Atrazine has a slight to moderate tendency to bio concentrate in microorganisms (U.S. EPA, 1996). The toxicity of toxicants to organisms is dependent on two event; penetration through biological membrane and interaction with the site of action (McFarland, 1970). Chemical effects on algae can directly affect the structure and function of an ecosystem resulting in oxygen depletion, decreased primary productivity, increased surface runoff, soil erosion and degradation (Fargasova, 1996).

2.4.2 Copper and cadmium

Excess of Cu in soil plays a cytotoxic role, induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis (Lewis *et al*., 2001). Exposure of plants to excess Cu generates oxidative stress and ROS (Stadtman and Oliver, 1991). Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules (Hegedus and Hovarth, 2001). Qian *et al*. (2007) stated that exposure to atrazine increased the level of malondialdehyde in *Chlorella vulgaris*, suggesting potential oxidative damage to the alga.

There is an agreement among scientist about the effect of cadmium on chloroplast ultra structure (Vassilev *et al*., 1995). Suarez *et al*. (2010) reported that heavy metals unlike other pollutants remain in the environment without degradation and wide spread of pollution by heavy metals that is generated by human activities has serious effects on human health and the environment. One ppm cadmium caused 60% decrease in growth, increased oxidation of lipids and increased oxidative damage to protein as shown by increased content of protein carbonyl groups (Collen *et al*., 2003).

A significant difference in growth of *Chlamydomona moewsii* as a result of the toxic effect was directly proportional to metal concentration; and inhibited biosynthesis of chlorophyll and carotenoids and reduced phosphorylation are most frequently observed symptoms of metal toxicity (El-Gamal, 2008). Cadmium ions can also produce free radicals which in turn, as a stressor can stimulate beta carotene synthesis. According to Lamai *et al*. (2005) toxicity symptoms of cadmium to *Cladophora fracta* showed damage and reduced number of chloroplasts, disintegrated cell wall and death. Cadmium is one of the most toxic metals to aquatic organisms and contaminations of these metals have been of wide concern in many aquatic systems (Wang and Robert, 2000). The presence of

cadmium ions in a nutrient medium resulted in the deformation of the green algae *Scenedesmus quadricauda* (Fargasova, 1993). Cadmium binds to organic molecules by forming bonds with Sulphur and Nitrogen, thereby inactivating proteins, and it is one of the three contaminants which poses the most threat to the environment (Awad and Chu, 2011).

Afkar *et al*. (2010) reported that inhibitory and stimulatory effect of heavy metals depends on concentration, different organisms have different sensitivities to some metals and some organisms may be less or more damaged by different metals. According to Qian *et al*. (2010) copper and cadmium independently inhibits photo system II activities and CO_2 assimilation but synergistically increased ROS contents to disrupt chlorophyll synthesis and inhibit cell growth in *Chlorella vulgaris*. Various sources of input such as mining milling and surface finishing industries have contributed to metal pollution in the environment (Wang and Robert, 2000). The co-existence of a multiplicity of metal ions is often considered to give rise to distinct interactive effects (Mathad *et al*., 2006)

Distinct dosage- dependant inhibitory effects of cadmium ions were found in individual growth and reproductive processes of *Scenedesmus quadricauda* both singly as well as in combination. $CdCl_2$ concentrations below 15µmol/L were not cytotoxic for intensively growing cultures of *S. quadricauda* (Bisova *et al*., 2003). UV-B and cadmium caused strong inhibition on activities of photo system II and the whole electron transport chain in *Plectonema boryanum*, whereas photo system I was the least affected (Prasad and Zeeshan, 2005).

Valavanidis *et al*. (2006) suggested that the balance between environmental pollution and antioxidant defence (enzymatic and non enzymatic) in biological systems can be used to assess toxic effects under stressful environmental conditions by different classes of chemical pollutants. Cellular antioxidant defence systems in biological systems when exposed to environmental pollutants are depleted but levels of this Antioxidant in living organisms may also redress the imbalance caused by the oxidative damage (Winston and Di Giulio, 1991).

2.4.3 Photosynthetic pigment

Fifty percent of Herbicides act via inhibition of photosynthesis at the photo system-I (PSI) and PSII levels by replacing PSI's ultimate electron acceptor or by blocking PSII-catalyzed photosynthetic electron transport. Pigments have often been used as biomarkers of exposure to different class of herbicides in autotrophic plants including algae (He *et al*., 2012). Carotenoid protects chlorophyll and photosynthetic membrane from photo oxidative damage. Therefore decline in carotenoid content could have serious consequences on chlorophyll, as well as thylakoid membrane which may lead to the reduction in photosynthetic efficiency of *Plectonema boryanum* (Prasad and Zeeshan, 2005).Total chlorophyll contents of *Cladophora fracta* exposed to lead and cadmium at higher concentrations decreased significantly from those of controls after two days of exposure. (Lamai *et al*., 2005).

2.4.4 Effect on antioxidant enzymes

Oxidative stress has become an important subject for terrestrial and aquatic toxicology (Ames *et al*., 1993). Regulated production of free radicals in organisms and maintenance

of “redox homeostasis” are essential for the physiological health of organisms. SOD is generally accepted as a first defence line protector against ROS and it is responsible for catalyzing the dismutation of oxygen to form oxygen and hydrogen peroxide which are decomposed by Catalase and Peroxidase into oxygen and water (Torres *et al*., 2008). According to Andersen *et al*. (2006) Glutathione-s-Transferase exhibited clear logical response to copper, the response being greater in higher exposure group. In addition to antioxidant enzymes, photosynthetic organisms reduce concentrations of ROS with low molecular weight compounds such as ascorbate, glutathione, flavonoids, tocopherols and carotenoids (Collen *et al*., 2003).

The activities of the antioxidant enzymes, Superoxides dismutase, Peroxidase and Catalase also increased markedly in the presence of glufosinate. Protein, carbohydrates, photosynthetic pigments levels, uptake and assimilation of nitrates, photosynthetic oxygen evolution and respiration, and growth of *Scenedesmus species* were inhibited by copper and zinc. (Tripathi and Guar, 2006).

Other auxiliary H_2O_2 removing mechanism is the production of volatile halocarbons (i.e bromoform, chlorofrom and trichloroethylene) from vanadium-bromoperoxidase (VBPx) in cell walls of seaweeds affected by both biotic and abiotic stress (Torres *et al*., 2008). .The H_2O_2 dependent production of volatile halocarbons is considered to be part of alga chemical defences against occasional epiphyte attack as well as protection from grazing fish and invertebrates. Inside compartment of plant and algal cell such as cytossol, chloroplast and peroxissomes NO from NO synthase (NOS) or from nitrate reductase (NR) can react with O_2 radicals to form the powerful oxidizing agent $ONOO^-$ (Figure 2.1).

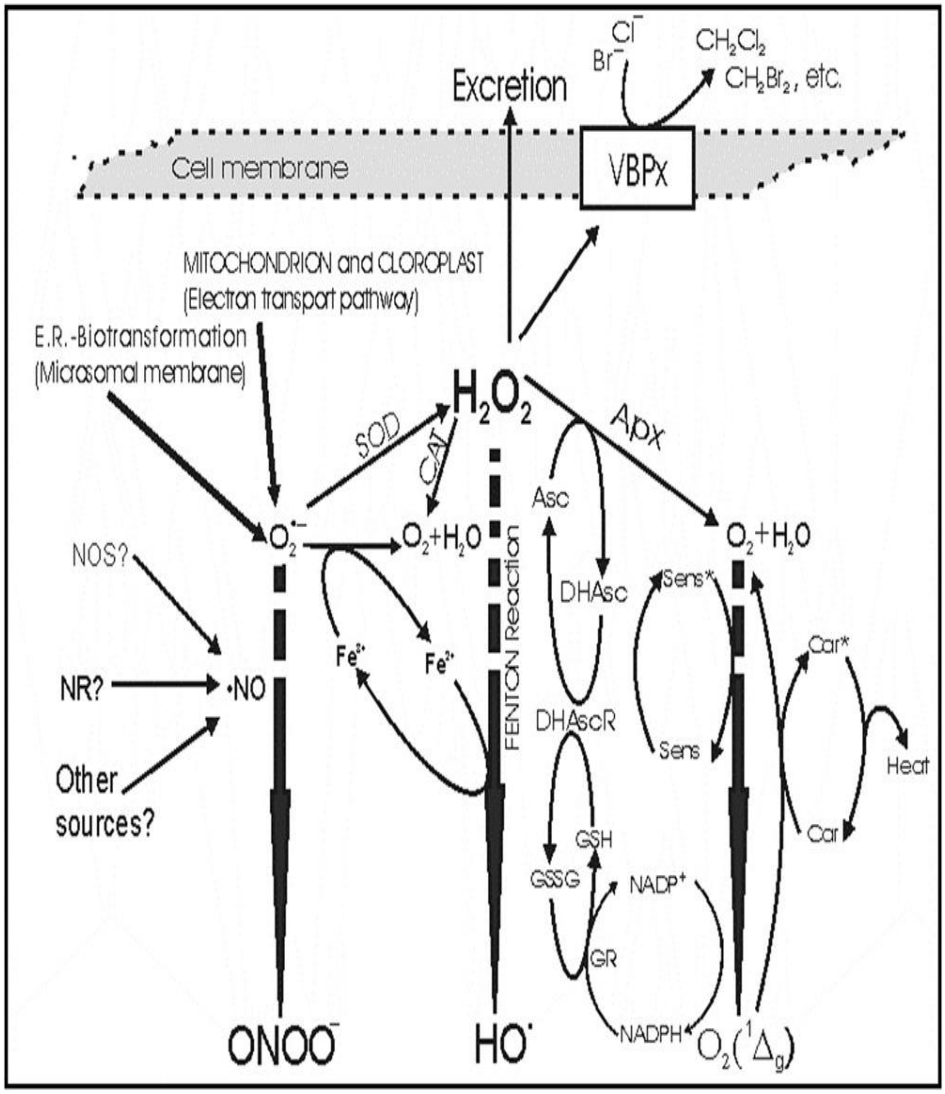


Figure 2.1. Scheme showing the relationship between Antioxidant Defenses and free radical productions in photosynthetic organism (Torres *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Algal Culture Conditions

The fresh water green algae *Scenedesmus quadricauda* was chosen as the test organism. The microalgae were isolated from the Department of Biological Sciences Fish Pond, Ahmadu Bello University, Zaria, Nigeria. The microalgae were cultured in the Organization for Economic Corporation and Development medium (OECD) (1984).The media were sterilized with autoclave at 121^{o}C for 30 minutes. Culture was maintained at 23± 2^{o}C under continuous lighting at an intensity of 120µmol m^{-2} s^{-1} with white fluorescent lamps. Cultures were shaken daily to prevent clumping. The pH of the culture media was adjusted to 7 using HCl and NaOH. The algal cell culture densities were then monitored spectrophotometrically (Spectrumlab 752s) at 500nm and microscopically using cell counts.

The culture medium consists of the following chemical components in Table 3.1

3.2 Cadmium Copper and Atrazine experiment

Cadmium (Cd) was provided as $CdCl_2$ at concentrations of 0, 1mg/L, 2mg/L and atrazine at 0, 0.005, 0.05, 0.5 mg/L concentrations. The atrazine and cadmium concentrations used here are environmentally relevant as the lowest concentration approximates to the maximum allowed level in water. In this study, *Scenedesmus quadricauda* were exposed

to atrazine and cadmium which are commonly detected in aquatic environment. The treatment combinations of atrazine, cadmium and copper which *Scenedesmus quadricauda* was exposed is shown in Table 3.2 and Table 3

Table 3.1 Composition of OECD culture medium (1984)

S/N	COMPOUND	CONCENTRATIONS(mg/L)
1	NH_4Cl	15mg/L
2	$MgCl_2.6H_2O$	12mg/L
3	$CaCl_2.2H_2O$	18mg/L
4	$MgSO_4.7H_2O$	15mg/L
5	KH_2PO_4	1.6mg/L
6	$FeCl_3.6H_2O$	0.08mg/L
7	$Na_2EDTA.2H_2O$	0.1mg/L
8	H_3BO_3	0.185mg/L
9	$MnCl_2.4H_2O$	0.415mg/L
10	$ZnCl_2$	$3x10^{-3}$mg/L
11	$CoCl_2.6H_2O$	$1.5x10^{-3}$mg/L
12	$CuCl_2.2H_2O$	10^{-5} mg/L
13	$Na_2MoO_4.2H_2O$	$7x10^{-3}$mg/L
14	$NaHCO_3$	50mg/L

Table 3.2: Experimental combinations of atrazine and cadmium to which *Scenedesmus quadricauda* was exposed.

	No Metals	1mg/L (M1)	2mg/L(M2)
No Herbicide	control	M1	M2
0.005mg/L(H1)	H1	H1M1	H1M2
0.05mg/L(H2)	H2	H2M1	H2M2
0.5mg/L(H3)	H3	H3M1	H3M2

H- Herbicides (Atrazine).

M- Metals (Cadmium).

H1- 0.005mg/l

H2- 0.05mg/l

H3- 0.5mg/l

M1- 1mg/l

M2- 2mg/l

H1M1- 0.005/1mg/l

H2M1- 0.05/1mg/l

H3M1- 0.5/1mg/l

H1M2- 0.005/2mg/l

H2M2- 0.05/2mg/l

H3M2- 0.5/2mg/l

Table 3.3: Experimental combinations of atrazine and copper to which *Scenedesmus quadricauda* was exposed.

	No Metals	1mg/L (M1)	2mg/L(M2)
No Herbicide	control	M1	M2
0.005mg/L(H1)	H1	H1M1	H1M2
0.05mg/L(H2)	H2	H2M1	H2M2
0.5mg/L(H3)	H3	H3M1	H3M2

H- Herbicides (Atrazine).

M- Metals (copper).

H1- 0.005mg/l

H2- 0.05mg/l

H3- 0.5mg/l

M1- 1mg/l

M2- 2mg/l

H1M1- 0.005/1mg/l

H2M1- 0.05/1mg/l

H3M1- 0.5/1mg/l

H1M2- 0.005/2mg/l

H2M2- 0.05/2mg/l

H3M2- 0.5/2mg/l

3.3 Preparation and Application of Herbicide and Heavy metal

Atrazine (99.8%) and cadmium (Cd) used in this study was of analytical grade and obtained from reputable local chemical stores in Nigeria. Stock solution of 1 mg/mL was prepared by dissolving atrazine in acetone. 0, 0.005, 0.05, 0.5 mg/L concentrations of atrazine was prepared from stock solution by serial dilution. Selection of these concentrations was based on 96h EC50 of atrazine that was obtained in preliminary experiment on determining the acute toxicity. This range of atrazine holds within environmentally realistic levels. The final concentration of acetone in the treatments and each control was 0.1% (v/v) and presumed to have little influence on the alga (Qian *et al*. 2008).

Concentrations of cadmium 0, 1mg/L, 2mg/L was prepared from $CdCl_{2.}$ (1g/L stock solution).Toxicity of cadmium was assessed independently in the preliminary experiments that was used to generate the concentration-response curve for *Scenedesmus quadricauda*. From the curve, two concentrations of Cd 1mg/L and 2mg/L were selected to determine their individual and combined effects with the herbicide atrazine. Fresh weight of algae was inoculated into each 500 mL Polycarbonate Erlenmeyer flask containing the growth medium and various concentrations of the Cd /atrazine treatments. Algae culture in the medium without any of the pollutant served as the control, and there were three replicate of each treatment. The microalgae were harvested on a daily basis for 4 days.

4.4 Determination of Growth, Biomass and Phenotypic Plasticity

Growth was determined according to the method of Liu *et al*., (2008) using optical density at 500 nm with a Sherwood colorimeter 257 (Sherwood scientific Ltd. Cambridge, England).

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

Where

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

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

Direct microscopic counts using a Zeis microscope was used to determine cell density with the aid of an improved Neubauer haemocytometer. Dry weight determination was done using Whatman GF/C filters (5 cm diameter) that was dried for 2 h at 60°C, cooled in desiccators and weighed to the nearest mg. The filters were used for filtering 20 mL of the samples. The filters then dried again for 24 h at 60°C, cooled in desiccators and weighed. Dry weight was calculated in mg L^{-1} DW as the difference between the initial dry weight of the filter without micro algal biomass and the final weight of the filter with the retained micro algal biomass. Phenotypic plasticity of *Scenedesmus quadricauda* was evaluated by quantifying the number of cells per coenobium in 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 single cells (absence of coenobium) under each treatment condition.

3.5 Photosynthetic Pigments Determination

Chlorophyll *a* was extracted using acetone according to the method of Shoah and Lium, (1973). Algal sample 5ml 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 were then be refrigerated at -20^{o}Cfor 3h. After refrigerating, the supernatants were removed. Chlorophyll *a*, quantification was done using absorbance measured at 470, 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 Well burn (1994):

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

3.6 Biochemical Composition Determination

3.6.1 Total proteins

For the Protein extraction 5mL of the algal sample was taken, centrifuged for 10000 rpm for 15 minutes, the supernatant discarded. 1.5mL of 0.05M of phosphate buffer (pH 7.8) was added, squashed on ice and centrifuged at 10000 rpm for 15 minutes. The supernatant obtained was used for protein and antioxidant enzymes assay. Determination of the total protein content of the microalgae cells followed the procedure of Bradford (1976) with bovine serum albumin (BSA) as standard. To every 500µl of the supernatant, 2.5mL of Bradford reagent (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 10 to 400 μg mL^{-1}. This value was used to plot the total protein standard calibration curve.

3.7 Antioxidant Enzyme Extraction and Assays

3.7.1 Assay of peroxidase

The activity of Peroxidase was measured by the method of Reddy *et al*. (1995).

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 (3.0mL) and enzyme extract (0.1 mL) was pipette into a cuvette. The spectrophotometer was adjusted to read zero at 430 nm followed by the addition of 0.5 ml of 1% H_2O_2 and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes. One unit of Peroxidase activity was defined as the change in absorbance per minute at 430 nm.

Peroxidase activity (units/mg) = ΔA_{510}/mins/(6.58×ml enzyme/mL reaction mixture)

Where,

A = Absorbance

3.7.2 Assay of glutathione-s- transferase (GST)

The determination of GST activity was carried out using the method of Habig *et al*. (1974).

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 (Habig *et al*., 1974).

Reagents

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

2. Reduced glutathione (1mM)

3. Phosphate buffer (0.1M, pH 6.5)

Procedure

GST activity was determined by monitoring the change in absorbance at 340 nm in a spectrophotometer. The assay mixture contained 10µl of GSH, 10µl of CDNB and phosphate buffer in a total volume of 990µl.10µl of enzyme extract was added to the mixture and the readings was recorded against distilled water blank after every one minute for minimum of five minutes. 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 nmoles of CDNB conjugated per minute.

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

ΔA_{340}= A_{340}(final readings) - A_{340}(initial readings)/reaction times(mins)

ΔA_{340} /minsample-ΔA_{340}/minblank

The above equation was used 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.

3.7.3 Assay of superoxide dismutase (SOD)

A Spectrophotometer (Spectrumlab 752s) was used to determine the activity of Superoxide dismutase following the method of Misra and Fridovich (1972).

Principle

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

Reagents

1. Potassium phosphate buffer (500 mM, pH 7.8)

2. Methionine (450 µM)

3. Riboflavin (53 mM)

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

5. Potassium cyanide (200 µM)

Procedure

The incubation medium contained, 0.3mL of 6.7 mM potassium phosphate buffer (pH 7.8), 0.3ml of 45 µM methionine, 0.3ml of 5.3 mM riboflavin, 0.3mL of 84 µM NBT and 0.8mL of the enzyme extract in a final volume of 2.0 mL. The amount of homogenate added to this medium was kept below one unit of enzyme to ensure sufficient accuracy. The tubes were placed 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. The maximum activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT. The percentage inhibition was then calculated as follows;

% inhibition = Absorbance of experimental samples – Absorbance of control samples

x100/(absorbance of control samples)

3.7.4 Assay of catalase

Catalase activity was determined by the method of Luck (1974).

Principle

The UV 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 0.067 M (pH 7.0)

2. Hydrogen peroxide in phosphate buffer (2mM)

Procedure

To the experimental cuvette, 2.9mL of H_2O_2 phosphate buffer was added, follow by the rapid addition of 0.1ml Catalase enzyme extract and mixed thoroughly. The samples were read against a control containing 3mL H_2O_2 phosphate buffer without enzyme extract. The time interval required for a decrease in absorbance by 0.05 units was recorded at 240 nm. The enzyme solution containing H_2O_2 free phosphate buffer was served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

The Catalase activity was calculated as follows:

Catalase activity (units/mL enzyme) = (3.45) / (Min)(0.1)

Where,

3.45 correspond to the decomposition of 3.45 micromoles of hydrogen peroxide in a 2.0 ml of reaction mixture.

0.1= Volume of enzyme used (in milliliters)

3.8 Determination of Metal and Herbicide Concentration in The Medium and Metal accumulation in Algal Cells.

Total dissolved and accumulated cadmium and copper was determined using Atomic Absorption Spectrophotometry (AAS). Atrazine in water was determined by simultaneous Spectrophotometric determination of atrazine (Hernandez *et al.*, 2005).

3.9 Data Analyses

The significant difference among the different treatments was determined by two-way analysis of variance (ANOVA). Where significant differences exist, Turkey's post hoc test was used for separation of means. A significant correlation between the growth, biomass production, biochemical composition and physiological responses of the microalgae with the treatment conditions was determined using a correlation based principal components analysis (PCA).

CHAPTER FOUR

4.0 RESULT

4.1 Growth and Biomass Production

Scenedesmus quadricauda showed a decreased in growth as the concentrations of cadmium/atrazine increased, in addition to their combinations. The lowest growth of the microalgae was observed at 0.5mg/L atrazine and 2mg/L cadmium treatment combination. Biomass production (chlorophyll *a*, dry weight and cell density) decreased with increasing atrazine/cadmium concentrations. Chlorophyll *a* (Figure 4.1), Dry weight production per cell (Figure 4.2) and cell density (Figure 4.3) were significantly reduced ($p < 0.05$) with increasing concentrations of atrazine/cadmium concentrations and their various combinations. The greatest effect of atrazine and cadmium was observed at the 96th hour (day4) after exposure.

4.2 Phenotypic Plasticity

There was reduction in the number of cells per coenobium as cadmium and atrazine concentrations increased. The number of single cells increased over the experimental period regardless of the treatment combination. However, the proportion of single cell production tended to increase with increasing time of exposure and cadmium/atrazine stress (Figure 4.4). The 0.5 mg/l atrazine and 2mg/cadmium treatment combination had

the lowest number of 2 cells per coenobium (Figure 4.5). Three and four cells per coenobium significantly ($p<0.05$) decreased with the combination of atrazine and cadmium stress on day 3 and day 4 of the experiment (Figure 4.6). Control had the highest number of 3 and 4 cells per coenobium (Figure 4.7).

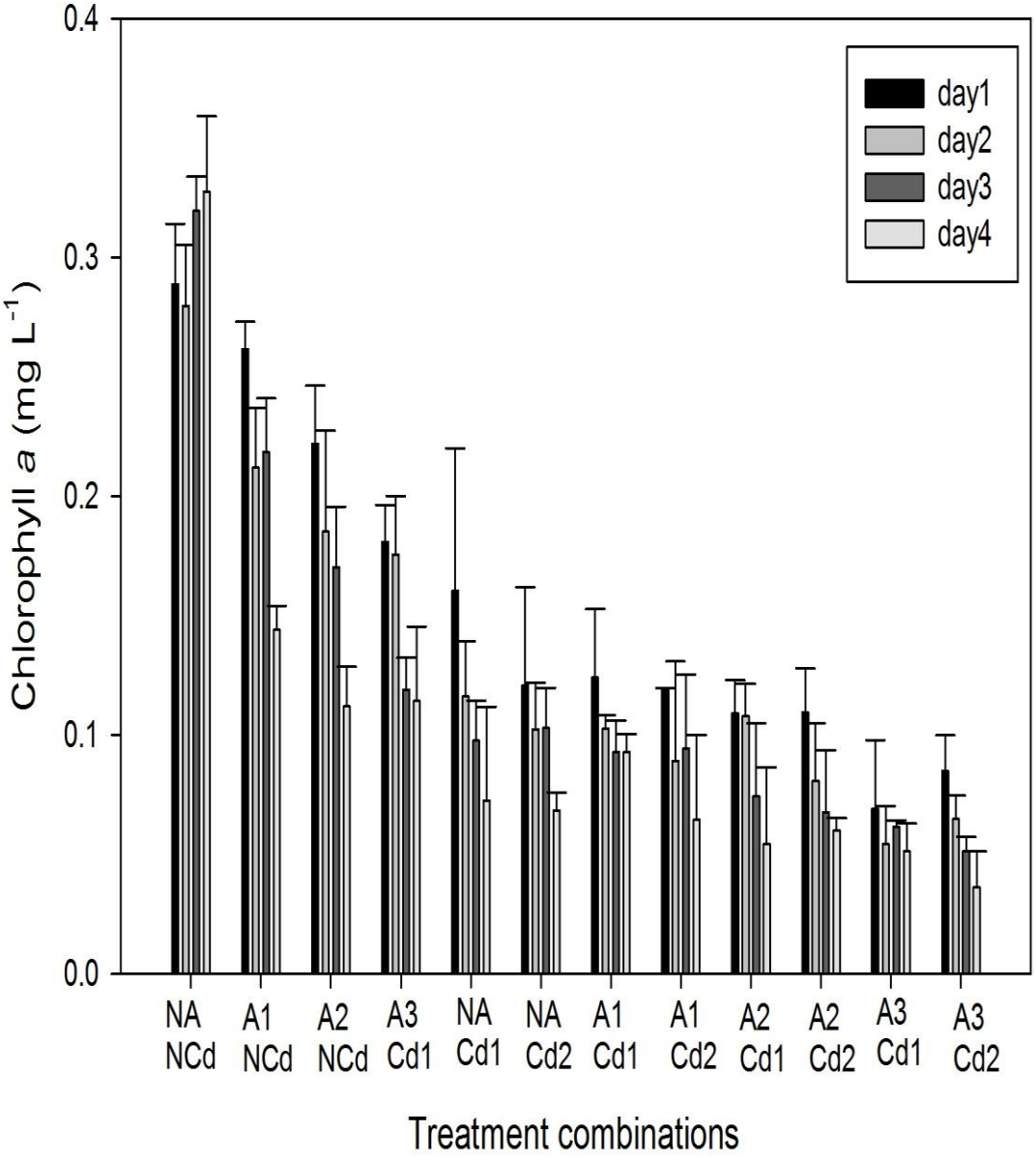


Figure 4.1: Chlorophyll *a* production by *Scenedesmus quadricauda* at different cadmium and atrazine concentrations. Error bars represent standard deviation for *n* =3.

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

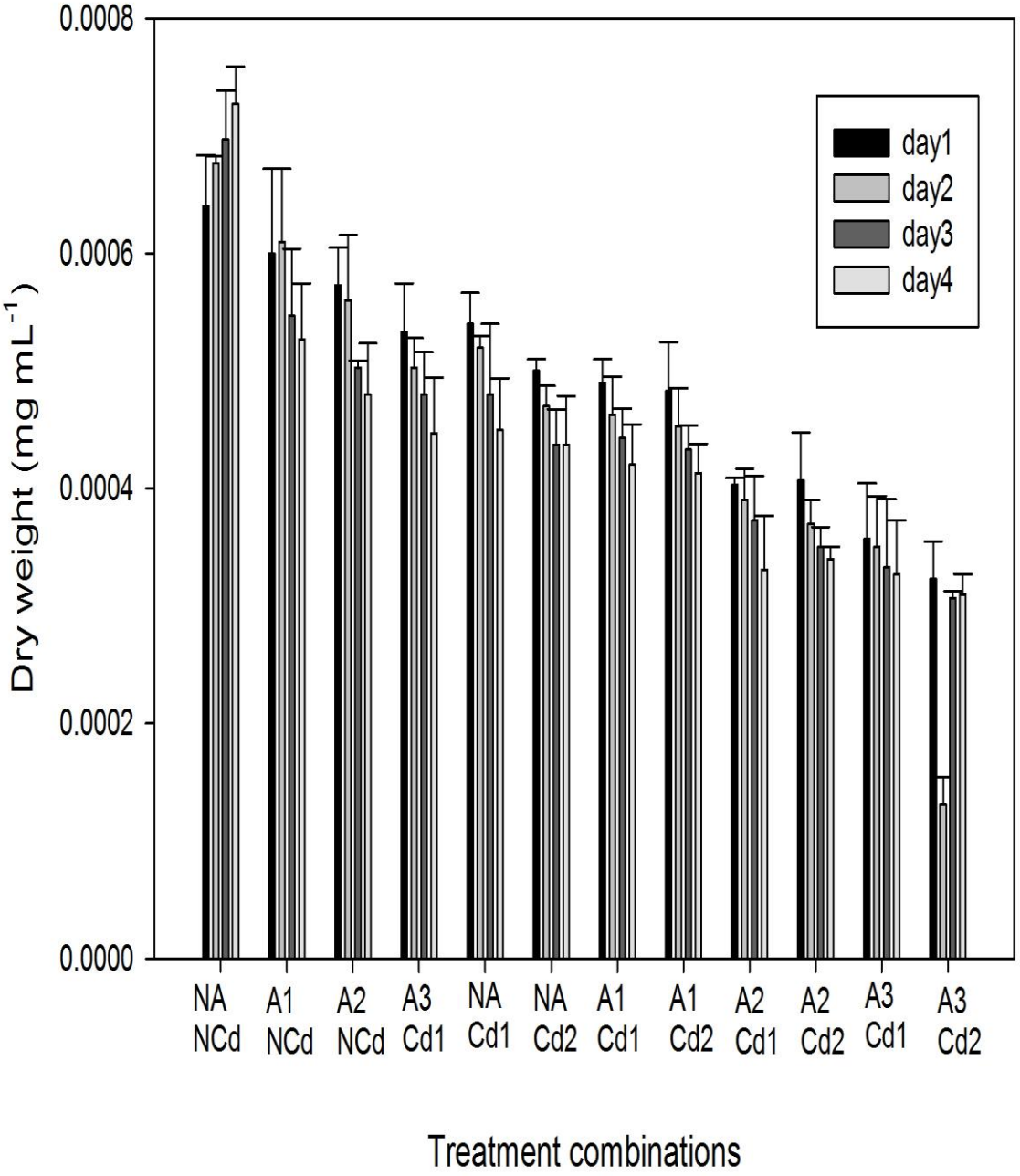


Figure 4.2: Dry weight of *Scenedesmus quadricauda* at different cadmium and atrazine concentrations. Error bars represent standard deviation for *n* =3.

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

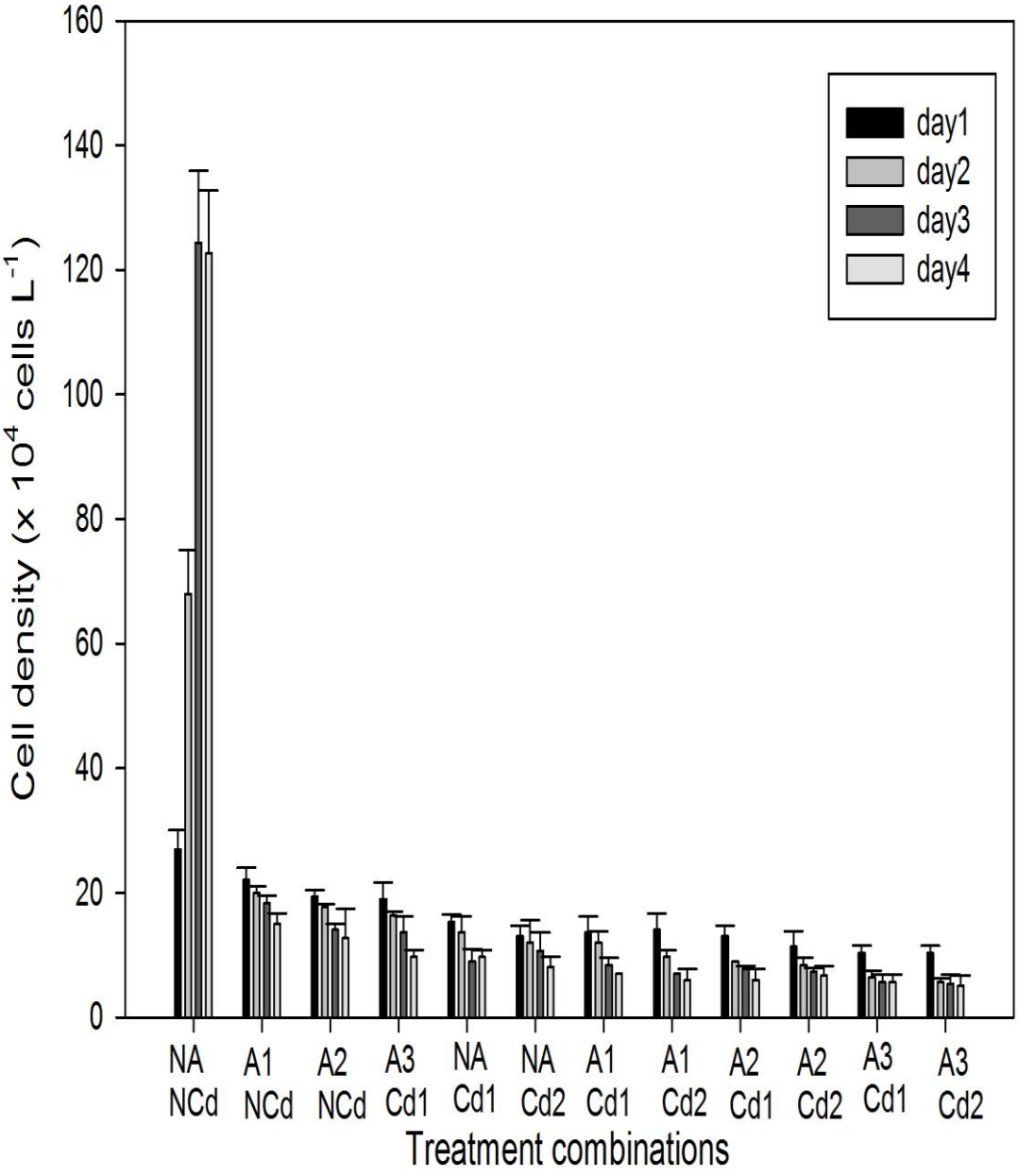


Figure 4.3: Cell density (x10^4 cells per ml) of *Scenedesmus quadricauda* at different cadmium and atrazine concentrations. Error bars represent standard deviation for *n* =3.

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/

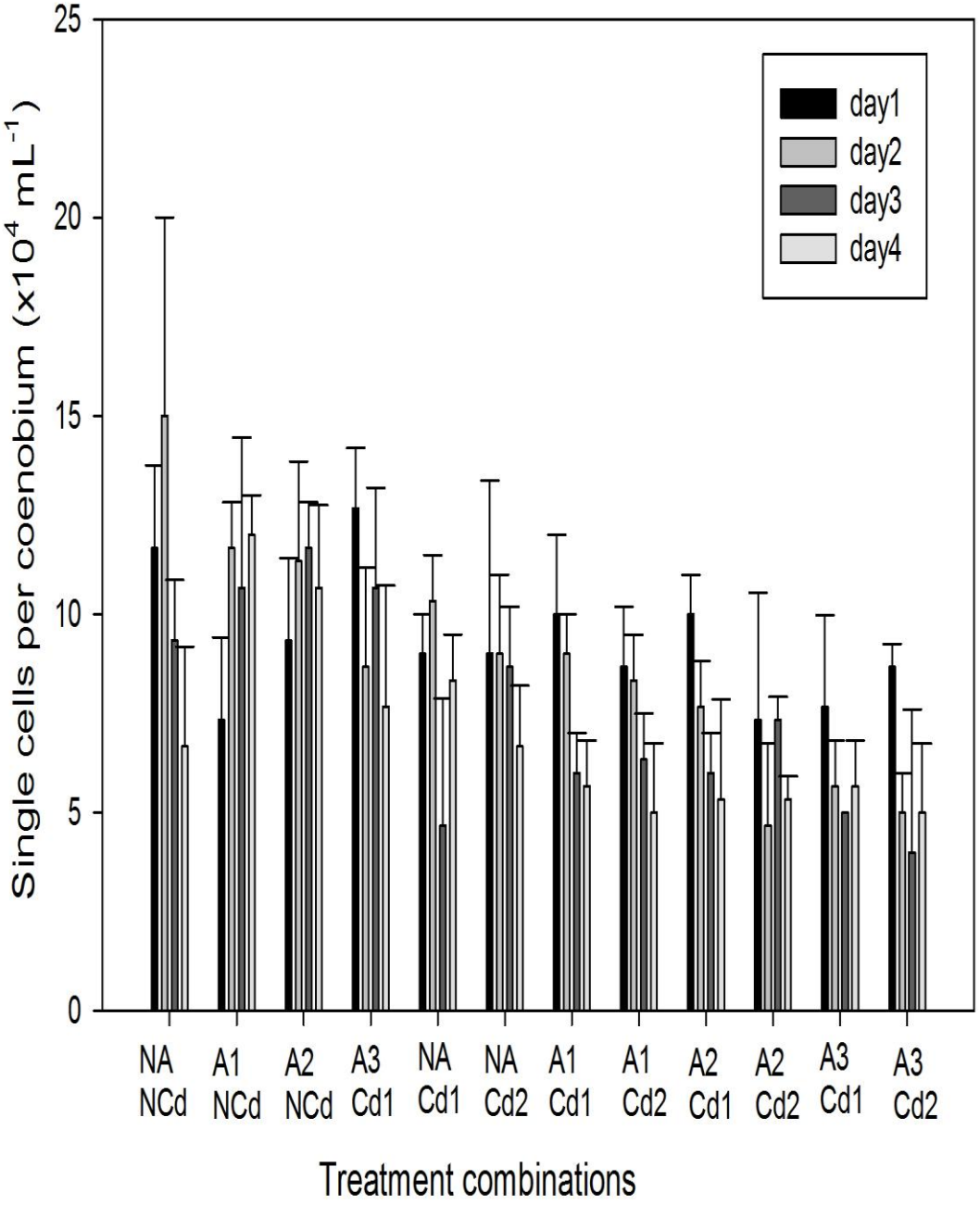


Figure 4.4: The formation of single cells by *Scenedesmus quadricauda* as a function of different cadmium and atrazine concentrations (values are means± SD for *n* =3).

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

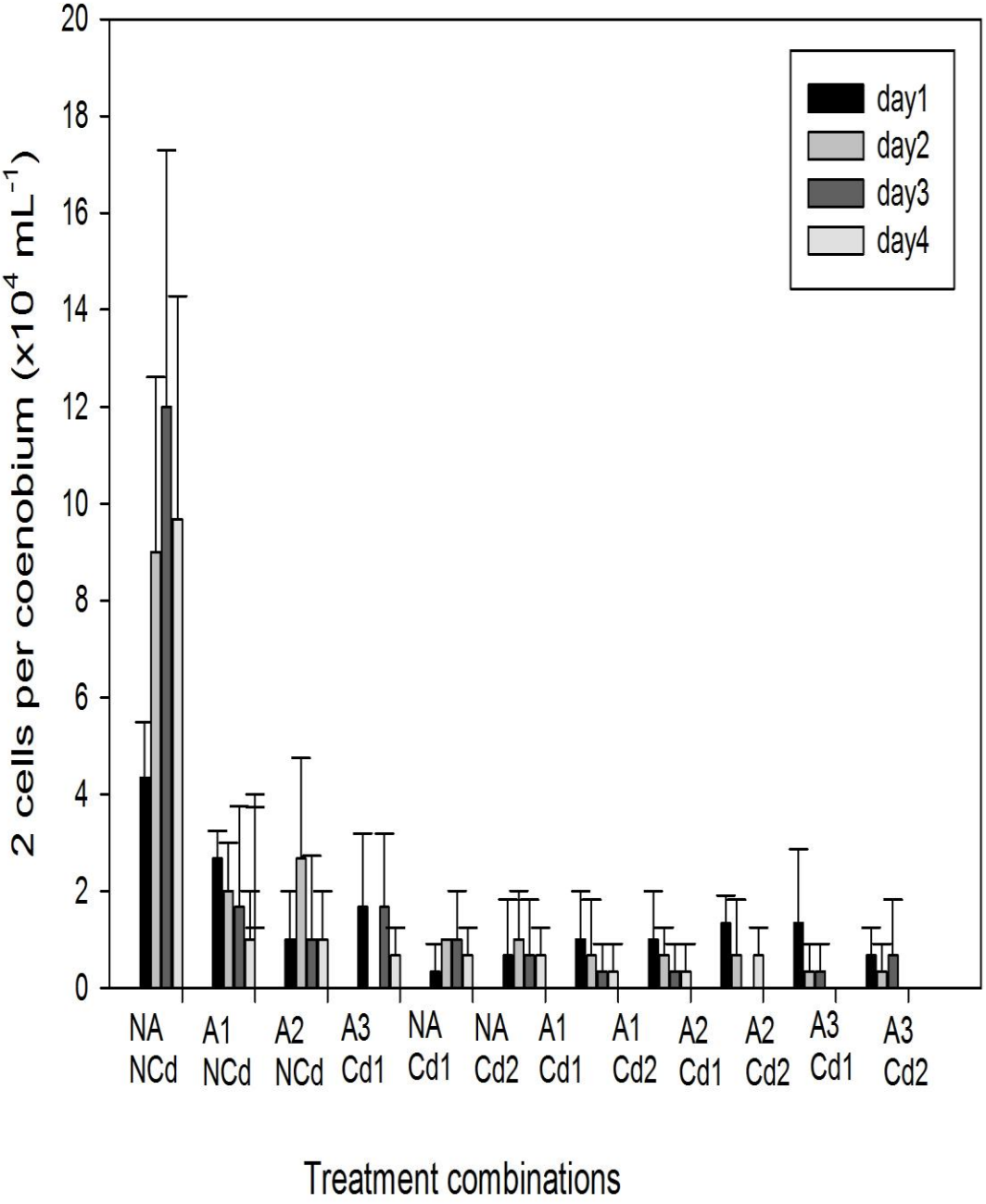


Figure 4.5: Two-celled coenobium formation by *Scenedesmus quadricauda* at different cadmium and atrazine concentrations (values are means ±SD for *n* =3).

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

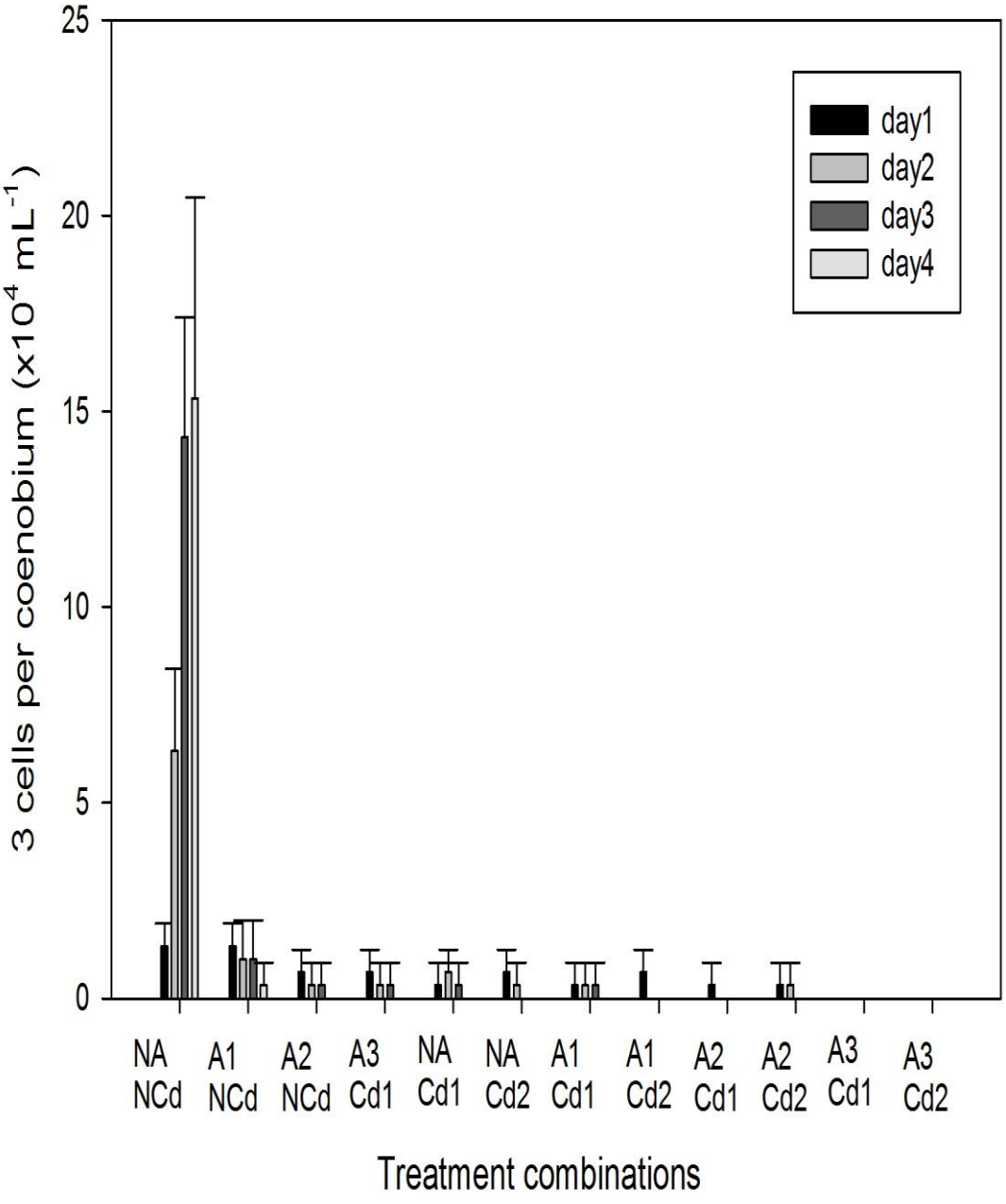


Figure 4.6: Three-celled coenobium formation by *Scenedesmus quadricauda* at different cadmium and atrazine concentrations (values are means ±SD for *n* =3).

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-

0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

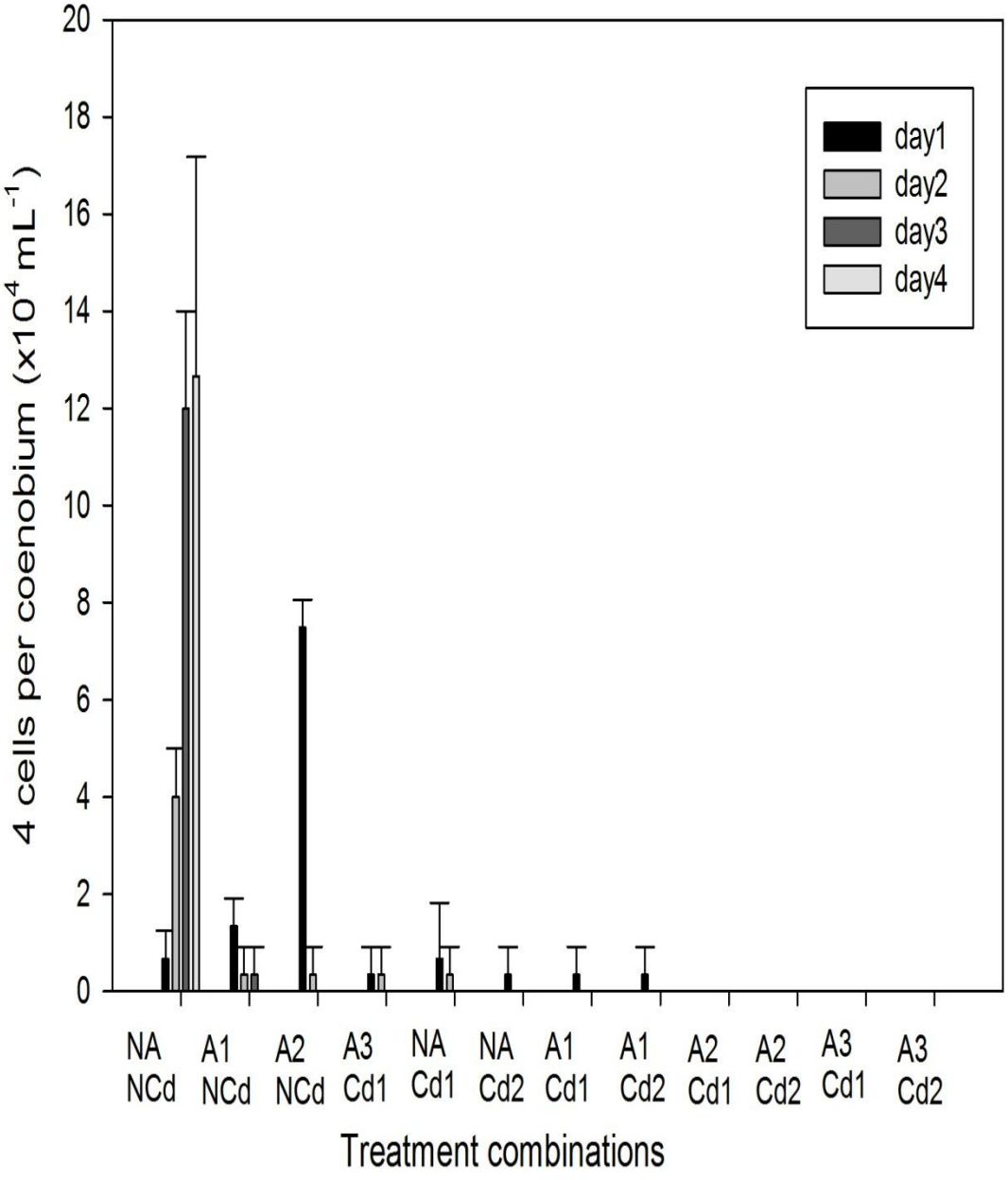


Figure 4.7: Four-celled coenobium formation by *Scenedesmus quadricauda* at different cadmium and atrazine concentrations (values are means ±SD for *n* =3).

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

4.3 Antioxidant Enzyme Activities

The lowest catalase activity was observed in the control and as atrazine and cadmium stress increased, the activity of the enzyme increased. The treatment 0.5mg/l atrazine and 2mg/l cadmium on day 4 had the highest catalase activity (Figure 4.8) Peroxidase (POD) activity significantly increased ($p < 0.05$) with increasing atrazine and cadmium concentrations (Figure 4.9). The lowest POD activity was observed in the control. Superoxide dismutase (SOD) and glutathione S transferase (GST) activities increased with the progression of the experiment. Day 1 showed the lowest GST and SOD activities, while the highest activities were observed on day 4 of the experiment (Figure 4.10, Figure 4.11). As to be expected, the lowest activities of CAT, POD, GST and SOD were observed in the control treatment ($p<0.05$).

4.4 Effect of Cadmium and Atrazine on Total Protein Content of *S. quadricauda*

The lowest protein content was recorded in the control, while in the presence of cadmium and atrazine the total protein concentration increased. Furthermore, protein concentration increased with increasing cadmium and atrazine concentrations and continued to increase in additive manner when both the metal and herbicide were combined. Day 4 of exposure showed the highest total protein content; demonstrating that protein content increased with increased with the progression of exposure experiment (Figure4.12)

4.5 Correlations of Growth, Biomass Production, Phenotypic Plasticity and Antioxidant Enzyme Activities with Atrazine and Cadmium Stress.

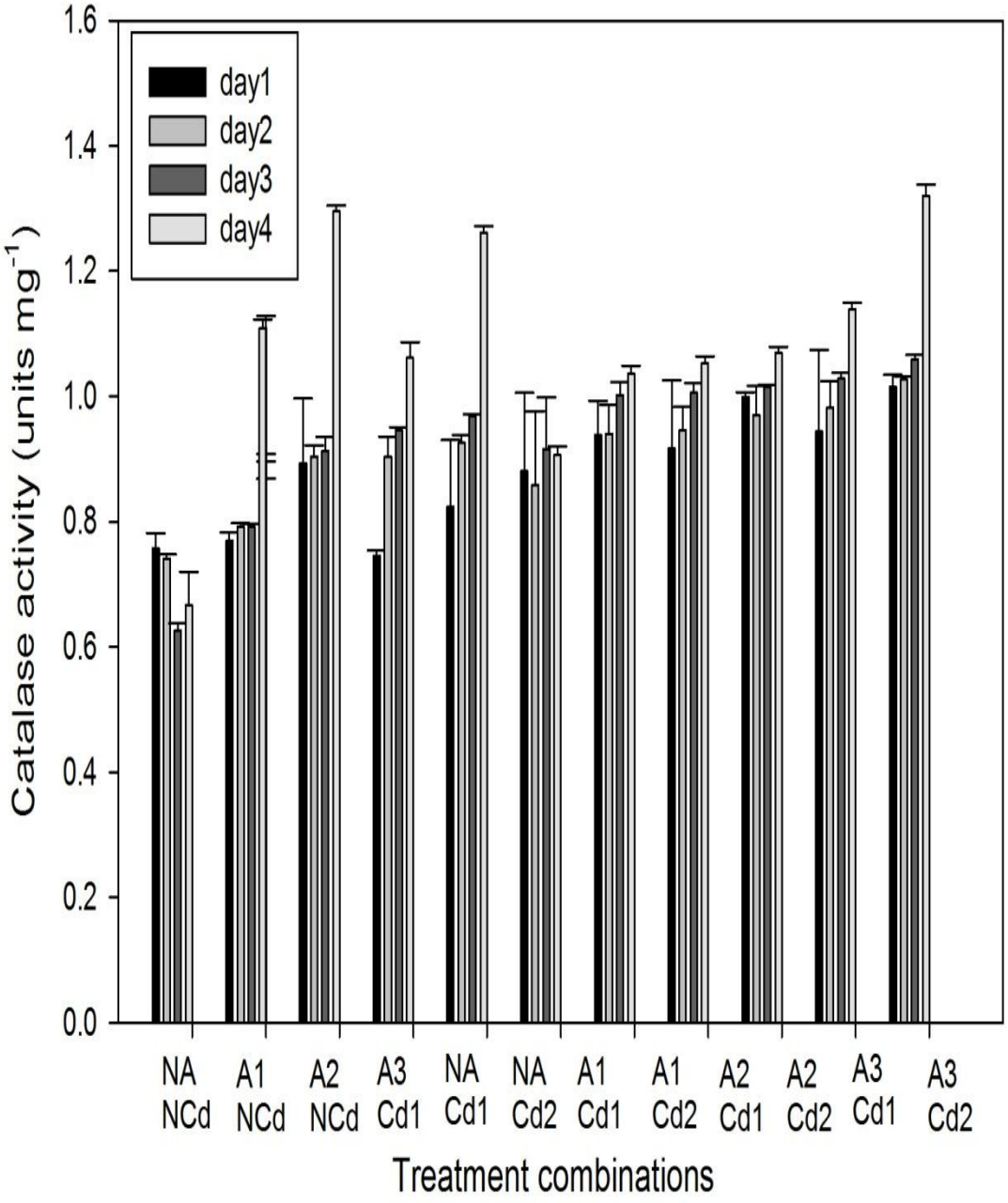
The first two principal components accounted for over 70% of the total variation observed in the PCA (Figure 4.13). SOD, POD, CAT, Protein and GST activity had a significant positive correlation/relationship with cadmium and atrazine concentrations in addition to the exposure time. Free cells, dry weight, chlorophyll a, 2 cell coenobium, cell density, 3 cell coenobium and 4 cell coenobium were negatively correlated with atrazine and cadmium. Biomass production (cell density, dry weight and chlorophyll a production) was negatively associated with atrazine/cadmium and exposure time of the experiment.

4.6 The Effect of Cu and Atrazine on Growth and Biomass of *S. quadricauda*

Scenedesmus quadricauda growth decreased under Cu and atrazine stress. The highest concentration of Cu and atrazine (2mg/L and 0.5mg/L) and their combination had the most significant and severe effect on the growth rate of this microalgae. Growth changes can be seen in (appendices 2). Chlorophyll *a* concentration was reduced with increasing Cu and atrazine concentrations compared to the control without Cu and atrazine. The highest Cu/atrazine combined treatment (2/0.5mg/L, respectively) had the lowest chlorophyll *a* content (Figure 4.14). Dry weight per cell (Figure 4.15) and cell density (Figure 4.16) were also inhibited by increased Cu and atrazine concentrations, which was further reduced with the progression of the exposure experiment ($p<0.05$). There was a slight increase in the chlorophyll *a* content, dry weight and cell density under a low copper concentration (1mg/L).

4.7 The Effect of Cu and Atrazine on The Phenotypic Plasticity of *S. quadricauda*

There was a reduction in the number of cells per coenobium with increased Cu and atrazine concentration. Single cells were seen across all the treatments but were less in the control as the experiment progressed (Figure 4.17). Two cells per coenobium decreased with increased Cu and atrazine concentration (Figure 4.18). The highest quantity of three cells per coenobium was observed in the control on day 4 (Figure 4.19). Coenobium with four cells were dominant in the control treatment (Figure 4.20). The Cu/atrazine (2/0.5mg/L, respectively) treatment had the lowest formation of four cells coenobium by the end of the experiment.



Figures 4.8: Catalase activity of *Scenedesmus quadricauda* as a function of different cadmium and atrazine concentrations.

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

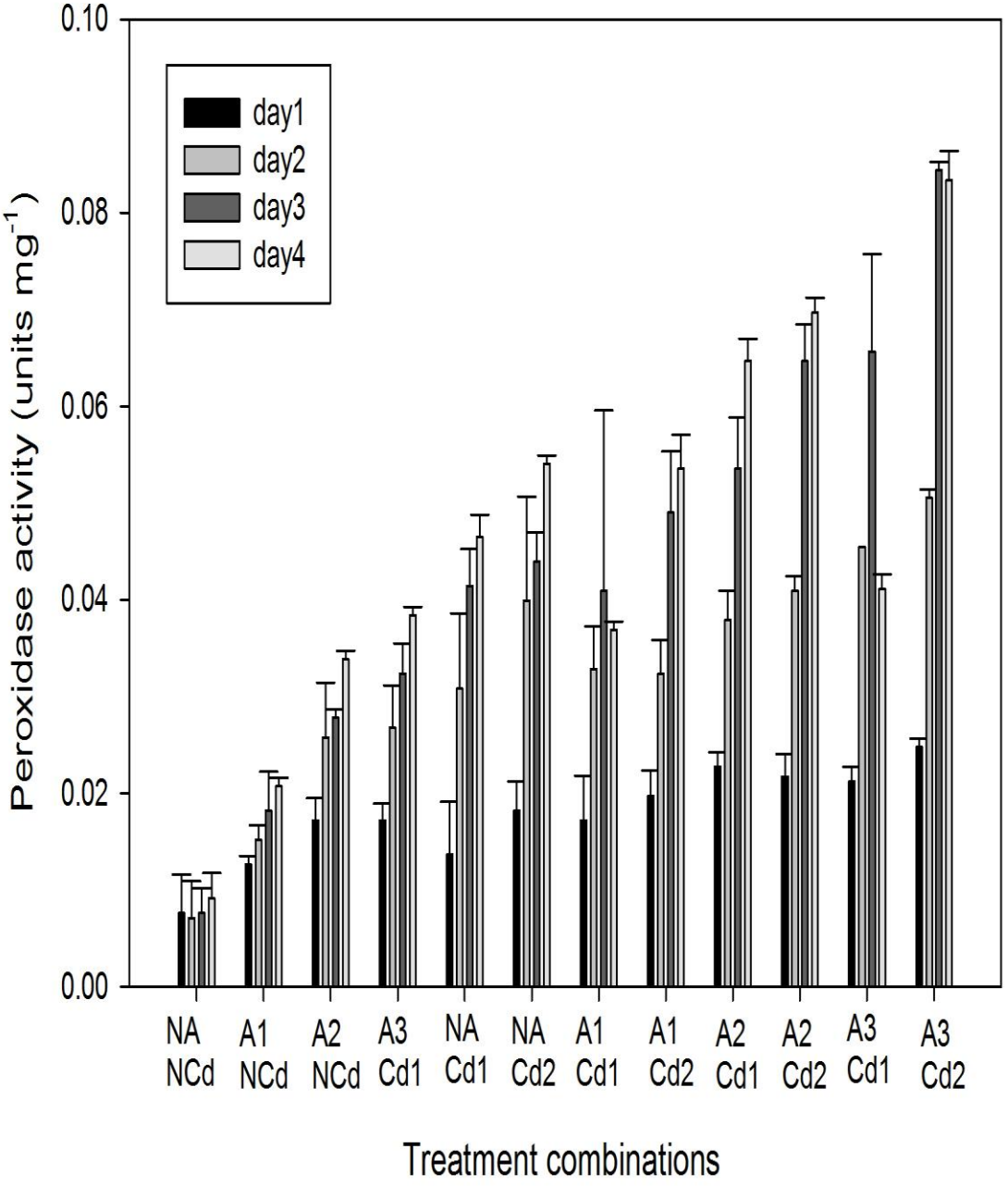


Figure 4.9: Peroxidase activity of *Scenedesmus quadricauda* after exposure to different cadmium and atrazine concentrations.

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

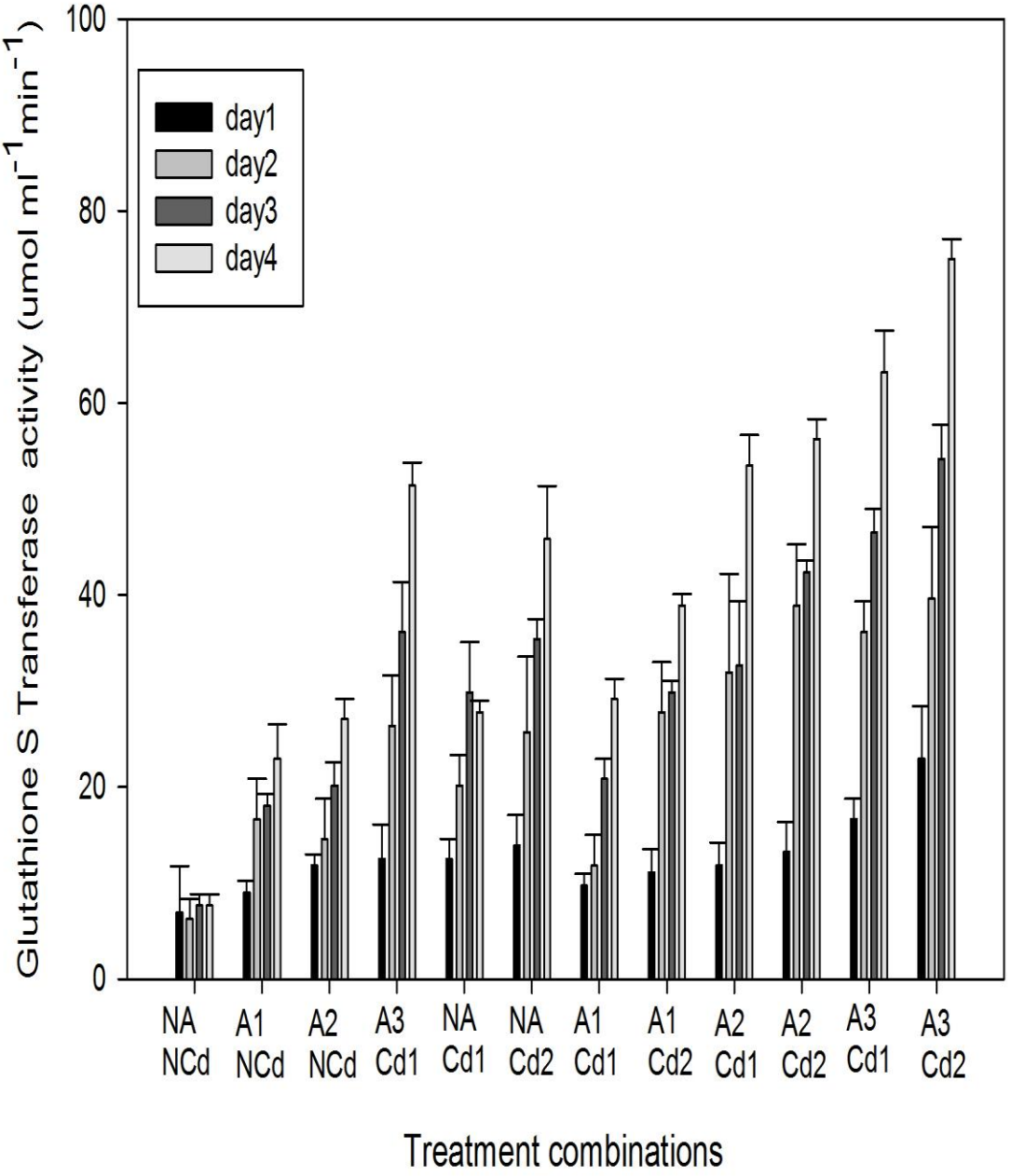


Figure 4.10: Gluthathione –S- Transferase activity of *Scenedesmus quadricauda* at different cadmium and atrazine concentrations

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

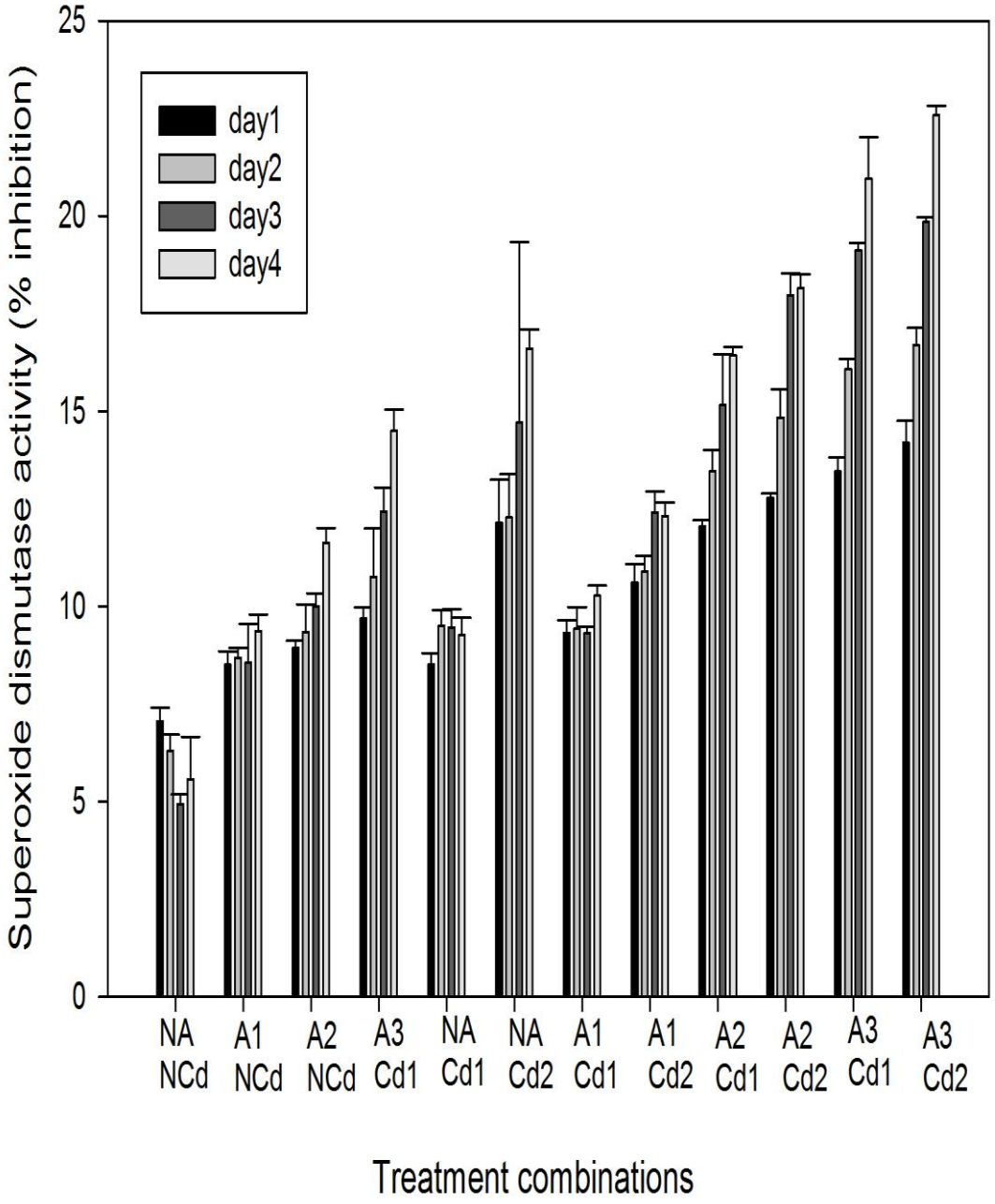


Figure 4.11: Superoxide dismutase activity (%inhibition) of *Scenedesmus quadricauda* at different cadmium and atrazine concentrations.

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

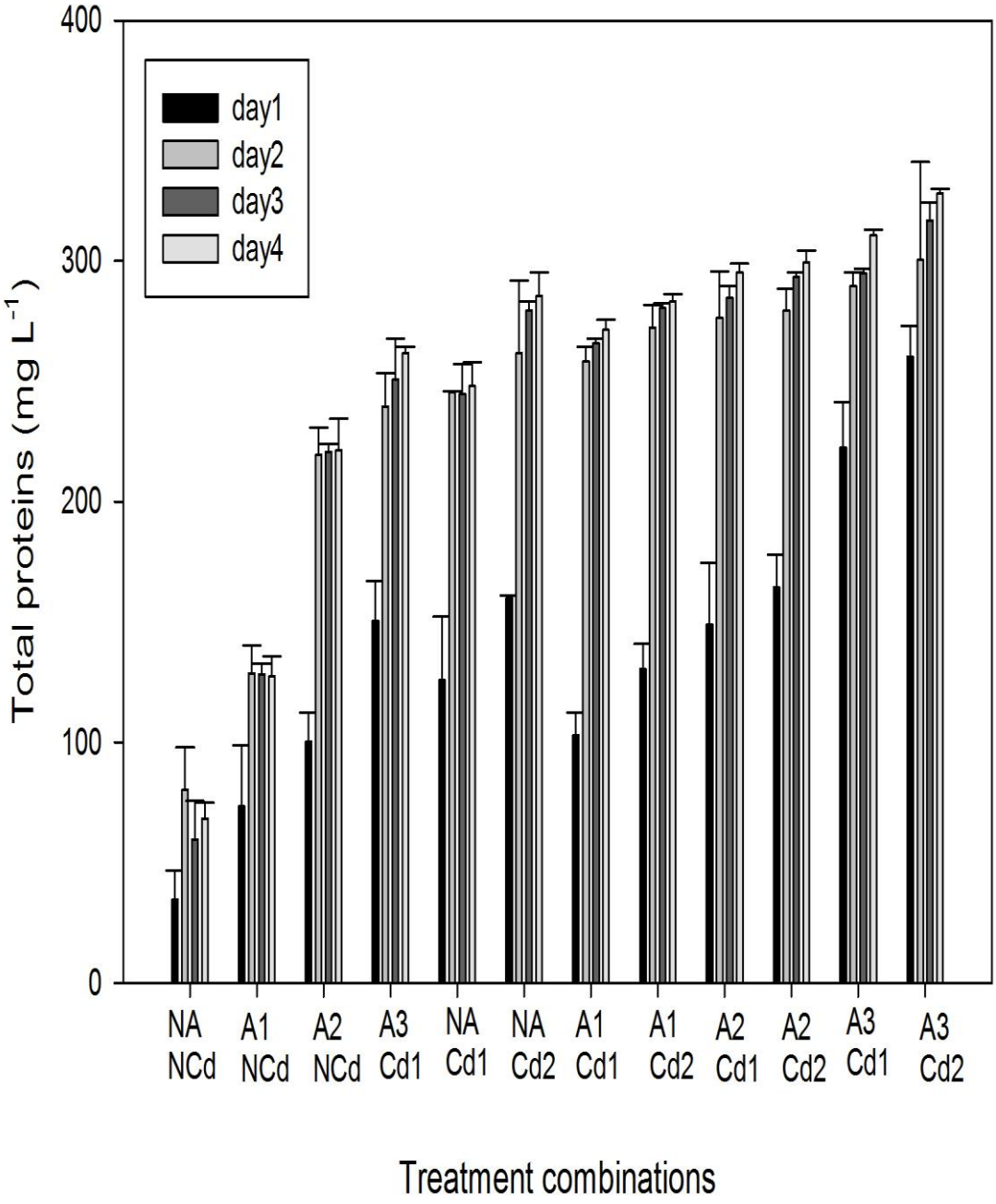


Figure 4.12: Total Protein (µg/l) of *Scenedesmus quadricauda* at different cadmium and atrazine concentrations

Abbreviations

NA/NCd- control A1/NCd- 0.005mg/l A2/NCd- 0.05mg/l A3/NCd- 0.5mg/l

NA/Cd1- 1mg/l NA/Cd2- 2mg/l A1/Cd1-0.005/1mg/l A1/Cd2-0.005/2mg/l

A2/Cd1- 0.05/1mg/l A2/Cd2- 0.05/2mg/l A3/Cd1- 0.5/1mg/l A3/Cd2- 0.5/2mg/l

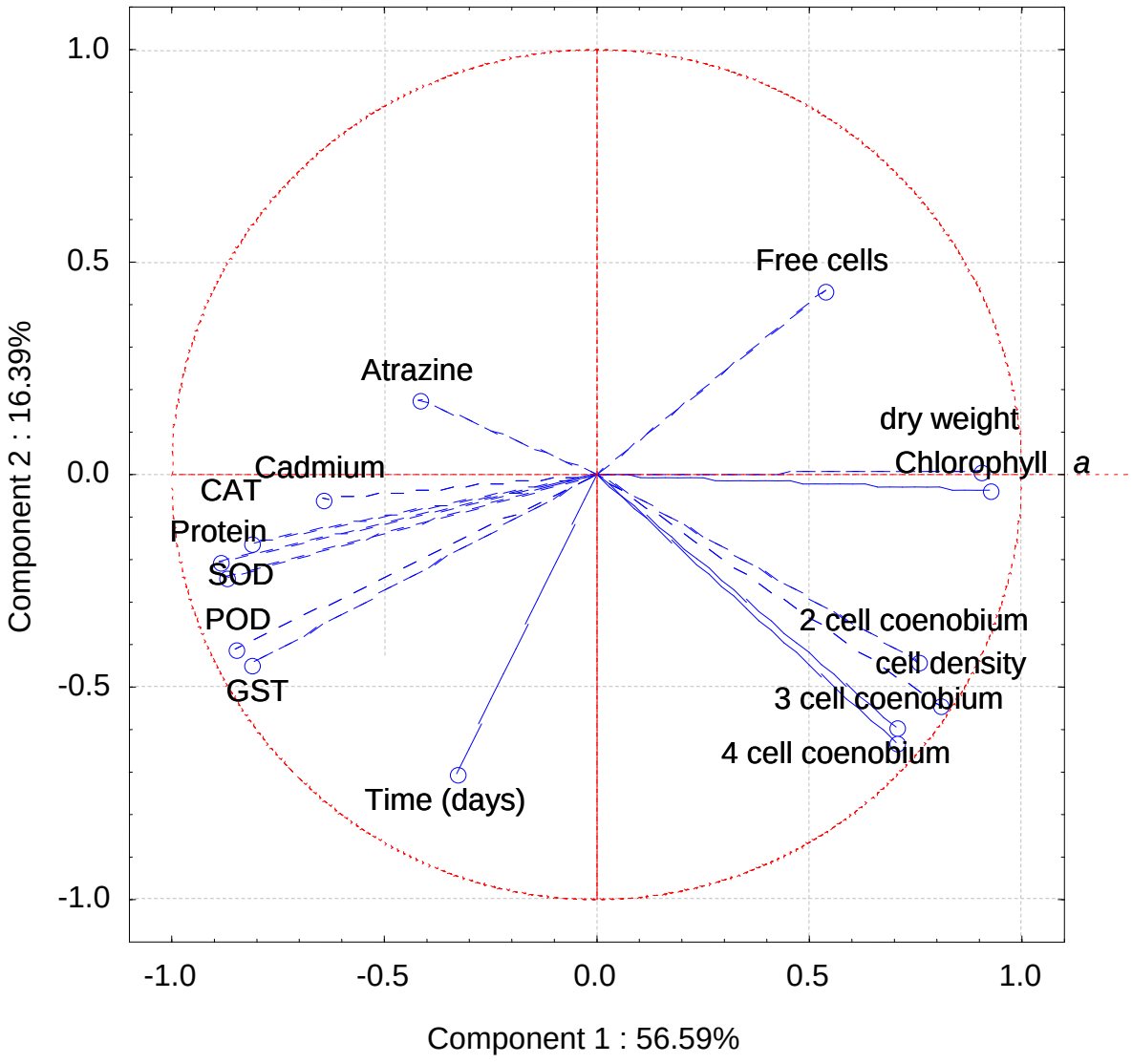


Figure 4.13: PCA showing the relationship between the various parameters measured for *Scenedesmus quadricauda* after exposure to different Cd and atrazine concentrations.

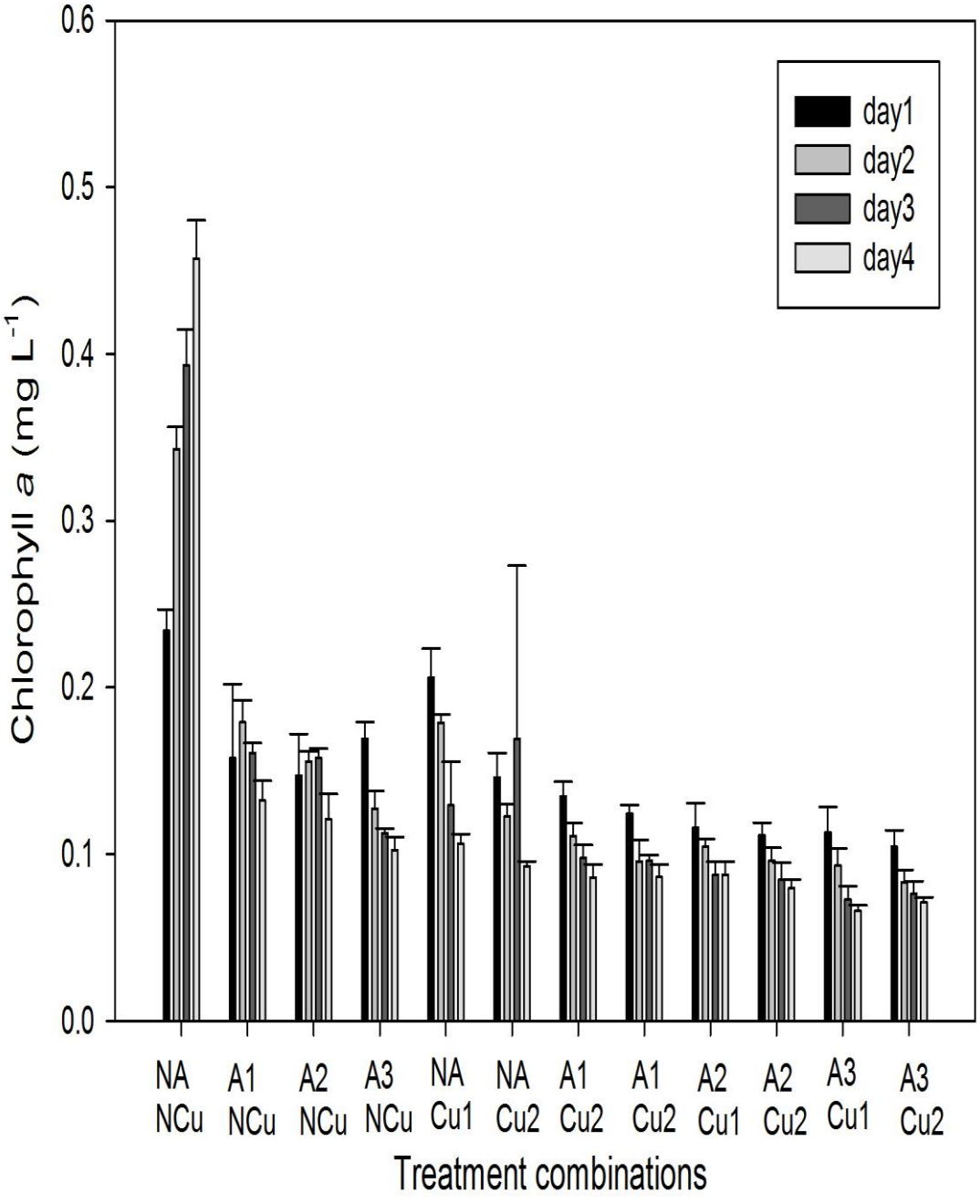


Figure 4.14: Chlorophyll *a* production by *Scenedesmus quadricauda* at different Cu and Atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

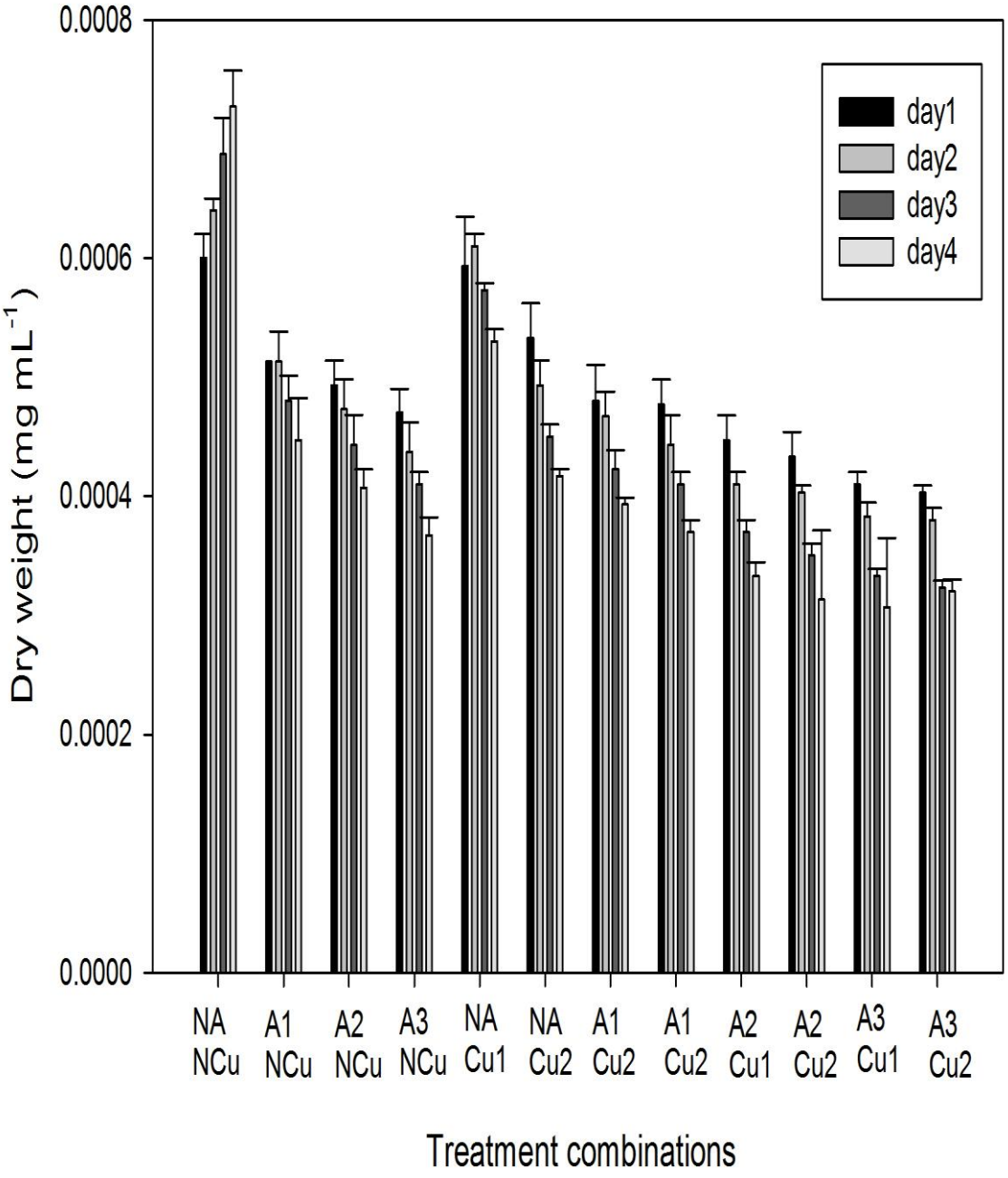


Figure 4.15: Dry weight of *Scenedesmus quadricauda* at different Cu and atrazine concentrations

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

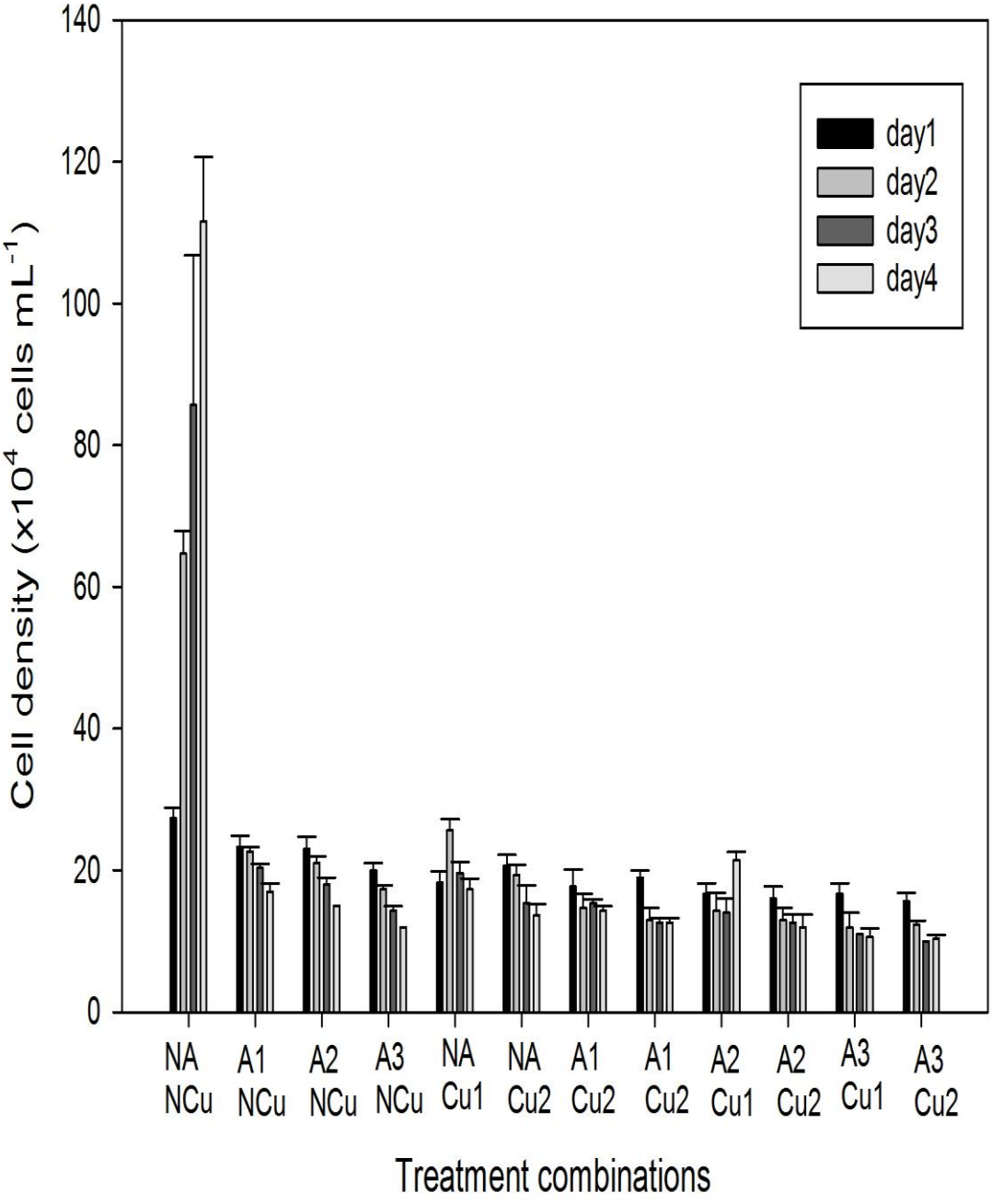


Figure 4.16: Cell counts (x10^4 cells per ml) of *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

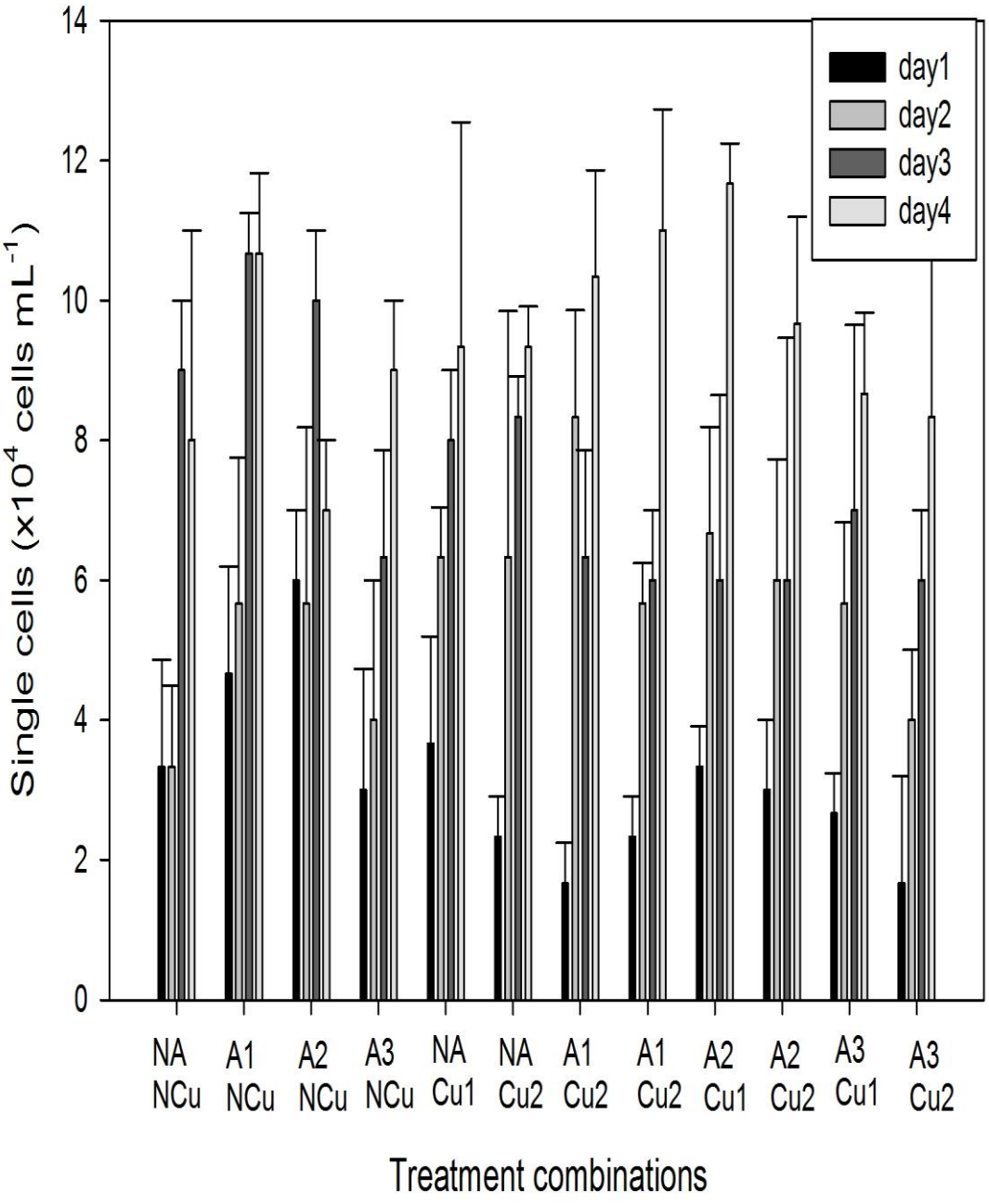


Figure 4.17: Single cells formation by *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

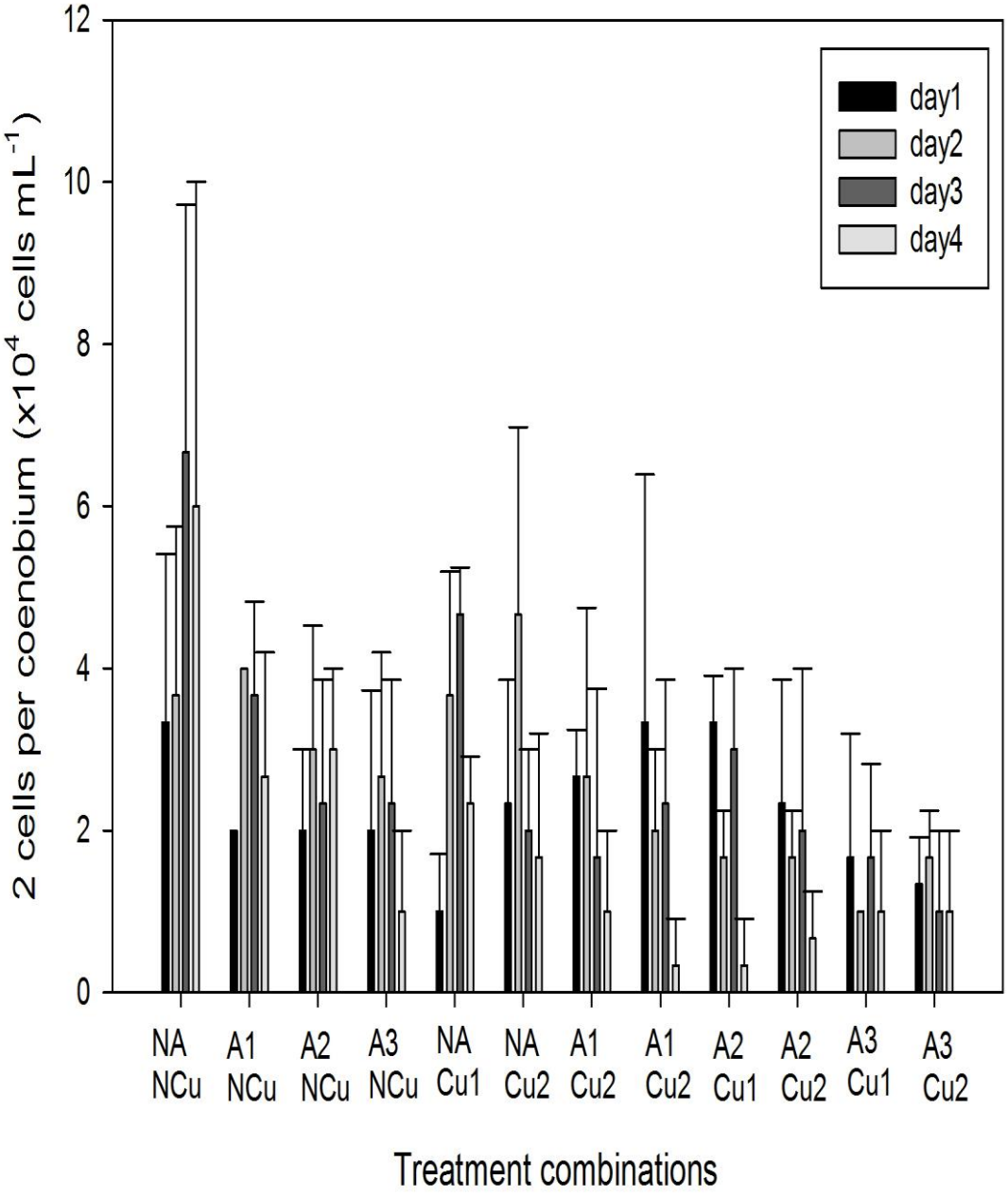


Figure 4.18: Two-celled coenobium formation by *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

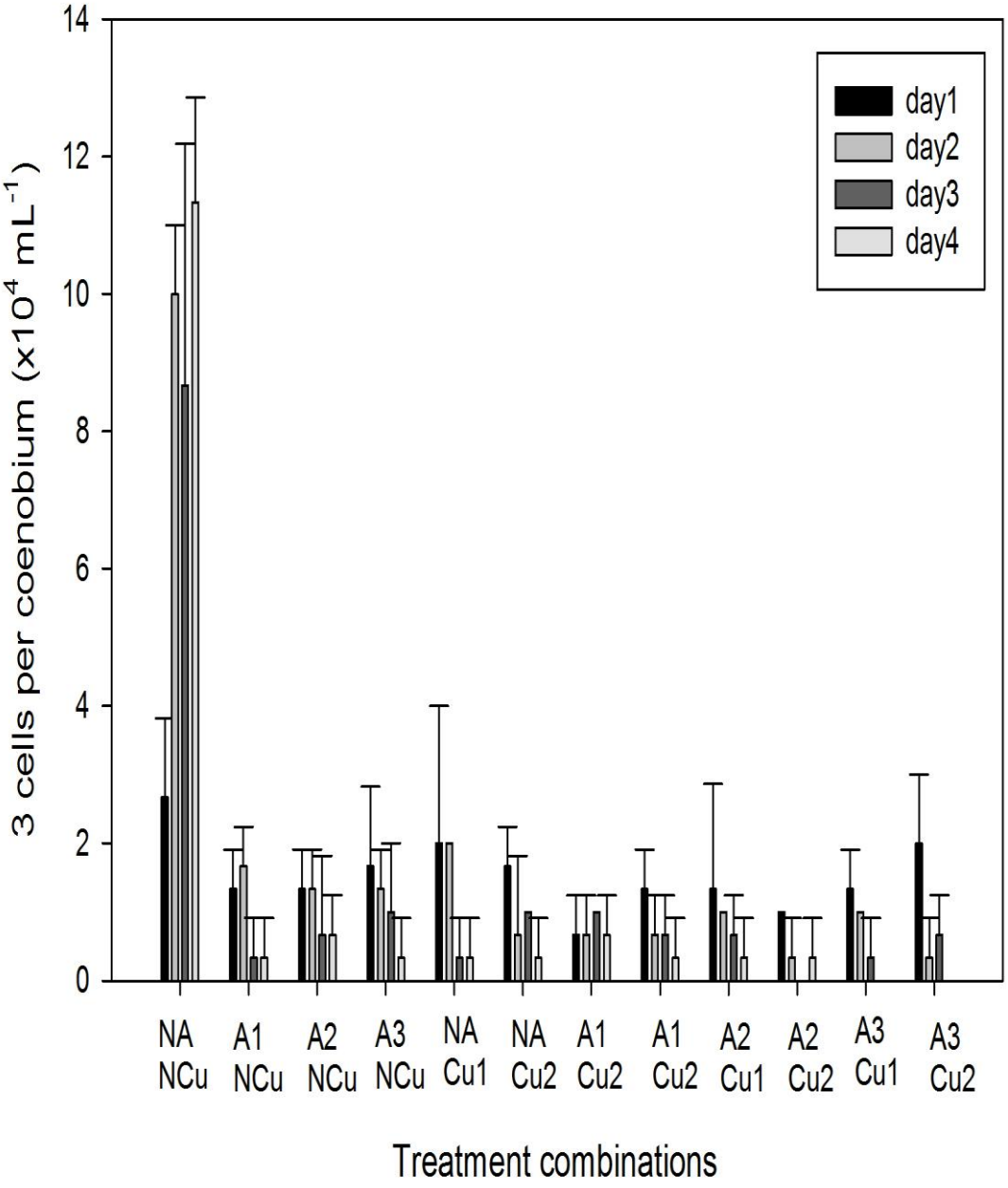


Figure 4.19: Three-celled coenobium formation by *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-

0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

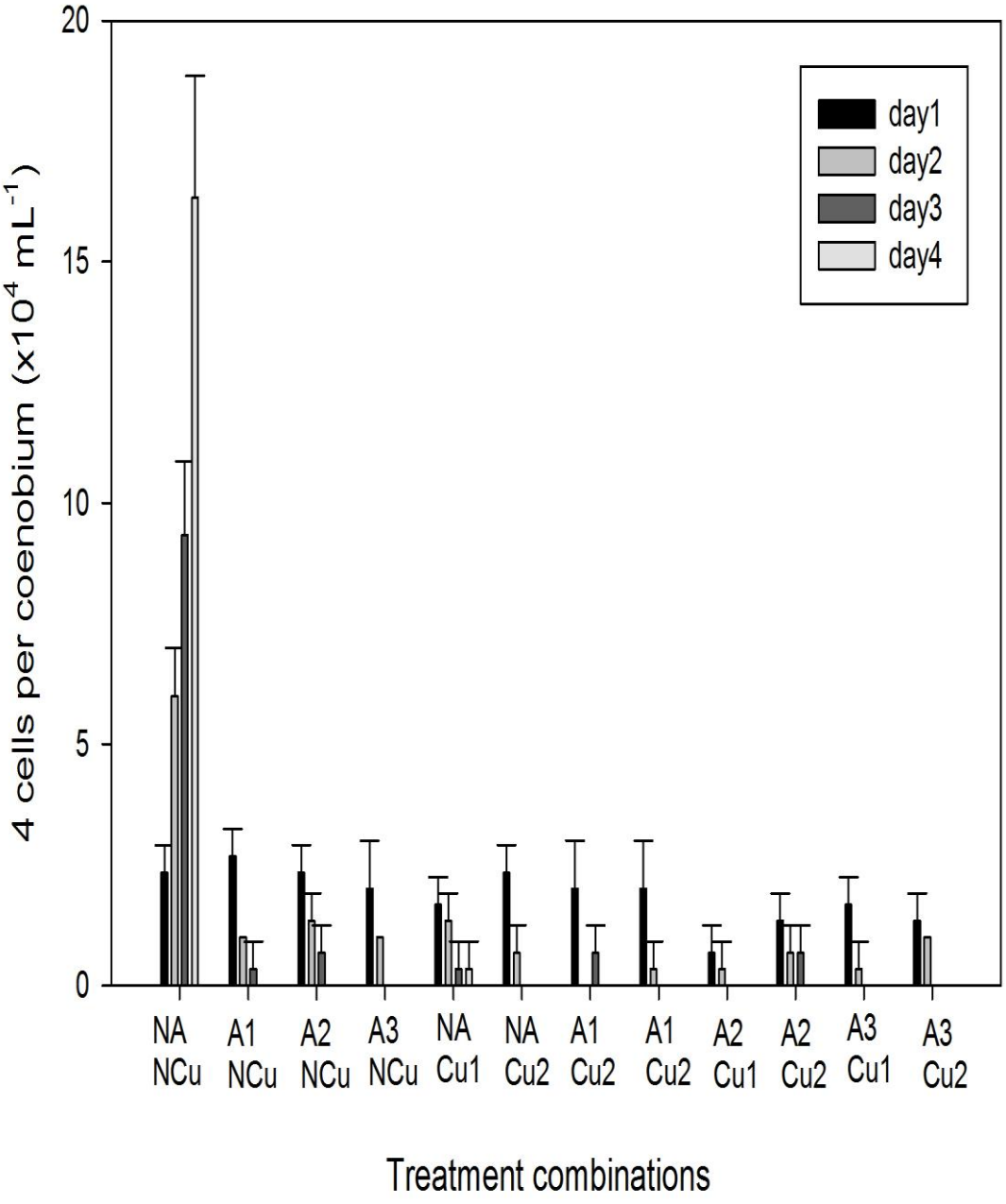


Figure 4.20: Four-celled coenobium formation by *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

4.8 Effect of Cu and Atrazine on The Antioxidant Enzymes of *Scenedesmu quadricauda*

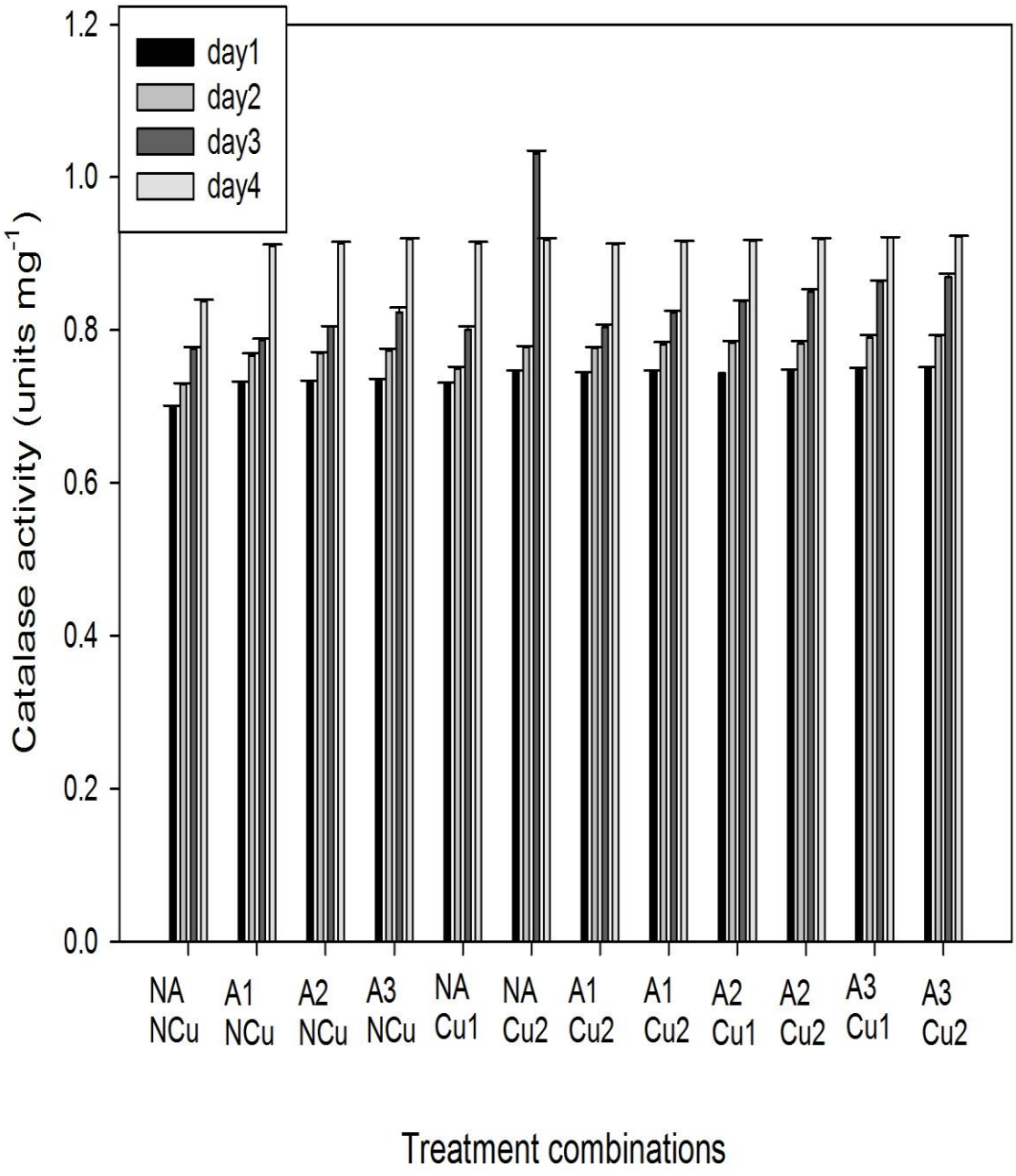
There was a significant increase in catalase (CAT) activity with increasing Cu/atrazine concentrations. The highest CAT activity was recorded at the highest Cu concentration (2mg/L, Figure 4.21). The intermediary copper concentration triggered higher activity of catalase on day 3 of the experiment but decreased on the 4thday. Increased concentrations of Cu and atrazine stimulated higher peroxidase activity, while the control treatment had the lowest activity (Figure 4.22). Glutathione S-transferase activity significantly increased as Cu and atrazine concentrations increased (Figure 4.23). The control treatment showed lower superoxide dismutase activity compared to the treatments with individual and combination of the metal and herbicide (Figure 4.24).

4.9 Effect of Cu and Atrazine on Total Protein Content of *S. quadricauda*

The highest protein content was recorded in the combined treatment having 2 mg/L copper and 0.5mg/L atrazine on day4 (Figure 4.25). In general, protein concentration increased with increasing copper and atrazine concentration. The control was characterized by having the lowest total protein.

4.10 Correlations of Growth, Biomass Production, Phenotypic Plasticity and Antioxidant Enzyme Activities Under Atrazine and Cu Stress

SOD, POD, CAT, Protein, single cell formation and GST activity positively correlated significantly with copper, Atrazine concentrations and time of exposure. Dry weight, chlorophyll *a*, 2 cell coenobium, cell density, 3 cell coenobium and 4 cell coenobium were negatively correlated/associated with atrazine and copper(Figure 4.26). The first two principal components accounted for over 60% of the total variations of the PCA.



Figures 4.21: Catalase activity of *Scenedesmus quadricauda* as a function of different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

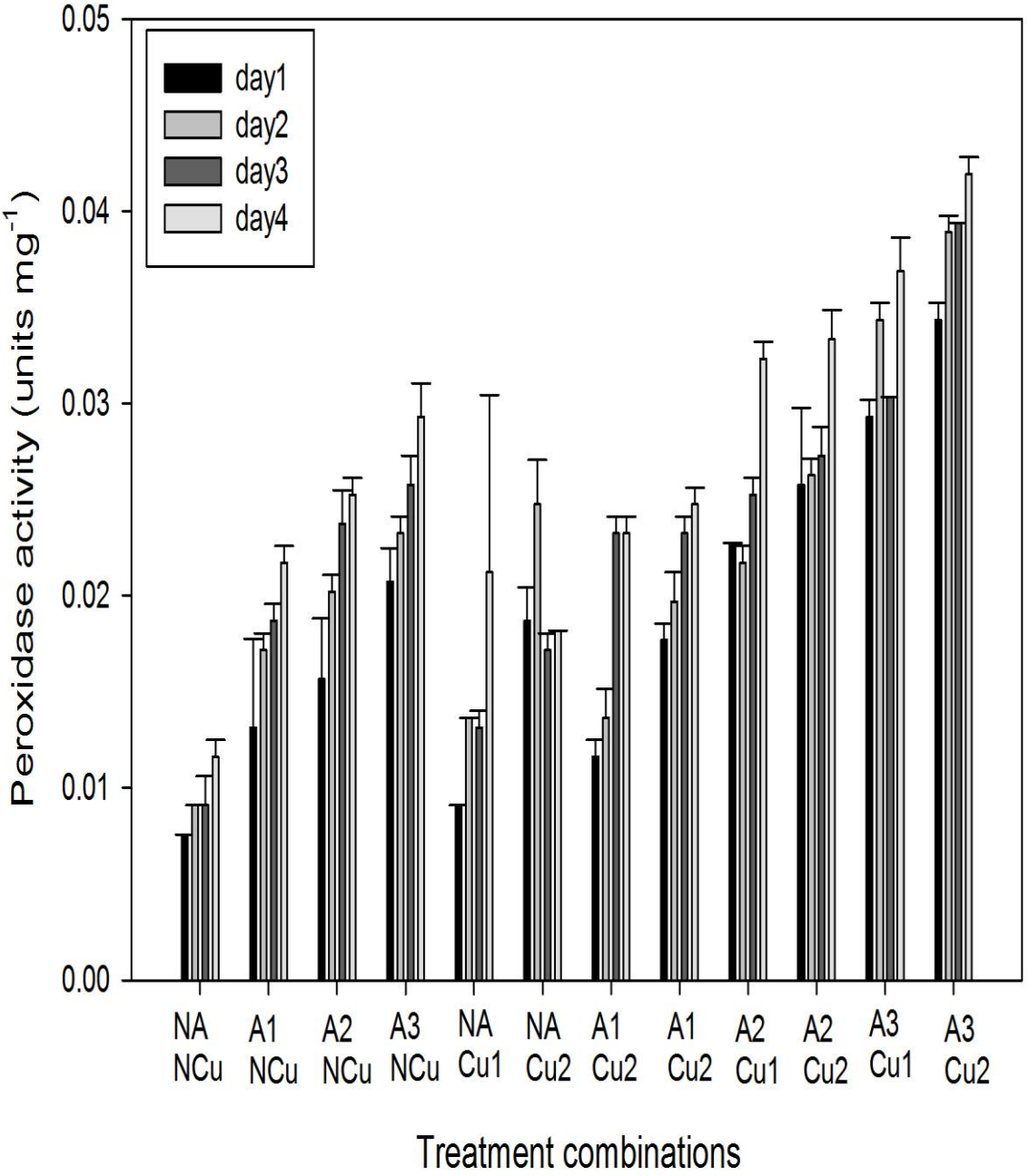


Figure 4.22: Peroxidase activity of *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

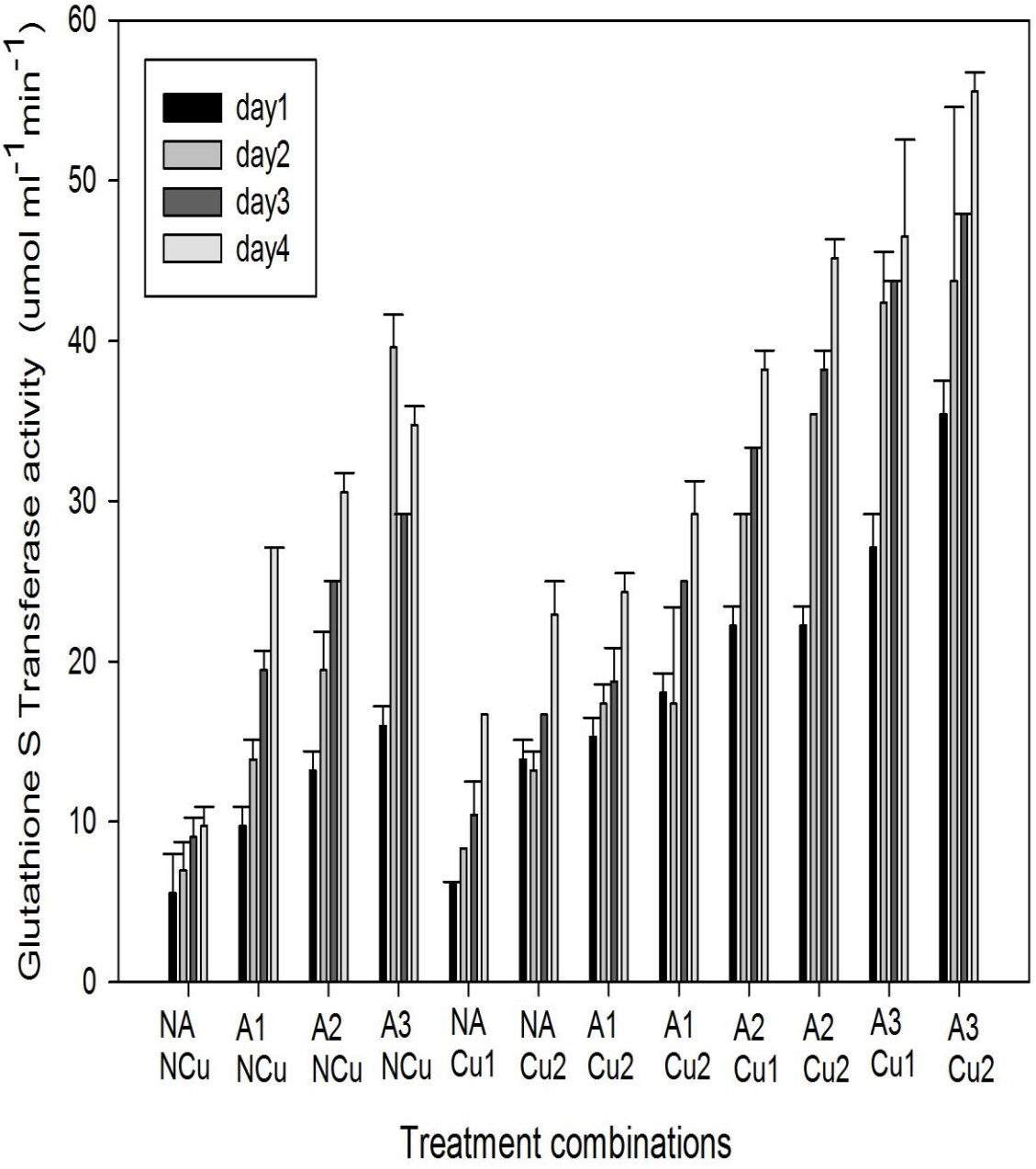


Figure 4.23: Glutathione-S-Transferase activity of *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

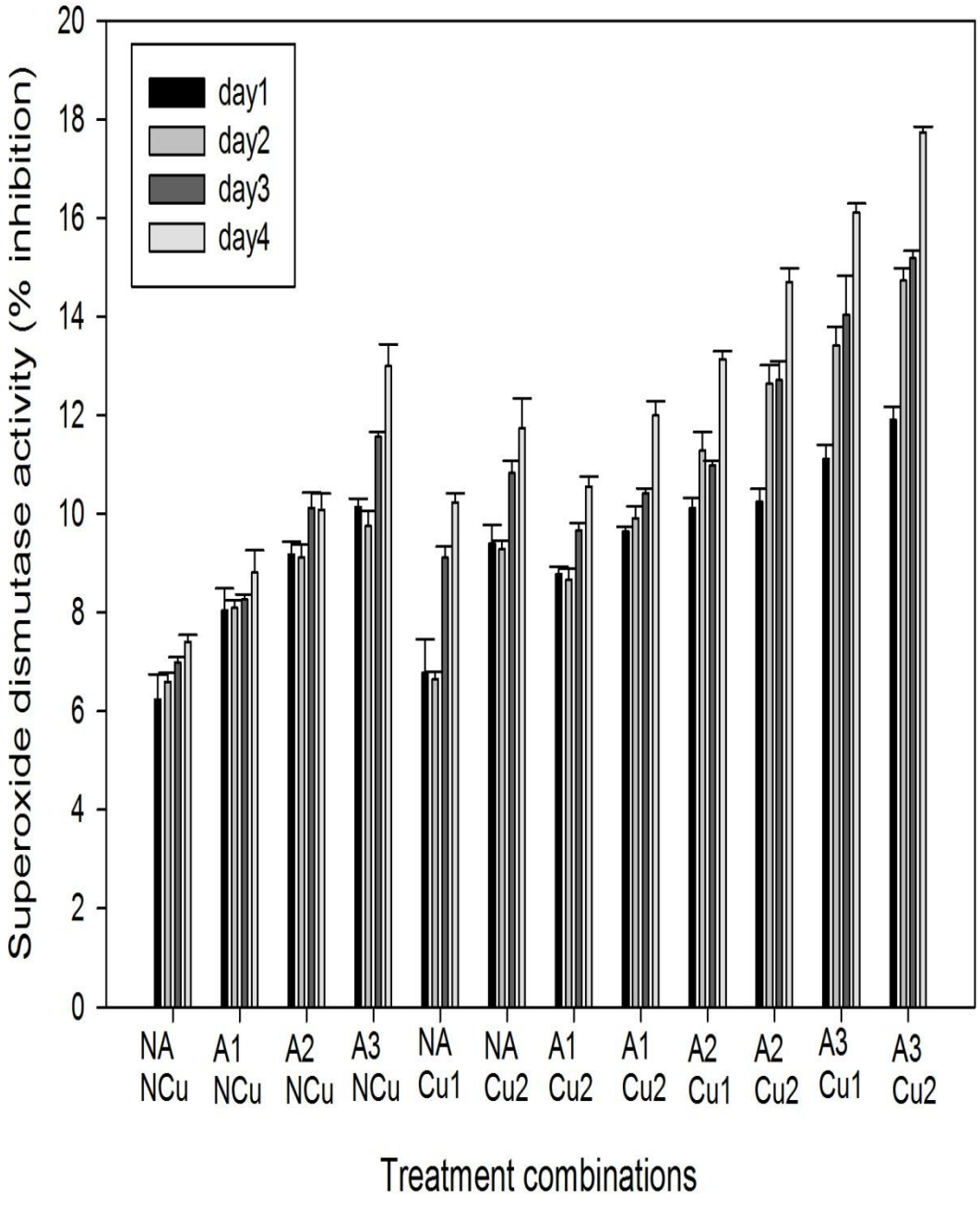


Figure 4.24: Superoxide dismutase activity (%inhibition) of *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-

0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

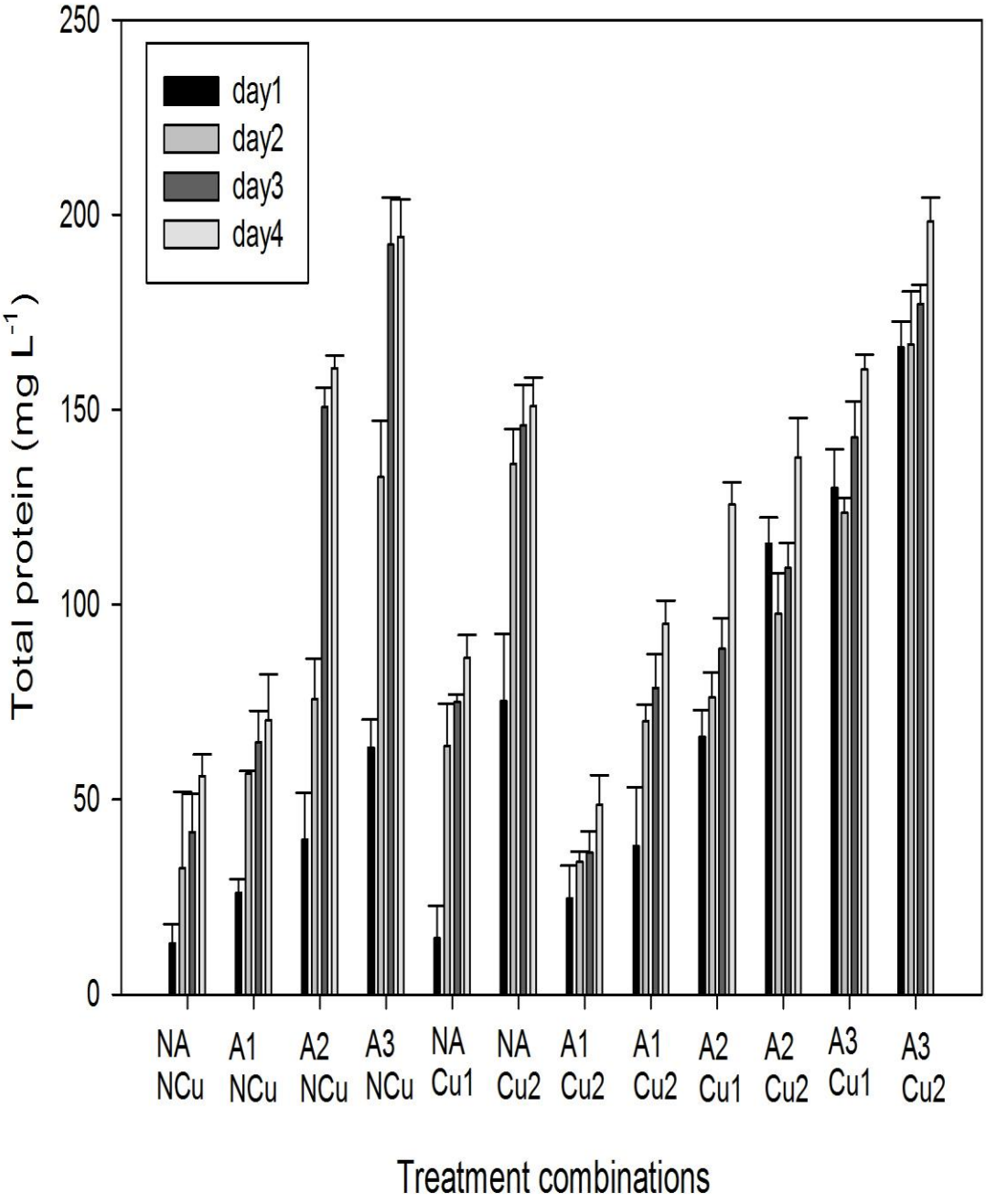


Figure 4.25: Total protein production of *Scenedesmus quadricauda* at different Cu and atrazine concentrations.

Abbreviations

NA/NCu- control A1/NCu- 0.005mg/l A2/NCu- 0.05mg/l A3/NCu- 0.5mg/l

NA/Cu1- 1mg/l NA/Cu2- 2mg/l A1/Cu1-0.005/1mg/l A1/Cu2-0.005/2mg/l

A2/Cu1- 0.05/1mg/l A2/Cu2- 0.05/2mg/l A3/Cu1- 0.5/1mg/l A3/Cu2- 0.5/2mg/l

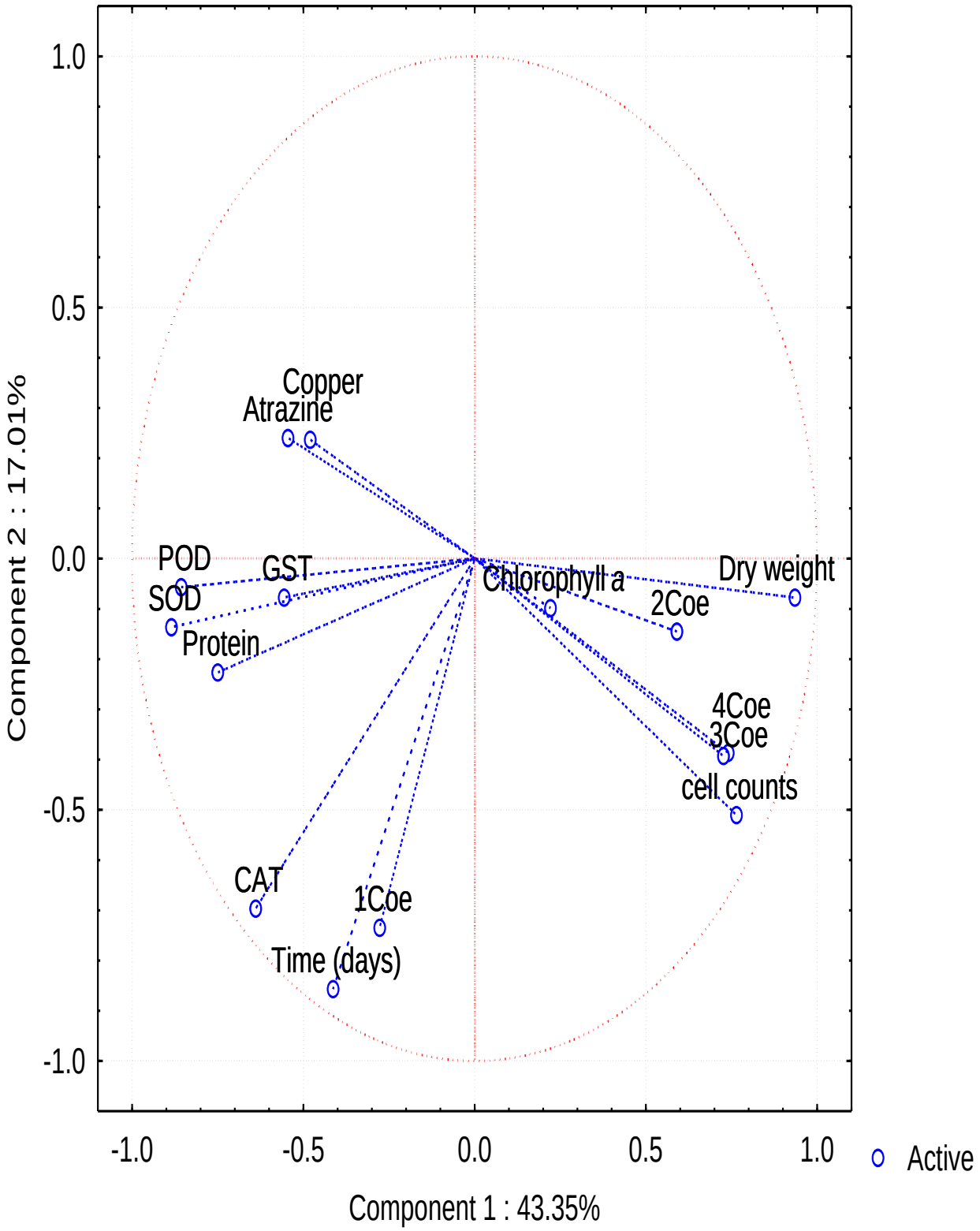

1.0
0.5
0.0
-0.5
-1.0
Component 2 : 17.01%
Copper
Atrazine
POD
GST
SOD
Protein
Chlorophyll a
Dry weight
2Coe
4Coe
3Coe
cell counts
CAT
1Coe
Time (days)
-1.0
-0.5
0.0
0.5
1.0
Component 1 : 43.35%
Active

Figure 4.26: PCA charts for the various parameters measured for *Scenedesmus quadricauda* at different Cu and atrazine concentrations
Coe- Coenobium

CHAPTER FIVE

5.0 DISCUSSION

5.1 Effect on Growth and Biomass Production

5.1.1 Growth, dry weight and chlorophyll *a* content

The presence of cadmium/atrazine and copper/atrazine caused a significant reduction in the growth of *S. quadricauda*, which is similar to the findings of Junghans *et al.* (2003).These authors showed that mixture of toxic chemicals (chloroaceta-nilide) demonstrated additive and synergistic inhibitory effects that were considerably higher than those of the individual components on microalgae. However, He *et al*. (2012) indicated that joint action of two herbicides showed antagonism to *Scenedesmus obliquus*. The decreased dry weight and chlorophyll *a* concentrations recorded in this study in the presence of different copper and cadmium combinations may be because these metals are capable of destroying and decreasing microalgae chlorophyll content. This is mainly because of the reduction and oxidation of various components involved in the biosynthesis pathway of pigments (Battah 2010). According to Wang and Dei (2001)the reduction in cell density observed in the presence of increasing metal content and combination with atrazine may be due to the blocking of the intake of other nutrients into the cell. This is because of the binding of metal ions to the cell nutrient transport systems. The negative effect of Atrazine on the production of chlorophyll *a* obtained in the present study agrees with the results of He *et al.,* (2012), who showed a decrease in chlorophyll *a*

concentration in cultures exposed to atrazine. Also Ma *et al.* (2006) showed that decreased chlorophyll *a* concentrations can lead to a decline in photosynthesis, thereby affecting growth rate and biomass concentrations in the cultures. According to Suarez *et al*., (2010) the cellular density of *Chlamydomonas moewusii* showed a concentration depended response when the microalgae was exposed to different cadmium concentrations. Investigations using different algal species as test organisms showed that algae vary greatly in their responses to different compounds. Algal cells exposed to heavy metals may suffer serious physiological alterations that can be readily observed via changing growth, pigment and biomass production (Chia *et al.*, 2013). So also this agrees with the findings in this study, where the growth and biomass production (cell number, dry weight production and chlorophyll *a*) of *D. quadricauda* slowed down with exposure to environmentally relevant concentrations of $CdCl_2$and $CuCl_2$. Furthermore, Bisova *et al*. (2003), Bajguz (2011), Tripathi and Gaur 2006) observed that growth rate, protein content and chlorophyll production of the microalgae decreased with increasing cadmium concentrations.

The mechanisms of toxicity of metal compounds (including Cu and Cd) on photosynthetic processes are still subject to discussions they are capable of decreasing the activity of photosystem II (PSII). Baron *et al*., (1995) showed that Cu has a direct impact on photosynthesis by inhibiting photosynthetic electron transport, especially in PSII.

In the chloroplast compartment, the presence of cadmium ions has been demonstrated to cause the destruction of chloroplasts and DNA-containing structures (Cepak *et al*., 2002). The effect of metal ions on plants includes the disruption of many physiological functions such as water uptake, respiration, mineral nutrient uptake and photosynthesis (Burzynski

and Zurek, 2007). Cadmium altered the activity of the oxygen-evolving complex in pea and broad bean, ultimately causing the disassembly of their PSII (Geiken *et al*., 1998; Herbette *et al*., 2006).

5.2. Atrazine, Copper and Cadmium Effect on Phenotypic Plasticity

Morphological variations provided an alternative means of evaluating the effect of toxic compounds to microalgae. Coenobial structure was significantly affected with increased in concentration and time of exposure to atrazine, copper and cadmium. A decrease in the number of cells per coenobium with increase in copper concentrations has also been shown by (Lombardi *et al*. (2007) in *Scenedesmus incrassatulus* and *Scenedesmus acuminatus* where there were transformations from 4-coenobia to 3-coenobia, 2-coenobia and unicellular forms. The effect of copper, cadmium and atrazine on phenotypic plasticity and morphological changes obtained in this study was similar to the findings of Peña-Castro *et al*., (2004). The authors exposed *Scenedesmus incrassatulis* to increasing concentrations of heavy metals (cadmium, copper, and chrominum). Zekiye and Omer (2012) reported similar findings when *Acutodesmus obliqus* and *Desmodesmus subspicatus* were exposed to increasing Zn concentrations. Therefore this indicates that coenobium structure is a very sensitive parameter that responds to herbicides and heavy metals variations. This parameter can be used as an important biomarker for monitoring the effect of toxic substances in the aquatic environment. He *et al.* (2012) showed that when treated with high concentrations of atrazine and butachlor, the micro algal cells demonstrated obvious changes in morphology, the four-piece structure of the *Scenedesmus* were destroyed and algal cells enlarged. Contrary to our findings,

Mohammed and Markert (2006) observed that at that Lead (Pb) concentrations had no effect on the morphology of *Scenedesmus*.

5.3 Effect on Total Protein Content of *Scenedesmus quadricauda*

The results from our study showed that total protein content of *Scenedesmus quadricauda* increased with increasing copper, cadmium and atrazine concentrations.Combinations of these heavy metals with the herbicide also stimulated higher protein production in the microalgae. There by demonstrating that the combination of the metals with the herbicide has an additive effect on protein production of the microalgae. This may also be as a result of low activation of protease to oxidize the protein content. This is similar to the result of Deng *et al*.(2012) who showed that Irgarol 1051 at 0.01 mol L^{-1} caused a significant increase in soluble protein content after 96 h of exposure ($P < 0.05$), up to 1.2 times greater than that in the untreated group. Also Branco *et al.* (2010) reported that protein content tended to increase as cadmium concentration in the medium was raised; at the lowest cadmium concentration changes in protein content were not significant, whereas at 0.2 and 0.3mg significant differences were observed.

Contrary to our findings, total amino acids content of the algal *Chlorella vulgaris* were significantly decreased with increasing stress(Fayez and Elfattah2007;Bajguz 2011; Kumar *et al* 2010).

5.4 Antioxidant Enzyme Activities of *Scenedesmus quadricauda*

5.4.1 SOD, Catalase, POD, and GST activities

This present study demonstrated an up regulation of the important antioxidant enzymes SOD, POD, CAT and GST in *Scenedesmus quadricauda* after exposure to Cd/atrazine and Cu/atrazine stress. This increased activity of these enzymes is indicative of the toxic effect of cadmium, copper and atrazine. The up-regulation of antioxidant enzyme activities means there was an increased ROS content, which are able to disrupt chlorophyll synthesis and inhibit cell growth (Qian *et al.* 2009).Many studies have shown that reactive oxygen species are produced when algal was exposed to different pollutants (Qian *et al*. 2009). Reactive oxygen species (ROS)are formed and degraded by all aerobic organisms, leading to either physiological concentrations required for normal cell function or excessive quantities, a state called oxidative stress (Pinho and Ladeiro 2012). In agreement with our findings, Li *et al*.,(2006) showed that copper at the greatest dose caused a similar increase in antioxidant (GSH) and antioxidant enzymes (SOD, CAT and GPX) compared to the control, which was accounted for a circumstantial evidence for enhanced production of free radicals under metal stress.

In agreement with our results, SOD activity have been demonstrated to increase after *Ditylum brightwellii* was exposed to copper and *Tetraselmis gracilis* to cadmium (Rijstenbil *et al*., 1994; Okamoto *et al*., 1996; Qian *et al*., 2008).Thus the increased activities of this enzyme suggests increased production of H_2O_2. To cope with metal stress, algae possess mechanisms to protect themselves from metal injury and poisoning, these mechanisms include extra cellular and intracellular sequestration of metal ions (Tripathi *et al*, 2006). Lee and Shin (2003)also found that catalase activity increased slightly when the microalga *Nannochloropsis oculata* was exposed to cadmium stress. A number of studies further supported our findings that when faced with stresses, these

enzymes are synthesized in high quantity to reduce the effect of ROS and further reduced oxidative damage (Collen *et al*., 2003;Qian *et al*. 2007;Branco *et al*. 2010; Qian *et al*. 2010).

The interaction between the metals and atrazine generally had an additive effect on the activity of most of the enzymes investigated in this study.Consequences of ROS formation include the gradual peroxidation of lipid structures (Baryla *et al*., 2000), oxidative DNA damage (Kasprzak,2002) and photosynthetic apparatus damage (Dewez *et al*., 2005). Devos *et al*. (1991) showed that Cu disturbed the integrity of thylakoid membranes. Yruela (2005) showed that ROS interfered with the biosynthesis of photosynthetic machinery and decreased the photosynthetic rate. He further stated that the effects of higher copper and zinc concentrations on growth of microalgae observed could be associated with the increased in lipid peroxidation, measured as MDA production compared to the control.

Contrary to our findings, Deng *et al*. (2012) showed that strong environmental stresses may cause the inactivation of CAT. The high suppression of CAT activity under high concentrations of Irgarol 1051 and Diuron indicated that antioxidant defence enzymes such as CAT might be an important site of action for Irgarol 1051 and Diuron in microalgae. The mechanism of defence of *Scenedesmus quadricauda* to Cd/atrazine and copper/atrazine toxicity is associated with the activities of these antioxidant enzymes as they showed a significant association with increasing Cd/atrazine and Cu/atrazine concentrations and the time of exposure. The increased activity of SOD with increased number of atrazine exposure time may also be due to the pivotal role it plays as the first line of defence against ROS, dismutating superoxide to $H_2O_2$and O_2. A similar increased

in SOD activity has been reported in *Gracilaria tenuistipitata* (Collen *et al*., 2003). CAT, POD SOD and GST activities increased throughout the experimental period, indicating that these enzymes played a more important role in the regulation of oxidative stress in *Scenedesmus quadricauda*, therefore it may be considered as stress biomarkers. Increased GST activity may imply that the enzyme may have contributed to the conjugation of glutathione via a sulphydryl group to copper and cadmium to reduce their toxicity or completely detoxify them (Pinto *et al.*, 2003).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

In summary, investigation on the effect of cadmium (Cd), copper (Cu) and atrazine on the microalgae *Scenedesmus quadricauda* showed that growth of the microalgae was inhibited with increasing metal and herbicide concentrations. Decrease in the biomass (cell density, dry weight and chlorophyll *a*) production was observed as herbicides concentration increased from 0.005 to 0.5mg/l and metals from 1 to 2mg/L ($p<0.05$). SOD, POD, CAT and GST activities generally increased with increased in cadmium, copper and atrazine concentrations and with time of exposure (24h, 48h, 72h, and 96h). The enzyme activities showed significant positive correlation with Cd/atrazine and Cu/atrazine stress. The combined effect of these contaminants was greater than their individual effect. Therefore, individual and combined toxicity of copper, cadmium and atrazine depends on the concentration and duration of exposure.

6.2 Conclusion

It is therefore concluded that increased concentrations of atrazine, copper and cadmium increased the stress on *Scenedesmus quadricauda*. Cd/ atrazine and Cu/atrazine concentrations led to oxidative stress on Scene*desmus quadricauda* which was observed by changes in the activities of CAT, POD SOD and GST ($p< 0.05$). The combined effect of atrazine/Cd and atrazine/Cu on the biomass production showed that as the treatment concentrations increased, biomass, dry weight and chlorophyll a decreased while antioxidant enzymes increased. PCA analysis indicated that time of exposure and treatment combination show significant negative correlation with biomass production,

phenotypic plasticity and growth of the microalga; significant positive correlation with antioxidant enzyme activities and protein content.

6.3 **Recommendation**

Scenedesmus quadricauda is a fresh water microalgae used in eco-toxicological studies of aquatic environments. It is a common non-target species exposed to both heavy metals and herbicides pollution extensively. However, there is only a limited amount of toxicity data available for the interactions of different herbicide and heavy metal mixtures. Nowadays, there is increased use of chemicals, fertilizers, metals and herbicides in the environment and these substances have significant effect on aquatic organisms. Therefore, more studies are needed to assess the toxic effects of chemicals to local species in Nigeria. Government should assist in the provision of policies with appropriate regulations of the quantity of chemicals (herbicides and heavy metals) released into the aquatic environments. Our results also highlighted the importance of studying heavy metals and herbicides toxicity interactions in parallel with their individual effects. Single species exposure to metals and herbicide cannot provide enough information about toxicity of microalgae in aquatic environments. Therefore, studies in multi- species exposure should be investigated to get further information of the effects of metals and herbicides on fresh water microalgae.

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APPENDICES

Appendices 1: Growth absorbance of *Scenedesmus quadricauda* with different Cadmium and Atrazine concentration and time of exposure, values are mean ±SD.

		NA/NCd	A1/NCd	A2/NCd	A3/NCd	NA/Cd1	NA/Cd2	A1/Cd1	A1/Cd2	A2/Cd1	A2/Cd2	A3/Cd1	A3/Cd2

day1	Mean	0.0133	0.0100	0.0087	0.0077	0.0057	0.0047	0.0050	0.0053	0.0047	0.0040	0.0037	0.0037
	SD	0.0006	0.0010	0.0006	0.0006	0.0006	0.0006	0.0010	0.0012	0.0006	0.0010	0.0006	0.0006
day2	Mean	0.0250	0.0093	0.0077	0.0063	0.0050	0.0043	0.0043	0.0033	0.0030	0.0023	0.0017	0.0017
	SD	0.0030	0.0006	0.0006	0.0006	0.0010	0.0015	0.0006	0.0006	5.31E-19	0.0006	0.0006	0.0006
day3	MEAN	0.0483	0.0066	0.0053	0.0050	0.0030	0.0037	0.0030	0.0020	0.0017	0.0023	0.0013	0.0013
	SD	0.0067	0.0006	0.0006	0.0010	0.0010	0.0012	0.0006	0.0000	0.0006	0.0006	0.0006	0.0006
day4	MEAN	0.0573	0.0053	0.0043	0.0033	0.0033	0.0023	0.0020	0.0017	0.0013	0.0020	0.0017	0.0013
	SD	0.0064	0.0006	0.0015	0.0006	0.0006	0.0006	0.0000	0.0006	0.0006	0.0010	0.0006	0.0006

Appendices 2: Growth absorbance of *Scenedesmus quadricauda* with different Cu and Atrazine concentration and time of exposure, values are mean ±SD.

		NA/Ncu	A1/NCu	A2/NCu	A3/ A3/ NCu	NA/C u1	NA/Cu 2	A1/Cu 1	A1/Cu2	A2/Cu1	A2/Cu2	A3/Cu1	A3/C
day 1	Mean	0.0113	0.0093	0.0083	0.0075	0.0080	0.0057	0.0057	0.0063	0.0057	0.0053	0.0063	0.0063
	SD	0.0015	0.0012	0.0006	0.0007	0.0010	0.0012	0.0006	0.0006	0.0006	0.0006	0.0006	0.0015
day 2	Mean	0.0210	0.0073	0.0070	0.0053	0.0117	0.0053	0.0037	0.0040	0.0040	0.0033	0.0033	0.0037
	SD	0.0010	0.0006	0.0010	0.0006	0.0015	0.0012	0.0012	0.0000	0.0010	0.0006	0.0006	0.0006
day 3	MEAN	0.0400	0.0057	0.0047	0.0033	0.0070	0.0037	0.0040	0.0040	0.0040	0.0033	0.0030	0.0027
	SD	0.0020	0.0006	0.0006	0.0006	0.0010	0.0006	0.000	0.0000	0.0010	0.0006	5.31E-19	0.0006
day 4	MEAN	0.0560	0.0050	0.0040	0.0037	0.0053	0.0040	0.0043	0.0040	0.0037	0.0037	0.0033	0.0030
	SD	0.0063	0.000	0.000	0.0006	0.0006	0.000	0.0006	0.000	0.0006	0.0006	0.0006	5.31E-19

Appendices 3: Analysis of variance for the effect of Atrazine and Cadmium on Biomass Production and antioxidant enzymes response of *Scenedesmus quadricauda*. 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- GST = specific activity of Glutathione –S transferases; CAT = specific activity of Catalase; POD = specific activity of Peroxidase, SOD = specific activity superoxide dismutase.

Parameters	**Atrazine**	**Cadmium**
Chl a	28.50 (**0.0001**)	185.27(**0.0001**)
Dry weights	18.73 (**0.0001**)	103.77(**0.0001**)
Cell counts	27.66 (**0.0001**)	47.64 (**0.0001**)
1 celled coenobium	9.05 (0.3729)	31.34 (**0.0001**)
2 celled coenobium	22.08 (**0.0001**)	41.93 (**0.0001**)
3 celled coenobium	21.25 (**0.0001**)	29.38 (**0.0001**)

4 celled coenobium	19.54 (**0.0001**)	25.60 (**0.0001**)
Protein	28.71 (**0.0001**)	124.44(**0.0001**)
GST	9.56 (**0.0001**)	29.40(**0.0001**)
CAT	13.54 (**0.0001**)	23.18(**0.0001**)
POD	6.77 (**0.0003**)	69.31(**0.0001**)
SOD	8.06 (**0.0001**)	42.38(**0.0001**)

Appendices 4: Biomass production and Antioxidant Enzymes of *Scenedesmus quadricauda* as a function of different Cadmium and Atrazine concentrations. Columns with same letters are not significantly different (P>0.05)

	Atrazine Conc. (mg/l)				Cadmium Conc. (mg/l)		
	NoA	0.005	0.05	0.5	NoCd	1	2
Chl a	0.1674^{a}	0.129^{b}	0.112^{b}	0.100^{c}	0.208^{a}	0.090^{b}	0.082^{b}
Dry weight	0.0005^{a}	0.0047^{b}	0.0044^{b}	0.0042^{c}	0.0006^{a}	0.0004^{b}	0.00039^{b}
Cell count	35.361^{a}	12.722^{b}	11.056^{b}	10.139^{b}	33.729^{a}	9.458^{b}	8.771^{b}
Protein	176.000^{d}	206.694^{c}	231.444^{b}	256.194^{a}	147.792^{c}	242.792^{b}	262.167^{a}
SOD	9.812^{b}	12.285^{a}	12.887^{a}	13.910^{a}	9.144^{c}	12.587^{a}	14.940^{b}
CAT	0.864^{c}	0.946^{b}	0.958^{b}	1.029^{a}	0.087^{c}	0.952^{a}	1.023^{b}
POD	0.029^{c}	0.035^{a}	0.038^{a}	0.043^{a}	0.020^{c}	0.040^{a}	0.047^{b}

GST	20.718[c]	28.183[b]	25.637[b]	35.475[a]	18.446[c]	28.385[b]	35.677[a]

Appendices 5: Biomass production and Antioxidant Enzymes of *Scenedesmus quadricauda* as a function of hours of exposure of Atrazine concentrations. Columns with same letters are not significantly different (P>0.05)

	Hour of exposure of Atrazine			
	24hr	48hr	72hr	96hr
Chl a	0.154194[a]	0.130936[b]	0.122488[b]	0.099891[c]
Dry weight	0.00048750[a]	0.00047333[a]	0.00044861[a]	0.00043722[c]
Cell count	15.694[a]	16.556[a]	19.194[a]	17.833[a]
Protein	139.472[b]	237.611[a]	243.250[a]	250.000[a]
SOD	10.6066[c]	11.5235[c]	12.8279[a]	13.9364[a]
CAT	0.87360[b]	0.90618[b]	0.93075[b]	1.08561[a]
POD	0.017803[c]	0.032155[b]	0.044108[a]	0.048864[a]

GST	12.674[d]	24.653[c]	31.134[b]	41.551[a]

Appendices 6: Analysis of variance for the effect of Atrazine and Copper on Biomass production and Antioxidant Enzymes response of *Scenedesmus quadricauda*. 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- GST = specific activity of Glutathione –S transferase; CAT = specific activity of Catalase; POD = specific activity of

Peroxidase, SOD = specific activity superoxide dismutase.

Parameters	**Atrazine**	**Copper**
Chl a	1.01 (0.3906)	1.01(0.3662)
Dry weights	177.44 (**0.0001**)	83.25(**0.0001**)
Cell counts	19.66 (**0.0001**)	22.35 (**0.0001**)

1 celled coenobium	4.01 **(0.0001)**	1.77 (0.1736)
2 celled coenobium	10.19 (**0.0001**)	9.02(**0.0002)**
3 celled coenobium	16.11 (**0.0001**)	16.91 (**0.0001**)
4 celled coenobium	12.15 (**0.0001**)	14.43 (**0.0001**)
Protein	126.39 (**0.0001**)	45.64(**0.0001**)
GST	21.51 (**0.0001**)	2.13(0.1228)

CAT	50.12 (**0.0001**)	70.40(**0.0001**)
POD	213.39 (**0.0001**)	93.65(**0.0001**)
SOD	265.79 (**0.0001**)	192.71(**0.0001**)

Appendices 7: Biomass production and Antioxidant Enzymes of *Scenedesmus quadricauda* as a function of different Copper and Atrazine concentrations. Columns with same letters are not significantly different (P>0.05)

	Atrazine Conc. (mg/l)				Copper Conc. (mg/l)		
	NoA	0.005	0.05	0.5	NoCu	1	2
Chl a	19.88[a]	0.12[a]	0.11[a]	0.10[a]	14.95[a]	0.11[a]	0.10[a]

Dry weight	0.057[a]	0.045[b]	0.041[c]	0.038[d]	0.051[a]	0.044[b]	0.041[c]
Cell count	36.94[a]	16.89[b]	15.75[b]	13.53[b]	32.33[a]	15.73[b]	14.27[b]
Protein	74.22[c]	53.58[d]	104.64[b]	153.97[a]	85.63[b]	81.042[b]	122.396[a]
SOD	8.44[d]	9.40[c]	11.19[b]	13.22[a]	8.96[c]	10.66[b]	12.040[a]
CAT	0.79[d]	0.808[c]	0.817[b]	0.826[a]	0.795[c]	0.815[b]	10.823[a]
POD	0.014[d]	0.019[c]	0.025[b]	0.032[a]	0.018[c]	0.022[b]	0.027[a]
GST	11.63[c]	19.618[b]	29.34[b]	42.77[a]	22.830[a]	24.783[a]	29.905[b]

Appendices 8: Absorbance at 595nm and Protein concentrations for Standard protein calibration curve.

Absorbance (595nm)	Protein Concentration (ug/ml)
1.389	0
1.391	10
1.424	20
1.461	40
1.493	60

1.536	80
1.548	100
1.560	150
1.610	200
1.674	250
1.786	300
1.894	350
1.990	400
2.01	450