ECOTOXICITY OF TITANIUM DIOXIDE NANOPARTICLE TO *CHLORELLA VULGARIS* BEYERINCK (TREBOUXIOPHYCEAE, CHLOROPHYTA) UNDER LIMITED NITROGEN CONDITIONS *IN VITRO*

BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER OF SCIENCE DEGREE IN BOTANY.

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APRIL, 2016.

DECLARATION

I declare that this dissertation entitled "**ECOTOXICITY OF TITANIUM DIOXIDE NANOPARTICLE TO *CHLORELLA VULGARIS* BEYERINCK (TREBOUXIOPHYCEAE, CHLOROPHYTA) UNDER LIMITED NITROGEN CONDITIONS *IN VITRO***" was carried out by me in the Department of Biological Sciences. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Suleiman Dauda ---------- ----------

Signature Date

CERTIFICATION

This dissertation entitled "**ECOTOXICITY OF TITANIUM DIOXIDE NANOPARTICLE TO *CHLORELLA VULGARIS* BEYERINCK (TREBOUXIOPHYCEAE, CHLOROPHYTA) UNDER LIMITED NITROGEN CONDITIONS *IN VITRO***" by Suleiman Dauda meets the regulations governing the award for the degree of M.Sc. Botany of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this dissertation to my parents for their unending care, love, support, and prayers.

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ABSTRACT

The broad application of titanium dioxide nanoparticles in many consumer products has resulted in the release of substantial amounts into aquatic system, which serve as the terminal sink for nanomaterials. These titanium dioxide nanoparticles may induce some unexpected toxic effects to aquatic organisms such as microalgae. This study was carried out to evaluate the toxicity of limited nitrogen and titanium dioxide nanoparticles to the microalgae *Chlorella vulgaris*. The nanoparticles were prepared in Organisation for Economic Cooperation and Development (OECD) algal test medium and also the nitrogen concentration in the algal test medium was adjusted to match limited environmental nitrogen level. The growth, biomass production, biochemical composition (Carbohydrate and protein content), and antioxidant response (Glutathione-s-transferase, peroxidase, and lipid peroxidation) of the algae were monitored over a 96h period. The results showed that limited nitrogen (2.8×10^{-6} M) decrease growth, biomass (dry weight, cell counts, chlorophyll content), carbohydrate content, and increase protein content, antioxidant enzyme activity and lipid peroxidation (malondialdehyde content) in the alga. Titanium dioxide nanoparicle treatments (0.2mg/L, 8mg/L, 16mg/L and 32mg/L) decrease growth, biomass, carbohydrate content and increase glutathione-s-transferase activity. The combination of limited nitrogen with titanium dioxide nanoparticle decrease growth, dry weight, chlorophyll content, and carbohydrate content of the alga. This result suggests that limited nitrogen and titanium dioxide nanoparticle treatments affects growth, biomass production, biochemical composition, induces oxidative stress and also induces the oxidation of lipids while titanium dioxide nanoparticle combined with limited nitrogen affect growth, biomass, and carbohydrate content of *Chlorella vulgaris* under white fluorescent light.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

BSA: Bovine serum albumin

CDNB: 1-Chloro-2, 4-dinitrobenzene

DNA: Deoxyribonucleic acid

DW: Dry weight

EC_{50}: 50% Effective concentration

EDTA: Ethylenediamine tetracetic acid

GSH: Reduced glutathione

GST: Glutathione-s-transferase

H_2O_2: Hydrogen peroxide

H_2SO_4: Sulphuric acid

MDA: Malondialdehyde

NMs: Nanomaterials

n-TiO_2: Titanium dioxide nanoparticles

OECD: Organisation for Economic Co-operation and Development

PCA: Principal Component Analysis

POD: Peroxidase

PUFA: Polyunsaturated fatty acid(s)

RNA: Ribonucleic acid

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RPM: Revolutions per minute

TBA: Thiobarbituric acid

TCA: Trichloroacetic acid

TiO_2: Titanium dioxide

UV: Ultraviolet

UV-VIS: Ultraviolet-Visible light

V/V: Volume by volume

W/V: Weight by volume

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Microalgae are a very important component of the aquatic ecosystem; they are a group of fast growing unicellular or simple multicellular microorganisms that have the ability to fix CO_2 while capturing solar energy with efficiency 10 to 50 times greater than that of terrestrial plants (Wang *et al.,* 2008). They also have higher biomass production compared to energy crops (Wang *et al.,* 2008). They inhabit the pelagic as well as benthic environments of the hydrosphere in a variety of forms. This variation is more pronounced in green algae, which taxonomically belongs to different phyla, such as Prochlorophyta, Volvocophyta, Euglenophyta, Chlorophyta, and Charophyta (Graham and Wilcox, 2000).

Chlorella sp belongs to the Division Chlorophyta, class Chlorophyceae, order Chlorococcales, and family Chlorococcaceae.

Titanium dioxide, also known as titanium (IV) oxide or Titania is the naturally occurring oxide of titanium, the ninth most abundant element in the world. It is five times less abundant than iron but 100 times more abundant than copper (IARC, 2010). Generally titanium dioxide is sourced from Ilmenite ore, rutile and anatase, which are mined from deposits located throughout the world. Ilmenite ore is the widest spread of titanium dioxide bearing ore in the world. Rutile (TiO_2) and Ilmenite ($FeTiO_3$) are commonly found as accessory minerals in plutonic and metamorphic rocks but occur also as detrital

minerals in beach sands. The industrial applications of titanium dioxide can be utilized either in its bulk form or in the nanoparticulate form, and the global production of titanium dioxide for all uses is in the millions of tons per year (Diebold, 2003). As a bulk material, titanium dioxide (TiO_2) is primarily used as a pigment because of its brightness, high refractive index, and resistance to discolouration (Diebold, 2003).

Nanoparticles are loosely defined as manufactured materials that are smaller than 100 nanometer in at least one dimension (Nowack and Bucheli, 2007). They have been of scientific interest for several decades, but are now being used in a wide range of commercial applications (Kulacki and Cardinale, 2012).

While many of the anticipated applications of nanomaterials remain to be seen, titanium dioxide (TiO_2) nanoparticles are already widely used (Hartmann *et al*., 2010). Nanoparticles have different properties than their bulk counterparts as they present a very large surface area-to-volume ratio (D'Agata *et al*., 2014) and when particle size shrinks, there is potential for enhanced toxicity to biota, even if the material is relatively inert in bulk form (Farrè *et al*,. 2009). Many applications of titanium dioxide nanoparticles are based on the ability of the particles to absorb UV-light and their photo catalytic activity, which has been found to increase with decreased particle size (Gupta and Tripathi, 2011). In the cosmetics industry the use of nanosized particles in creams is increasing. Because of the small particle size, they can be more easily absorbed through the skin, as in moisturizers, pigment, thickener, and used in sunscreens as a UV absorber.

Nitrogen is an important element for growth of algae. It is essential in protein synthesis and in pigment construction. The protein composition in algae fluctuates with nitrogen

availability and hence the population density may rise and fall. Since proteins are vital to growth of algae, excessive nitrogen concentrations in the form of nitrogen for example can cause algal bloom. Nitrogen and phosphorus are often the primary limiting nutrients for aquatic algal production (Dodds *et al*., 2002), because they are frequently in short supply relative to other cellular growth requirements. Accrual of algal biomass, and the overall ecosystem productivity, may be controlled by the type and intensity of nutrient limitation (Dodds *et al.,* 2002).

Oxidative stress results when production of reactive oxygen species (ROS) overwhelms antioxidant defense systems. According to Sies, (1991), it is 'a disturbance in the prooxidant–antioxidant balance in favour of the former, leading to potential damage'. In principle, oxidative stress can result from: Diminished antioxidants, like the enzymes glutathione peroxidase and superoxide dismutase. Secondly, Increased production of reactive oxygen species and reactive nitrogen species (RNS), for instance by exposure to elevated levels of toxins that are themselves reactive species (e.g. nitrogen dioxide gas, NO_2.) or are metabolized to generate such species, or by excessive activation of 'natural' reactive oxygen species and reactive nitrogen species-producing systems. Antioxidant defense mechanisms include enzymes that catalyze reactions of ROS scavenging, such as catalase, ascorbate peroxidase, glutathione peroxidase and superoxide dismutase (Pinto *et al*., 2003), lipophylic compounds and free radical scavengers like carotenes and α-tocopherol, reducers like ascorbate and reduced glutathione (GSH) (Rahman, 2007). Reduced glutathione is the major water-soluble antioxidant in algal cells and it can directly reduce most of the ROS (Carvalho *et al.*,2004) or act as a cofactor or substrate in enzymatic reactions that contribute to control the cell ROS levels (Okamoto *et al*., 2001).

Oxidative stress has become an important subject for terrestrial and aquatic toxicology (Valavanidis *et al.*, 2006). One generally accepted mechanism of nano-titanium dioxide antimicrobial property is the generation of ROS, which can cause cell wall or cell membrane damage (Sadiq *et al*., 2011), such as lipid peroxidation.

Photocatalytic oxidation is also the mechanism for titanium dioxide nanoparticle degradation of organic pollutants in wastewater. Photocatalytic degradation is based on the formation of radicals such as hydroxyl radicals, superoxide radical anions and hydroperoxyl radicals, which serve as oxidizing species in the photo catalytic oxidation process (Ahmed *et al*., 2011).

Titanium dioxide nanoparticles generates ROS (mainly highly reactive hydroxyl radicals) in the presence of UV and oxygen (Navarro *et al*., 2008), so mechanisms other than oxidative stress might also contribute to n-TiO_2 toxicity in the dark (and possibly also under UV), as suggested by a recent study indicating that anatase n-TiO_2 can generate carbon-centered free radicals in the dark in the presence of dissolved oxygen (Fenoglio *et al*., 2009).

The consequences of oxidative stress includes, adaptation by the organism in the form of up regulation of antioxidant defense systems, tissue injury in the form of damage to all molecular targets such as DNA, proteins and lipids. Oxidative stress also leads to cell death, which occurs by either necrosis or apoptosis or both (Niizuma *et al*., 2009).

1.2 Statement of the Research Problem

The disturbance of algal habitats by humans can cause adverse effects to algae's function in carbon dioxide uptake (Talling 1976). Diminishing algal abundance will contribute to the rising levels of carbon dioxide in the atmosphere with major negative implications for the rest of the ecosystem. Because algae are primary producers and make up the base of the food chain, they are essential for the functioning of higher trophic levels. A decrease in algal abundance will impact the rest of the food chain.

The increasing presence of nanoparticles in many products has made it all the more likely that they will also be released into the aquatic environment. Scientists are concerned for the effects of the nanoparticles on biological systems because of their novel appearance in the environment and the lack of protective mechanisms in the course of biological evolution in living organisms (Valavanidis and Vlachogianni, 2010). The recent advances in nanotechnology and the corresponding increase in the use of nanomaterials in products in every sector of society have resulted in uncertainties regarding environmental impacts (Klaine *et al*, 2008).

During evolution, living organisms have adapted to the presence of natural nanoparticles in the environment. For synthetic nanoparticles however, it is recognized that their potential harmful properties on ecosystems have to be evaluated (Handy *et al*., 2008).

The fate and toxicity of engineered nanoparticles in natural waters therefore need to be studied as the situation is complicated by the existence of a very broad spectrum of engineered nanoparticles, differing in their chemical, physical and morphological

properties (Navarro *et al.*, 2008). But precisely these properties need to be taken into account in efforts to understand how nanoparticles act on organisms, since they influence the bioavailability of particles and the mechanisms of toxicity (Navarro *et al.*, 2008).

Mechanisms of nanomaterial toxicity include cellular damage due to oxidative stress, physical damage to the cell surface, dissolution at the cell surface, and impacts via bioaccumulation. The latter involves interaction with the cell surface for unicellular organisms and uptake across the gill and other external surface epithelia for higher organisms. Bioaccumulation via the food chain is also possible. (Batley and McLaughlin, 2010).

According to available literature, impacts of nanoparticles on nutrient availability in aquatic ecosystems have not been assessed in detail yet. The effect of titanium dioxide nanoparticle on the nutrient uptake by *Chlorella vulgaris* needs to be studied. However, according to Mahler *et al*. (2012), since nanopaticles interact with ions and other diverse components, it is very likely that they also interact with nutrients essential for aquatic organisms especially if these nutrients are present only in very low concentrations, such as for microelements. The formation of porous microstructures on the sediment surface after settling of nanoparticles might also influence the bioavailability of nutrients (Christian *et al*., 2008), and the adsorption of larger engineered nanoparticles aggregates to cell walls might also alter the cellular acquisition of essential nutrients, either through clogging of the walls or nutrient adsorption.

It is inevitable that scientists worry about environmental pollution by nano-scale manufactured materials since it looks that in the near future nanotechnology and nanosciences will produce a great variety of NMs with widespread use (Valavanidis and Vlachogianni, 2010).

As emerging economies (such as Nigeria's) vigorously pursue their developmental needs, it is obvious that there will be increased adoption of nanotechnologies. This means increased exposure of the various environmental compartments to pollution by nanoparticles, such as that of titanium dioxide (Nowack and Burcheli, 2007; Valavanidis and Vlachogianni, 2010). In addition, available literature indicate that the impact of nanoparticles on the dynamics of metabolism of nutrients such as nitrogen and the subsequent effect of this on physiological responses that support growth and reproduction of aquatic organisms such as microalgae is poorly understood.

1.3 Justification

Algae are one of the normally used model organisms for the toxicity examination of toxicants and nanoparticles as well, (Ji *et al*, 2011). As excellent aquatic models, microalgae are prevalent in lakes and seas, easy to culture and propagate, and sensitive to pollutants. *Chlorella vulgaris* is distributed widely in freshwater and seawater and has a short growth cycle, these attributes makes it relevant for aquatic eco-toxicity studies and it can be used to directly observe toxicity at the cellular level (Wong *et al*., 1997).

Bioavailability and toxicity of nanoparticles are largely unknown (Biswas and Wu, 2005). Some of the same special properties that make nanomaterials useful are also properties that may cause some nanomaterials to pose hazards to humans and the environment, under specific conditions (USEPA, 2007). Due to the increased use, titanium dioxide nanoparticles will inevitably reach the aquatic environment, where they have so far been traced from urban applications into receiving waters of urban runoff (Kaegi *et al*., 2008). Engineered nanoparticles might be released along the lifecycle of consumer products during their production, use, and disposal. Recent studies have demonstrated that for instance titanium dioxide nanoparticles and silver nanoparticles, which are used as whitening pigments and as bactericides in paints, leach from house facades releasing the particles in facade runoff and in urban storm water runoff which is discharged into receiving waters (Kaegi *et al.*, 2008; Kaegi *et al*. 2010).

These engineered nanoparticles have been shown to affect aquatic organisms, by being toxic to them, but despite a reasonable number of works demonstrating the toxicity of titanium dioxide nanoparticles on algae and some other aquatic organisms (Navarro *et al*., 2008, Aruoja *et al*., 2009; Sadiq *et al*., 2011; Chen *et al*., 2012; Suman *et al*., 2015), the ecotoxicological studies of this material together with nutrient concentrations, particularly under limited nitrogen exposure in experiments have not been carried out. The concentration of nutrients, particularly nitrogen and phosphorus in algae growth culture is considered to be a fundamental factor and has a direct influence on algal growth kinetics, which relates to nutrient removal and lipid accumulation (Xin *et al.*, 2010). This implies that it is important not only to investigate the toxicity of engineered nanoparticles alone

but also to reveal their interactions with other nutrient and how these interactions may influence the growth behavior and physiological responses of algae.

Against the backdrop of the information above, this study focused on effect of titanium dioxide nanoparticle and nitrogen limitation on the biomass production, some biochemical composition, and oxidative stress response of *Chlorella vulgaris in vitro.*

1.4 Aim of the Study

The aim is to evaluate the effect of titanium dioxide nanoparticles and nitrogen concentrations on the growth, biomass production, biochemical composition and oxidative stress response of *Chlorella vulgaris.*

1.5 Objectives

1. To determine the single and combined effects of limited nitrogen and titanium dioxide nanoparticles on the growth and biomass production of *Chlorella vulgaris*.
2. To determine the single and combined effects of limited nitrogen and titanium dioxide nanoparticles on the carbohydrate and protein content of *Chlorella vulgaris.*
3. To determine the single and combined effects of limited nitrogen and titanium dioxide nanoparticles on the antioxidant enzyme response of *Chlorella vulgaris.*

1.6 Hypotheses

1. The single and combined effects of limited nitrogen and titanium dioxide nanoparticles do not significantly affect the growth and biomass of *Chlorella vulgaris*.
2. The single and combined effects of limited nitrogen and titanium dioxide nanoparticles do not significantly affect the carbohydratrate and protein content of *Chlorella vulgaris.*

3. The single and combined effects of limited nitrogen and titanium dioxide nanoparticles do not significantly affect the antioxidant enzyme response of *Chlorella vulgaris.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Taxonomy and Morphology of *Chlorella vulgaris*

The Taxonomic classification according to Bock *et al*. (2011) is as follows:

Phylum: Chlorophyta

Class: Trebouxiophyceae

Order: Chlorellales

Family: Chlorellaceae

Genus: *Chlorella*

Species: *Chlorella vulgaris*

Chlorella is a unicellular, non-motile green alga which can be found both in fresh and marine waters. Its cells are solitary, very small (2-12 μm) and spherical, globular or ellipsoidal in shape. The cells are surrounded by a thin cellulose cell wall which encloses a parietal and cup shaped chloroplast with a pyrenoid (Sambamurty, 2005). The cells are devoid of flagella, stigma and contractile vacuole. *Chlorella* reproduces only asexually i.e. with the help of autospores. Each cell produces by successive divisions generally 4 autospores, sometimes 8 or16 autospores may be produced. The alga can grow in different conditions, that is it can grow in autotrophic, heterotrophic and mixotrophic conditions (Sambamurty, 2005)

According to Putt (2008) *Chlorella vulgaris* has the potential to double in cell number every 8 hours, provided nutrients and light are not limiting the growth at a temperature between 20-35 °C during autotrophic growth.

2.2 Environmental Factors Affecting Algal Growth

The results of algal growth inhibition tests are affected by the experimental conditions such as the light intensity, temperature, pH, type of nutrient medium. These experimental factors are discussed below.

2.2.1 Light

As with all plants, algae photosynthesize, i.e. they convert carbon dioxide into organic compounds especially sugars using the energy from light, therefore sufficient illumination is essential for the growth inhibition test. Only radiation that forms the visible light spectrum that is between 400 and 700 nm can be used by microalgae; this is called the 'Photosynthetic Active Radiation (PAR)', that is radiation utilisable in photosynthesis (Masojidek *et al.*, 2004)

As light is the source of energy for photosynthesis, the intensity, spectral quality and photoperiod need to be considered. Light intensity plays an important role, but the requirements vary greatly with the culture depth and the density of the algal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture (e.g. 1,000 lux is suitable for some small lab flasks, but 5,000-10,000 might be required for larger volumes). Algal growth rate increases with increasing light intensity up to a level where light saturation is reached. That level depends on the species, temperature and nutritional status of the algae and is relatively higher for green algae (Nyholm and Källqvist, 1989) such as *Chlorella vulgaris*.

Light may be natural from the sun or can be supplied artificially by fluorescent bulbs, tungsten-halogen lamps, and also light-emitting diodes (LEDs).

2.2.2 Temperature

Temperature determines the activity and reaction rates of intracellular enzyme, which will have an influence on algal photosynthesis, respiration intensity, affect the growth of microalgae. (Tan *et al*., 2009). With the light intensity changing, temperature is an environmental factor which indirectly affects growth of microalgae. According to a study by Takemura *et al*. (1985), when the temperature of water was lower than 4 °C the photosynthesis of microalgae was completely inhibited and when the temperature is between 4 °C to 11 °C photosynthesis is substantially inhibited. When the temperature is higher than 11℃ the relationship between temperature and growth of microalgae was linear.

Most processes of the algal metabolism are temperature-dependent, and the response to toxic substances is likely to be temperature-dependent as well. However, the studies published so far do not provide clear evidence as to how the toxicity of chemicals to algae depends on temperature.

2.2.3 pH

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2–8.7, though there are species that dwell in more acid/basic environments (Barsanti and Gualteiri, 2006)**.** Algal growth is affected by pH and failure to maintain an acceptable pH can result in complete culture collapse due to the disruption of many cellular processes. According to a study by Leavitt (1999), when the pH was lowered from 6.6 to 5.0, algal abundance increased. Algal abundance is usually decreased when

the pH is raised. Pendersen (2003) reported a reduction in growth among a group of pH tolerant algae when the pH exceeded 9.5.

In the algae culture; pH is balanced by the interplay between photosynthesis and respiration. Carbon dioxide fixation during photosynthesis liberates OH^- in the culture, which accumulates while the algae species grow and results in an elevated pH in the system. A similar reaction takes place, when bicarbonate is the inorganic carbon source (Grobbelaar, 2005). During respiration, the opposite reaction takes place, resulting in decreasing the pH in the culture.

Besides CO_2 and bicarbonate, nitrate and ammonium also influence the pH level in a culture. When ammonium is the sole nitrogen source of a culture, due to release of H^+ ions during assimilation a decrease in pH is obtained, whereas nitrate as major nitrogen source has the opposite

The pH and buffering capacity of the OECD test medium is determined by its carbonate system. The conversion of CO_2 to carbohydrates by photosynthesis is pH neutral (Arensberg *et al*., 1995):

2.2.4 Test medium

The test medium adopted for this study is the OECD TG 201 (OECD, 2011). The nutrient medium which is a mixture of micro and macronutrients is an essential component of algal growth inhibition test. As the test endpoint is the inhibition of the algal growth, the composition of the medium may have a strong effect on the test results (OECD, 2011).

Factors like pH, hardness, chelators and major cations have been found to impact toxicity results. Concentration of chelators may be particularly important when testing heavy metals, it has been demonstrated that EDTA greatly decreases the toxicity of metals due to the chelating properties (Debelius *et al*., 2010). For this reason, algal toxicity tests are sometimes carried out without EDTA in the test medium (Lin *et al.*, 2005). However, the algae may excrete secondary metabolites that act as chelators (Sheath, 1997) and therefore a small amount of a chelating agent is preferred in order to have a defined and more stable chelating capacity of the medium (Aruoja, 2011).

2.2.5 Nutrient

As a response to nutrient availability, microalgal cells undergo a series of metabolic acclimations, which often result in variations in cellular composition of macromolecules such as carbohydrates, lipids, amino acids, nucleic acids, pigments and proteins. Changes in these biochemical compounds and their specific ratios, including chlorophyll/protein, chlorophyll/RNA, RNA/DNA, and protein/carbohydrate under different nutrient conditions might help to indicate algal physiological state and nutritional status.

2.2.5.1 *Nitrogen*

Nitrogen (N) is an essential element for growth of microalgae and it is the second most important nutrient after carbon that contributes to algal biomass production (Collos and Berges, 2003).

Nitrogen is required by algae to manufacture amino acids, nucleic acids, chlorophyll, and other nitrogen-containing organic compounds. Nitrogen has a wide source. Some species

of microalgae can fix the nitrogen gas in the air through nitrogen fixation process for their own use. Microalgae can utilize different forms of nitrogen such as urea, nitrate and ammonium. However ammonium is the preferential form for many algae because it does not have to be reduced prior to amino acid synthesis, the point of primary intracellular nitrogen assimilation into the organic linkage (Graham and Wilcox, 2000; Barsanti and Gualteiri, 2006).

2.3 Ecotoxicology: Chemicals and the Environment

Truhaut (1977) defined ecotoxicology as the branch of toxicology concerned with the study of toxic effects caused by natural or synthetic pollutants, to the constituents of ecosystems in an integrated context. In the broadest sense ecotoxicology has been described as toxicity testing on one or more components of any ecosystem. This definition of ecotoxicology can be further expanded as the science of predicting effects of potentially toxic agents on natural ecosystems and on non-target species.

Because most chemicals introduced into the environment ultimately find their way into aquatic ecosystems, aquatic algal toxicity evaluations are particularly necessary because algae contribute approximately half of the global primary production as well as atmospheric oxygen (Aruoja, 2011). Algae play an important role in the aquatic ecosystem, not only producing biomass that forms the basic nourishment for food webs, but also contributing to the self-purification of polluted water. Changes in the structure and productivity of the algal community may induce direct structural changes in the rest of the ecosystem which affect the ecosystem by affecting water quality (Varol and Fucikova, 2015). The evaluation of the phytotoxicity of a chemical is an essential component of the ecological risk assessment since primary producers form an essential trophic level of any ecosystem, their growth response to existing and potential new environmental threats has to be clarified.

2.4 Nanoparticles in the Environment

The term nanoparticle describes a subset of natural or synthetic particles of the colloidal range between 1 and 100 nm that have been present in the environment since the formation of the earth or produced by human activity which have been used by mankind for thousands of years (Nowack and Bucheli, 2007; Hartland *et al*., 2013).

Nanoparticles are engineered to take advantage of properties specific to their tiny size and they are already finding uses in hundreds, possibly thousands, of consumer products (Sass, 2007). As more products containing nanomaterials are developed, there is greater potential for environmental exposure. Potential nanomaterial release sources include direct and/or indirect releases to the environment from the manufacture and processing of nanomaterials, releases from oil refining processes, chemical and material manufacturing processes, releases of nanomaterials incorporated into materials used to fabricate products for consumer use including pharmaceutical products, and releases resulting from the use and disposal of consumer products containing nanoscale materials (e.g., disposal of screen monitors, computer boards, automobile tires, clothing and cosmetics). (USEPA, 2007) Like most nanomaterials, the development and use of titanium dioxide nanoparticles epitomizes a growing problem – while an ever increasing number of new technologies and products are taking advantage of the unique properties of titanium dioxide nanoparticles, rarely have the potential hazards of these materials been effectively assessed (Kulacki and Cardinale, 2012).

2.4.1 Natural nanoparticles

An example of natural nanoparticles are colloids (defined as between 1-1000 nm) (Wagner *et al*., 2014). Colloids have been in the Earth for millions of years as a key

component in our ecosystem, being produced by microbial processes, sedimentation, natural combustion (in atmosphere), and other natural processes (Sengul *et al*., 2008).

2.4.2 Anthropogenic nanoparticles

Anthropogenic (incidental) nanoparticles have existed since the earliest days of civilisation. Anthropogenic nanoparticles can either be unintentionally formed as a by-product, mostly during combustion, or produced intentionally due to their particular characteristics. When anthropogenic nanoparticles are formed intentionally, they are often referred to as engineered or manufactured nanoparticles (Nowack and Bucheli, 2007). Example of engineered nanoparticle includes, fullerenes, metal oxides such as Titanium dioxide and Silver. However, anthropogenic emissions of inorganic nanoparticles have more than doubled the mass of natural nanoparticles into the atmosphere (Perez *et al*., 2009).

2.5 Ecotoxicology of Metal oxide Nanoparticles

Environmental release of nanoparticles into aquatic environment poses new environmental problems that need to be studied. Scientists have already formulated the areas of research which need further investigation: the hydrodynamic behaviour of small particles, synergistic effects with other chemical pollutants that will enhance their toxicity, routes of NPs uptake into living organisms, the significance of size and surface properties, and the effects of NPs on the aquatic ecosystems (Moore, 2006).

In the environment, NPs are regularly created as either weathering by products of minerals, as biogenic products of microbial activity, or as growth nuclei in super-saturated fluids (Wigginton *et al*., 2007).

Although the currently available information regarding production volumes is scarce and uncertain, based on market study reports and data on industry websites it can be concluded that metal oxide NPs are already manufactured in large scale for both industrial and household use. It has been estimated that 60,000 tonnes of titanium dioxide and 10,000 tonnes of zinc oxide NPs are produced per year worldwide (Aitken *et al*., 2006; Gottschalk *et al*., 2010), which makes them probably the most important nano-materials in terms of production volume as well as potential environmental impact.

Engineered nanoparticles might produce reactive oxygen species (ROS) upon their interaction with organisms or with agents present in the environment (e.g., ultraviolet radiation). ROS production is especially relevant in the case of engineered nanoparticles with photo catalytic properties such as TiO_2 upon ultraviolet (UV) exposure (Navarro *et al*., 2008)

2.5.1 Titanium dioxide nanoparticles

Titanium dioxide nanoparticles (n-$TiO_{2)}$ can be synthesized in from three crystalline phases: rutile, brookite and anatase. The anatase form being the most studied materials for photocatalysis and each of them presenting different properties and therefore different applications (Boccaccini *et al*., 2004). The increasing use of titanium dioxide nanoparticles are primarily driven by its photo activity, high refractive index, and transparency in the nanoparticulate form (Diebold, 2003). Titanium dioxide has received much attention for the application in the fields of photocatalytic activity (Lan *et al*., 2013), they can also be used as energy storage devices, in paints and coatings (Kaegi *et al*., 2008;

Blinova *et al*., 2010), in cosmetics, toothpastes, sunscreens, and will likely be used in the manufacture of the next generation solar cells (Klaine *et al.*, 2008).

Titanium dioxide nanoparticles between 20 and 300 nm were found to be detached from new and aged exterior facade paints by natural weather conditions and are then transported by facade runoff and are discharged into natural, receiving waters (Kaegi *et al*., 2008). According to Li *et al*. (2014), titanium dioxide nanoparticles may induce oxidative stress due to their photo catalytic properties, therefore, the balance between environmental pollutants and antioxidant defenses (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).

2.6 Enzyme Biomarkers

2.6.1 Antioxidants and oxidative stress in algae

Oxidative stress is understood as a situation of imbalance with an increased oxidants or decreased antioxidants levels. Oxidative stress implies the recognition of the physiological production of oxidants (oxidizing free-radicals and related species) and the existence of operative antioxidant defenses (Repetto *et al*., 2012). The imbalance concept recognizes the physiological effectiveness of the antioxidant defenses in maintaining both oxidative stress and cellular damage at a minimum level in physiological conditions (Boveris *et al*., 2008).

Antioxidant defenses are produced by either enzymatic or non-enzymatic processes. Enzymatic antioxidant defenses are provided by means of different antioxidant enzymes, such as peroxidase, glutathione-s-transferase, catalase, etc. Oxidative stress can be detected and quantified by measuring the production of such antioxidant enzymes. Lipid peroxidation is also another indicator of oxidative stress in organisms whereby lipids are oxidized through either enzymatic or non enzymatic means (Niki *et al*., 2005). Algae express oxidative stress through the increased production of oxidants and antioxidant enzymes (Valavanidis *et al*., 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Algal Culture Species

The microalga *Chlorella vulgaris* (UTEX# 2714) strain (Plate I) was obtained from the freshwater microalgae culture collection of University of Texas, USA.

3.2 Culture Media

Chlorella vulgaris was cultured in OECD medium (2011). Before culturing, the medium was sterilized by autoclaving at 121°C for 30 minutes. Cultures were maintained at 23±2°C under a 16:8 hrs lighting routine with an intensity of 1300 Lux with white fluorescent bulbs; white fluorescent bulbs are known to emit light within the 'Photosynthetic Active Radiation' (PAR) region, which is absorbed and utilized by microalgae. The cultures were periodically shaken manually daily to prevent clumping (Wei *et al.,* 2010). The pH of the culture medium was measured after preparation using a pH meter and adjusted to 7 using 0.1N HCl or 0.1N NaOH as required.

3.2.1 Preparation of culture media

Four stock solutions with constituents as shown in Table 3.1 were prepared. For each of the four stock solutions, the salts were dissolved one after the other in the appropriate

volume of distilled water until all the salts were completely dissolved. The stock solutions were then stored in the dark (Plastic bottles wrapped with aluminium foil) in the refrigerator, until required.

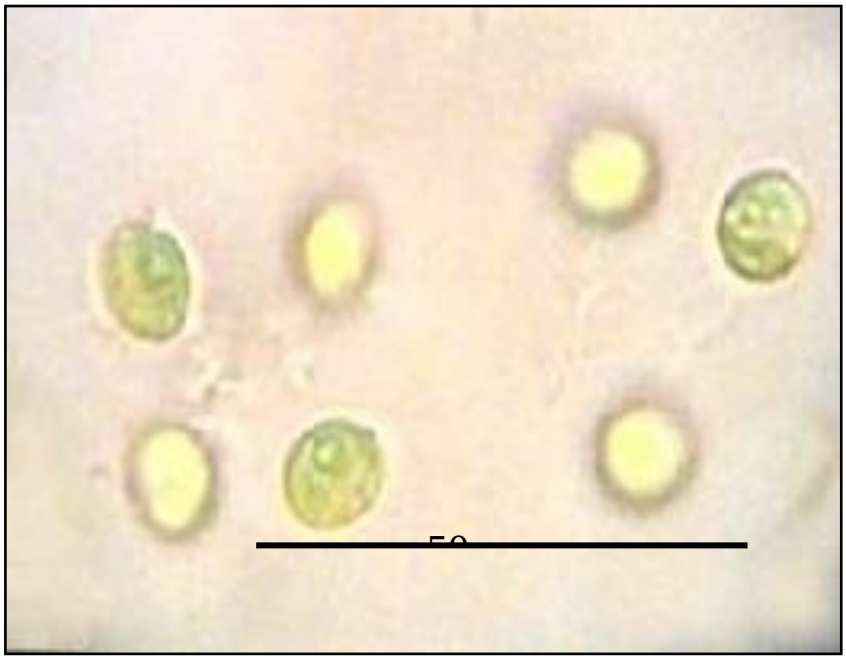

Plate I: *Chlorella vulgaris* cells at × 40 magnification

Table 3.1: Composition of OECD culture medium (2011).

Nutrient	**Concentration in stock**
Stock solution 1:macro nutrients	
NH_4Cl	1.5 g/L
$MgCl_2.6H_2O$	1.2 g/L
$CaCl_2.2H_2O$	1.8 g/L
Stock solution 2:iron	
$FeCl_3.6H_2O$ $Na_2EDTA.2H_2O$	64 mg/L
Stock solution 3:trace elements	
H_3BO_3	185 mg/L
$MnCl_2.4H_2O$	
$ZnCl_2$	415 mg/L
$CoCl_2.6H_2O$	3 mg/L
$CuCl_2.2H_2O$	1.5 mg/L
$Na_2MoO_4.2H_2O$	
Stock solution 4:bicarbonate	
$NaHCO_3$	50 g/L
pH	7

3.3 Titanium dioxide Nanoparticle Treatment

Dry Titanium (IV) oxide-anatase nano-powder was purchased from Sigma-Aldrich, USA; (CAS number 637254), particle size <25nm.

3.3.1 Preparation of nanoparticles dispersion and treatment

The titanium dioxide nanoparticles (n-TiO_2) powder was used to produce suspensions in OECD algal medium 201 (OECD, 2011). Titanium dioxide nanoparticle stock solution was prepared by suspending titanium dioxide nanoparticles powder in OECD medium in a concentration of 1000mgL^{-1}. The concentrations of titanium dioxide nanoparticle used were 32mg/L, 16mg/L, 8mg/L and 0.2mg/L. These concentrations were chosen after a preliminary EC_{50} determination, where an EC_{50} value of 15.60mg/L was recorded.

When the algal cultures were at their exponential growth phase, 145.2mL algal suspension was distributed into 500 mL Polycarbonate Erlenmeyer flask, then 4.8mL of the various concentrations of titanium dioxide nanoparticles were added to the algal suspension in the Erlenmeyer flask. Treatment was replicated thrice and ran for 96hrs.

3.4 Nutrient Source

Nitrogen was provided as NH_4Cl at 2.8×10^{-4}M (control) and 2.8×10^{-6}M which represent limiting environmental nitrogen concentration in aquatic environments (Reynolds, 2006). At each nitrogen concentration, n-TiO_2 concentrations of 0 mg/L, 0.2

mg/L, 8 mg/L, 16 mg/L, and 32 mg/Lwere added, while the controls had no n-TiO_2.

Experimental design shown in Table:3.2

Table 3.2: Experimental design

	2.8×10^{-4}mol/L (N0)	**2.8×10^{-6}mol/L (N-)**
No T	Control	N-
0.2mg/L(T1)	N0T1	N-T1
8mg/L (T2)	N0T2	N-T2
16mg/L(T3)	N0T3	N-T3
32mg/L(T4)	N0T4	N-T4

Key:

T: Titanium dioxide nanoparticles

N0: Control nitrogen

N-: Limited nitrogen

3.5 Growth and Biomass Determination

The specific growth rate (μ, d^{-1}) was calculated using the following equation:

$\mu = \ln(N_2/N_1)/t_2\text{-}t_1$,

where, N_1 was the dry weight at time t_1 and N_2 was the dry weight at time t_2.

3.5.1 Dry weight measurement

A Whatman's GF/C filter paper (5 cm diameter) was dried for 2 h at 60°C in an oven, cooled in a desiccator and weighed to the nearest mg with a weighing balance (Sartorius ED224S; AG Germany). These filter papers were used to filter 20, 10, 5, 2.5, and 1.25 mL of the samples, for the 10, 5, 2.5, and 1.25mL samples appropriate volume of distilled water was added to make it up to 20mL. The filter papers were dried again for 2 h at 60°C, cooled in a desiccator and weighed. Similarly 4, 2, 1, 0.5, 0.25mL of algal culture were collected, for the 2, 1, 0.5, 0.25mL samples, appropriate volume of distilled water was added to make it up to 4mL. The absorbance of each of these samples were read at 680nm.

Dry weight were calculated in mg L^{-1} DW as the difference between the initial dry weight of the filter paper without microalgal biomass and the final weight of the filter paper with the retained microalgal biomass. A dry weight calibration curve was plotted with the absorbance vs dry weight and the dry weight of the unknown samples were calculated from the fitted regression equation.

3.5.2 Cell counts (cells mL^{-1})

Algal cells (3mL) were collected daily and cell count was done with a Neubauer haemocytometer under a compound microscope.

3.6 Chlorophyll Determination

Chlorophyll a and total chlorophyll were extracted using 80% (v/v) acetone. 5mL of algae cell culture was collected and centrifuged at 4000 rpm for 10min. The supernatant was discarded and 3mL of 80% (v/v) acetone was added, mixed, and the container was wrapped with aluminium foil and refrigerated for 30 min. After refrigerating the containers were centrifuged at 4000 rpm for 10min, the supernatants were decanted and used for chlorophyll and carotenoids quantification. Chlorophyll a and total chlorophyll quantified by taking the absorbance of the supernatant at 653 and 666 nm with a UV-VIS Spectrophotometer (B.bran scientific and instrument company, England). Pigment concentrations were determined using the following equations provided by Németh (1998):

C_a (mg/L) $=17.12A_{666}- 8.86A_{653}$

C_T (mg/L) $= 2.57A_{666}+ 23.6A_{653}$

Where C_a is the content of chlorophyll a (mg/L) and C_T the content of total chlorophyll.

3.7 Biochemical Composition Determination

3.7.1 Total carbohydrates

Carbohydrates analysis was performed according to the phenol-sulfuric acid method (Liu *et. al.*, 1973) using glucose as standard. The glucose standard concentrations used were from 10, 30, 50, 80, 100 μg mL^{-1}. 1 ml of distilled water was added to a test tube to serve as the blank solution. For the test samples, 5mL aliquots were taken from the culture and centrifuged at 2000 rpm for 10 min to pellet the cells. The pellets obtained were re-suspended in 1mL of distilled water in a test tube. 1mL of 5% (w/v) aqueous phenol solution was added to the test tube containing the sample, all the test tubes containing the different glucose concentrations, and the blank reagent test tube. After thoroughly mixing, 5mL of concentrated H_2SO_4 was quickly added to all test tubes, directing the flow at the liquid surface to obtain a good mixing. The mixture was then left to stand at room temperature for 10 min and later centrifuged at 2000rpm for 10 min. The supernatant was decanted and read at 490nm against the reagent blank. Sample carbohydrate concentrations were obtained from a calibration curve of glucose with absorbance plotted against concentrations.

3.7.2 Protein extraction

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

Algal sample of 5mL was centrifuged at 4000 rpm for 15 minutes; the supernatant was then discarded and 1.5mL of pre-cooled 0.05M phosphate buffer (pH 7.8) was then added, crushed on ice and centrifuged at 4000 rpm for 15 minutes. The supernatant obtained was used for protein and antioxidant enzymes assay.

3.7.3 Total proteins

To determine the total intracellular protein content of *C. vulgaris* cells, the procedure of Bradford (1976) with bovine serum albumin (BSA) as protein standards with concentrations, 15.625, 31.25, 50, 80, 100μg mL^{-1} was used.

3.7.3.1 Bradford reagent preparation

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid (Bradford, 1976).

3.7.3.2 Protein Assay

To start the protein assay, 0.1 ml of the protein extract was pipetted into 12 x 100 mm plastic test tubes, then 3 ml of Bradford reagent was added to the test tube and the contents mixed by shaking. The absorbance at 595 nm was measured after 10 minutes in plastic disposable cuvettes against a reagent blank prepared from 0.1 ml of the phosphate buffer (pH 7.2) and 3 ml of Bradford reagent. The concentration of protein was plotted against the corresponding absorbance and the resulting standard curve was used to determine the amount of protein in the unknown samples.

3.7.4 Antioxidants enzyme determination

The antioxidant enzymes that were analysed include: glutathione-s-transferase, peroxidase and lipid peroxidation.

3.7.4.1 Assay of glutathione-s-transferase

The assay of glutathione S-transferase activity was performed using the method of Habig *et al*. (1974) with some modification. Glutathione S-transferase conjugates GSH with CDNB and the extent of conjugation is used as a measure of enzyme activity from the proportionate change in the absorption at 340 nm.

Reagents

1. 1-Chloro-2, 4-dinitrobenzene (CDNB) (30mM in 95% ethanol)

2. Reduced glutathione (GSH) (75mM in distilled water)

3. Phosphate buffer (0.1M, pH 6.5)

Procedure

The assay mixture contains 100 µl of GSH, 100 µl of CDNB, and 2.7mL phosphate buffer. The reaction was started by the addition of 100 µl enzyme extract to this mixture and

absorbance was recorded against blank after every one minute for five minutes. The blank contained 2.8ml phosphate buffer, 100 µl of GSH, 100 µl of CDNB. The complete assay mixture without the enzyme served as the control to monitor non-specific binding of the substrates.

One unit of GST activity is defined as the nmoles of CDNB conjugated per minute.

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

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

$$GST\ specific\ activity\ (Units/ml\ enzyme) = \frac{\Delta A_{340}/\text{min} \times 3.0 \times d.f.}{(9.6)(0.10)} = \text{µmol/ml}/min$$

3.0 = Total volume (in milliliters) of assay

d.f = Dilution factor

9.6 = Millimolar extinction coefficient of Glutathione-1-chloro-2,4-dinitrobenzene conjugate at 340 nm

0.10 = Volume (in milliliter) of enzyme used

$$Units/mg\ solid = \frac{\text{Units/ml enzyme}}{mg\ solid/ml\ enzyme}$$

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

ϵmM(mM/cm) = extinction coefficient for CDNB conjugate at 340nm = 0.0096.

V= reaction volume.

3.7.4.2 Assay of peroxidase

The activity of peroxidase in the algal samples was assayed using the method of Reddy *et al*., (1996) with some modifications. 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 by spectrophotometry 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 (3.0 ml) and enzyme extract (0.1 ml) was pipetted out 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 for 3 minutes.

The activity of enzyme was then calculated as follows:

$$Peroxidase\ activity\ \ (units/mg) = \frac{\Delta A_{430}/mins}{6.58 \times \frac{ml\ enzyme}{ml\ reaction\ mixture}}$$

Where,

A_{430} = Absorbance at 430nm

3.7.4.3 Assay of lipid peroxidation

The Malondialdehyde (MDA) concentration was measured according to the method described by Heath and Packer (1968). To 1ml of the algal extract, 2ml of 10% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) was added, then the mxture was heated in a boiling water bath for 15 minutes and quickly cooled in an ice-bath. The mixture was centrifuged at 3000 rpm for 5 minutes and the absorbance of the supernatant was read at 532 nm and 600nm. The concentration of MDA was calculated, using an extinction coefficient of 155 $mM^{-1}cm^{-1}$, using the following formula:

MDA equivalents (nmolcm-^{3}) =1000[A_{532}-A_{600}/155]

3.8 Data Analyses

Algal growth inhibition test was done in triplicates; the EC_{50} value (titanium dioxide concentration required to cause a 50% reduction in growth) was determined using the method described in OECD (2011). Repeated measures Analysis Of Variance (ANOVA)

using STATISTICA software (version 10) was used to test the significant differences among the different treatments of algal samples, where significant Tukey's post hoc test was used to separate the means. Significant correlations between the growth, biomass production, biochemical composition and physiological response of the algae with the treatment conditions was determined using a correlation based Principal Component Analysis (PCA). All analyses were done at 5% significance level.

CHAPTER FOUR

4.0 RESULTS

4.1 Growth and Biomass Production of *Chlorella vulgaris*

4.1.1 Dry weight

The dry weight (mg/L) showed an increase and was highest under the control nitrogen condition (2.8×10^{-4} M) as against the limited nitrogen condition (2.8×10^{-6} M) (Table 4.1). There was a decrease in the dry weights under the titanium dioxide nanoparticles treatments, and this was most pronounced under the treatments with limited nitrogen concentrations. There was a statistically significant difference ($P<0.01$) in dry weights between the control and limited nitrogen conditions, the control and the titanium dioxide nanoparticle treatments, and also there was statistically ($P<0.05$) significant interaction between the nitrogen and titanium dioxide nanoparticle concentrations.

4.1.2 Cell counts

The total cell count ($\times 10^4$ cells/mL) increased and was highest under the control nitrogen condition (2.8×10^{-4} M) as against the limited nitrogen condition (2.8×10^{-6} M) (Table 4.2). There was a decrease in the cell counts under the titanium dioxide nanoparticle treatments. There was a statistically significant difference ($P<0.05$) in cell counts between the control and limited nitrogen conditions, and between the control and the titanium dioxide nanoparticle treatments, but there was no statistically significant ($P>0.05$)

interaction between the nitrogen and titanium dioxide nanoparticle concentrations with regards to cell counts.

Table 4.1: Dry weight (mg/L) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen treatments

Treatments	**24h**	**48h**	**72h**	**96h**
Control	143.82±0.25^{a}	153.91±0.88^{a}	169.93±0.65^{a}	196.96±0.72^{a}
0.2 mg/L n-TiO_2	140.02±0.25^{a}	146.32±0.97^{ab}	164.93±0.25^{ab}	187.89±0.16^{bc}
8 mg/L n-TiO_2	135.94±0.58^{ab}	145.39±2.51^{ab}	151.87±3.98^{bc}	175.67±4.24^{c}
16 mg/L n-TiO_2	124.19±0.76^{bc}	145.48±0.72^{ab}	152.24±0.185^{bc}	180.67±1.63^{bc}
32 mg/L n-TiO_2	131.04±6.90^{abc}	148.63 ±0.81^{ab}	156.96±1.73^{abc}	192.80±0.25^{ab}
N-	79.09±2.58^{d}	87.52±3.21^{c}	88.17±7.52^{de}	106.69±5.91^{d}
N-0.2 mg/L n-TiO_2	62.33±1.28^{e}	76.87±0.61^{cd}	74.28±0.56^{ef}	81.04±0.40^{f}
N- 8 mg/L n-TiO_2	65.57±0.33^{de}	74.65±2.14^{cd}	76.22±1.60^{ef}	87.98±1.13^{ef}
N-16 mg/L n-TiO_2	52.89±0.32^{e}	76.32±0.67^{cd}	74.28±1.94^{ef}	99±0.16^{de}
N-32 mg/L n-TiO_2	54.37±2.45^{e}	72.06±0.89^{d}	63.72±0.74^{f}	85.20±3.24^{ef}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different (P<0.05).

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

Table 4.2: Cell counts ($\times 10^4$ cells/mL) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen treatments

Treatments	**24h**	**48h**	**72h**	**96h**
Control	298.44 ± 10.97^{a}	336.88 ± 19.07^{ab}	392.66 ± 10.21^{ab}	424.66 ± 16.13^{ab}
0.2 mg/L n-TiO_2	236.44 ± 10.97^{abc}	369.11 ± 25.83^{a}	369.78 ± 13.86^{abc}	402.22 ± 14.45^{b}
8 mg/L n-TiO_2	266.22 ± 9.16^{ab}	338.66 ± 22.01^{ab}	358.89 ± 7.53^{abc}	375.33 ± 19.96^{bc}
16 mg/L n-TiO_2	224.89 ± 15.86^{abc}	326.66 ± 18.67^{ab}	361.77 ± 22.62^{abc}	358.44 ± 10.98^{bc}
32 mg/L n-TiO_2	253.33 ± 25.39^{abc}	353.33 ± 5.01^{a}	418.22 ± 24.08^{a}	367.77 ± 21.24^{bc}
N-	111.99 ± 12.35^{cd}	93.55 ± 2.73^{c}	95.78 ± 8.54^{d}	87.77 ± 6.02d^{e}
N-0.2 mg/L n-TiO_2	90.88 ± 4.08^{cd}	85.33 ± 7.21^{c}	97.55 ± 2.56^{d}	86.44 ± 10.57^{de}
N- 8 mg/L n-TiO_2	69.33 ± 3.79^{d}	73.55 ± 2.51^{c}	77.55 ± 9.92^{d}	68.44 ± 2.12^{de}
N-16 mg/L n-TiO_2	59.55 ± 3.58^{de}	75.55 ± 7.91^{c}	90.44 ± 8.66^{d}	101.11 ± 9.41^{de}
N-32 mg/L n-TiO_2	65.33 ± 3.90^{d}	76.88 ± 7.75^{c}	87.55 ± 7.78^{d}	114.44 ± 8.97^{d}

Values represent mean ± standard error, n = 3 per treatment group; n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration); Means along the same column with different superscripts are significantly different ($P<0.05$).

4.1.3 Chlorophyll a

Chlorophyll a content (mg/L) was higher in the treatments with the control nitrogen concentration (2.8×10^{-4} M) than in treatments with limited nitrogen concentrations (2.8×10^{-6} M) (Table 4.3). There was relatively higher production of chlorophyll a among titanium dioxide nanoparticles treatment with control nitrogen than those with limited nitrogen. The least chlorophyll a production for all the treatments was recorded after 96h exposure time. The lowest chlorophyll a content was recorded under limited nitrogen concentration with the highest titanium dioxide nanoparticle concentration (32mg/L) after 96h. There was a statistically significant difference ($P<0.05$) in chlorophyll a content between the control and limited nitrogen conditions, and between the control and the titanium dioxide nanoparticle treatments, there was also a statistically significant ($P<0.05$) interaction between the nitrogen and titanium dioxide nanoparticle concentrations with regards to chlorophyll a content.

4.1.4 Total chlorophyll

Total Chlorophyll content (mg/L) was generally higher in the treatments with the control nitrogen concentration (2.8×10^{-4}M) than in treatments with limited nitrogen concentrations (2.8×10^{-6}M) (Table 4.4), except that in treatments with control nitrogen concentration least total chlorophyll contents were recorded after 96h. There was relatively higher total chlorophyll production among titanium dioxide nanoparticles treatment with control nitrogen than in those with limited nitrogen. The lowest total

chlorophyll content among the limited nitrogen concentration was recorded under the highest titanium dioxide nanoparticle concentration (32mg/L) after 96h.

Table 4.3: Chlorophyll a (mg/L) content of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen treatments

Treatments	**24h**	**48h**	**72h**	**96h**
Control	0.77 ± 0.04^{abc}	0.75 ± 0.09^{c}	0.99 ± 0.02^{b}	0.06 ± 0.01^{b}
0.2 mg/L n-TiO_2	0.43 ± 0.06^{cd}	1.02 ± 0.16^{ab}	1.26 ± 0.12^{ab}	0.06 ± 0.01^{b}
8 mg/L n-TiO_2	0.32 ± 0.02^{de}	0.91 ± 0.19^{abc}	1.13 ± 0.11^{ab}	0.08 ± 0.02^{b}
16 mg/L n-TiO_2	0.49 ± 0.01^{bcd}	1.23 ± 0.06^{a}	1.17 ± 0.06^{ab}	0.04 ± 0.03^{b}
32 mg/L n-TiO_2	0.43 ± 0.01^{cd}	0.83 ± 0.09^{abc}	0.26 ± 0.03^{c}	0.08 ± 0.03^{b}
N-	0.89 ± 0.04^{ab}	0.33 ± 0.09^{d}	0.17 ± 0.04^{c}	0.60 ± 0.19^{a}
N-0.2 mg/L n-TiO_2	0.66 ± 0.00^{abc}	0.53 ± 0.16^{cd}	0.14 ± 0.04^{cd}	0.13 ± 0.02^{b}
N- 8 mg/L n-TiO_2	0.70 ± 0.07^{abc}	0.14 ± 0.06^{de}	0.15 ± 0.02^{cd}	0.10 ± 0.01^{b}
N-16 mg/L n-TiO_2	0.62 ± 0.01^{bc}	0.09 ± 0.01^{f}	0.99 ± 0.04^{b}	0.12 ± 0.01^{b}
N-32 mg/L n-TiO_2	0.73 ± 0.01^{abc}	0.07 ± 0.00^{f}	0.13 ± 0.01^{cd}	0.01 ± 0.01^{b}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different ($P<0.05$).

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

Table 4.4: Total chlorophyll (mg/L) content of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen treatments

Treatments	**24h**	**48h**	**72h**	**96h**
Control	1.32 ± 0.08^{bc}	2.00 ± 0.02^{ab}	1.27 ± 0.012^{b}	0.08 ± 0.01^{b}
0.2 mg/L n-TiO_2	0.76 ± 0.16^{cd}	2.54 ± 0.31^{a}	1.68 ± 0.155^{b}	0.06 ± 0.01^{b}
8 mg/L n-TiO_2	0.39 ± 0.04^{d}	1.75 ± 0.11^{bc}	1.47 ± 0.153^{b}	0.08 ± 0.02^{b}
16 mg/L n-TiO_2	0.84 ± 0.08^{cd}	2.18 ± 0.14^{ab}	1.59 ± 0.090^{b}	0.04 ± 0.03^{bc}
32 mg/L n-TiO_2	0.85 ± 0.05^{cd}	2.19 ± 0.18^{ab}	0.32 ± 0.034^{c}	0.08 ± 0.03^{b}
N-	1.87 ± 0.11^{ab}	1.14 ± 0.35^{c}	0.35 ± 0.107^{c}	0.306 ± 0.190^{a}
N-0.2 mg/L n-TiO_2	1.35 ± 0.01^{bc}	1.04 ± 0.34^{c}	$0.40\pm0.092_{c}$	0.243 ± 0.047^{a}
N- 8 mg/L n-TiO_2	1.40 ± 0.11^{bc}	0.26 ± 0.12^{d}	0.36 ± 0.068^{c}	0.081 ± 0.009^{b}
N-16 mg/L n-TiO_2	1.29 ± 0.07^{bc}	0.31 ± 0.06^{d}	3.04 ± 0.107^{a}	0.059 ± 0.074^{b}
N-32 mg/L n-TiO_2	1.41 ± 0.01^{bc}	0.15 ± 0.02^{d}	0.32 ± 0.030^{c}	0.012 ± 0.029^{c}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different ($P<0.05$).

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

There was a significant difference ($P<0.05$) in total chlorophyll content between the control and limited nitrogen conditions, and between the control and the titanium dioxide nanoparticle treatments, there was also a statistically significant ($P<0.05$) interaction between the nitrogen and titanium dioxide nanoparticle concentrations with regards to total chlorophyll content.

4.1.5 Specific growth rate

The specific growth rate (μ, d^{-1}) of the limited nitrogen (2.8×10^{-6} M) treatment was lower than that of the control nitrogen (2.8×10^{-4} M) treatment after 24h of treatment (Fig. 1), it increased after 48h, but the increase was not sustainable and it decrease after 72h. It increased again after 96h but it was not different from the control. For the titanium dioxide nanoparticle treatment and the combination of limited nitrogen with titanium dioxide nanoparticle a similar pattern of growth was observed against the control (Fig. 2). There was a statistically significant difference ($P<0.05$) in specific growth rate between the control and limited nitrogen treatment (Table 4.5), the control and the titanium dioxide nanoparticle treatments, and there was a significant interaction between nitrogen and titanium dioxide nanoparticle in terms of specific growth rate.

4.2 Biochemical Composition of *Chlorella vulgaris*

4.2.1 Carbohydrate content

Carbohydrate content (µg/ml) increased and was higher under the control nitrogen condition (2.8×10^{-4} M) as against the limited nitrogen condition (2.8×10^{-6} M) (Table 4.6). There was a decrease in the carbohydrate content under the titanium dioxide nanoparticles treatments. There was a statistically significant difference ($P<0.05$) in carbohydrate content between the control and limited nitrogen conditions, between the control and the titanium dioxide nanoparticle treatments, there was also a statistically significant ($P<0.05$) interaction between the nitrogen and titanium dioxide nanoparticle concentrations with regards to carbohydrate content.

4.2.2 Protein content

Protein content (µg/ml) showed a steady increase both under the control nitrogen condition (2.8×10^{-4} M) and the limited nitrogen condition (2.8×10^{-6} M) (Table 4.7). There was a statistically significant difference ($P<0.05$) in protein content between the control and limited nitrogen conditions, but there was no significant difference ($P>0.05$) between the control and the titanium dioxide nanoparticle treatments. There was also no statistically significant ($P>0.05$) interaction between the nitrogen and titanium dioxide nanoparticle concentrations with regards to protein content.

4.3. Antioxidant Enzyme Activities

4.3.1 Glutathione-s-transferase

Glutathione-s-transferase activity (µmol/mL/min) showed a slight increase both under the control nitrogen condition (2.8×10^{-4} M) and the limited nitrogen condition (2.8×10^{-6} M) (Table 4.8), with increasing titanium dioxide nanoparticle concentrations. There was a statistically significant difference ($P<0.05$) in glutathione-s-transferase activity between the control and limited nitrogen conditions, between the control and the different titanium dioxide nanoparticle concentrations. There was no statistically significant ($P>0.05$) interaction between the nitrogen and titanium dioxide nanoparticle concentrations with regards to glutathione-s-transferase activity.

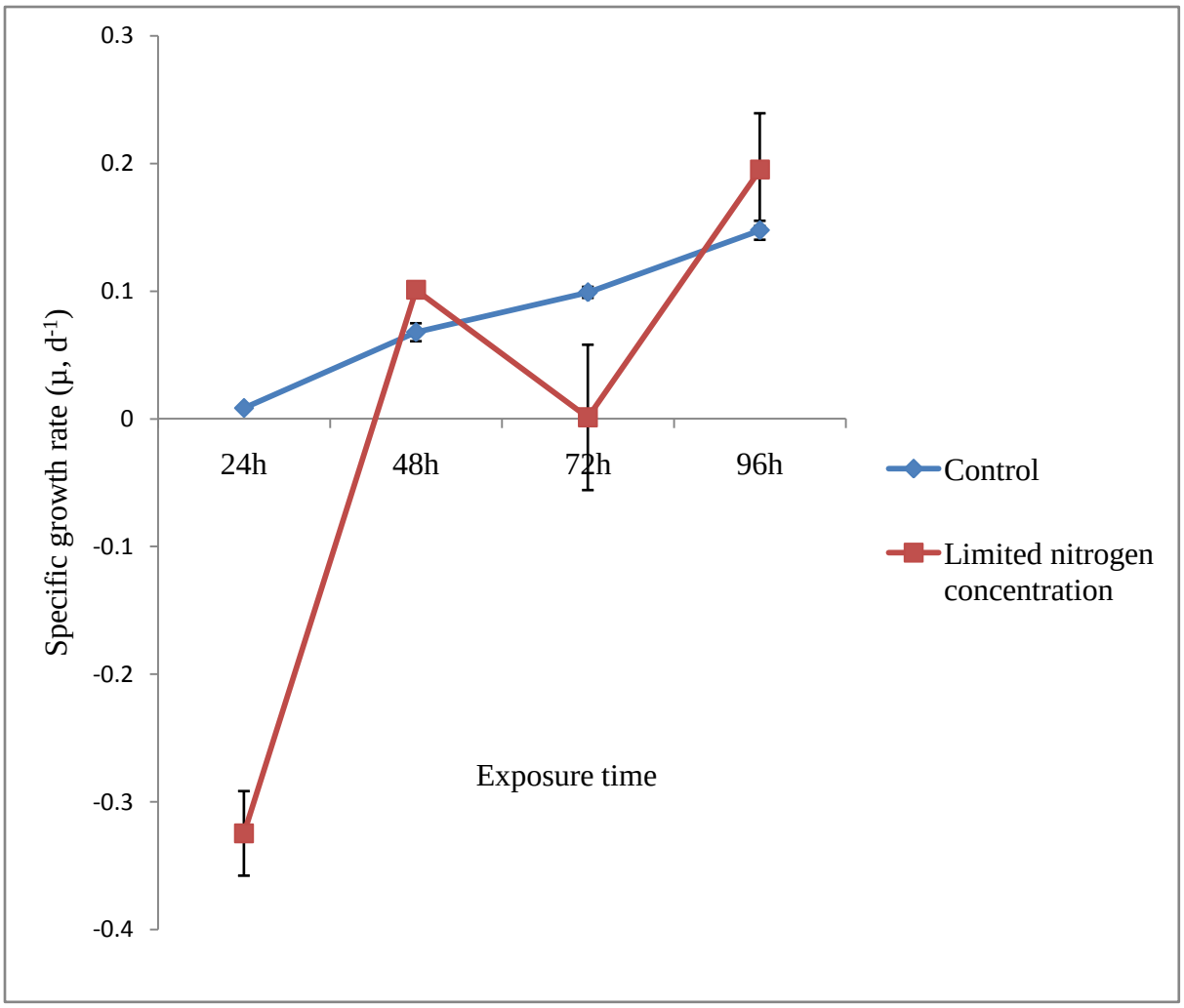

Fig 1: Specific growth rate (μ, d^{-1}) of *Chlorella vulgaris* at two nitrogen concentrations

Points represent mean ± standard error

Means are not significantly different ($P>0.05$)

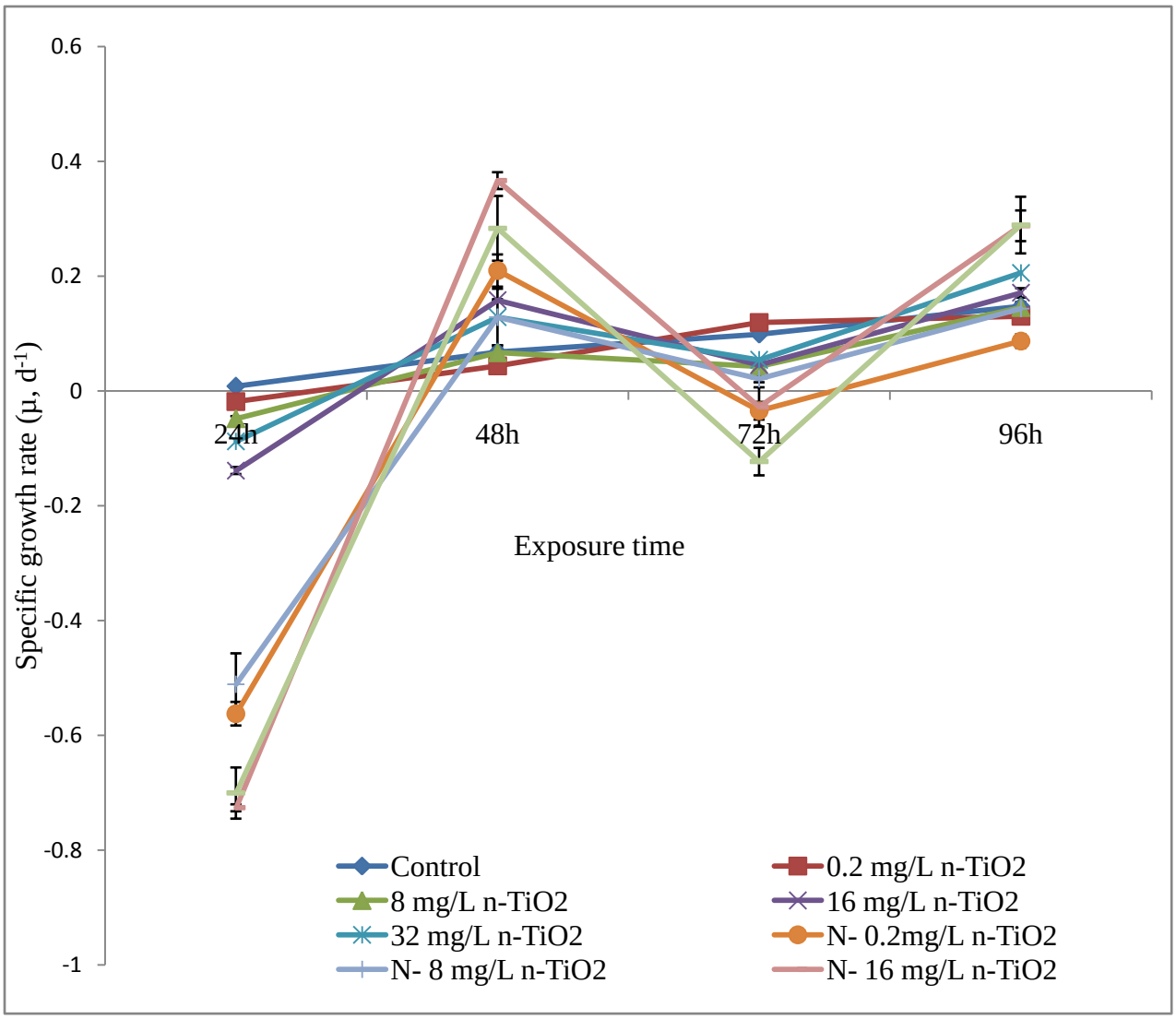

Fig 2: Specific growth rate (μ, d^{-1}) of *Chlorella vulgaris* under titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen

Points represent mean ± standard error

Means are not significantly different ($P>0.05$)

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

Table 4.5: Specific growth rate (μ, d^{-1}) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen treatments

Treatments	**24h**	**48h**	**72h**	**96h**
Control	0.0084 ± 0.002^{a}	0.0678 ± 0.007^{cd}	0.0990 ± 0.004^{bc}	0.1477 ± 0.007^{abcd}
0.2 mg/L n-TiO_2	-0.0184 ± 0.002^{ab}	0.0439 ± 0.005^{de}	0.1198 ± 0.005^{ab}	0.1304 ± 0.001^{cd}
8 mg/L n-TiO_2	-0.0479 ± 0.004^{ab}	0.0669 ± 0.013^{cd}	0.0432 ± 0.011^{bc}	0.1457 ± 0.005^{abcd}
16 mg/L n-TiO_2	-0.1384 ± 0.006^{bc}	$0.1583\pm0.002b^{cd}$	0.0454 ± 0.006^{bc}	0.1711 ± 0.008^{abc}
32 mg/L n-TiO_2	-0.0875 ± 0.054^{abc}	0.1288 ± 0.049^{cd}	0.0545 ± 0.006^{bc}	0.2057 ± 0.009^{abc}
N-	-0.3245 ± 0.033^{d}	0.1009 ± 0.004^{cd}	0.0011 ± 0.057^{bc}	0.1951 ± 0.044^{abc}
N-0.2 mg/L n-TiO_2	-0.5621 ± 0.021^{e}	0.2100 ± 0.028^{bc}	-0.0343 ± 0.015^{c}	0.0871 ± 0.011^{cd}
N- 8 mg/L n-TiO_2	-0.5110 ± 0.005^{e}	0.1288 ± 0.033^{cd}	0.0212 ± 0.009^{bcd}	0.1438 ± 0.008^{abcd}
N-16 mg/L n-TiO_2	-0.7260 ± 0.006^{f}	0.3666 ± 0.015^{a}	-0.0277 ± 0.034^{c}	0.2880 ± 0.027^{a}
N-32 mg/L n-TiO_2	-0.7003 ± 0.044^{ef}	0.2835 ± 0.056^{ab}	-0.1229 ± 0.024^{d}	0.2892 ± 0.049^{a}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different ($P<0.05$).

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

Table 4.6: Carbohydrate content (µg/ml) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen treatments

Treatments	24h	48h	72h	96h
Control	93.45±0.54^{a}	104.23±0.27^{a}	122.45±0.08^{a}	121.90±0.10^{a}
0.2 mg/L n-TiO_2	92.91±0.50^{a}	102.64±0.98^{a}	122.33±0.04^{a}	121.90±0.10^{a}
8 mg/L n-TiO_2	81.17±13.21^{a}	103.92±0.18^{a}	121.71±0.37^{a}	121.67±0.14^{a}
16 mg/L n-TiO_2	95.00±2.39^{a}	103.53±0.34^{a}	121.59±0.13^{a}	121.79±0.04^{a}
32 mg/L n-TiO_2	93.84±3.60^{a}	103.76±0.17^{a}	121.24±0.24^{a}	121.94±0.07^{a}
N-	90.62±5.20^{ab}	104.69±2.92^{a}	113.07±2.75^{ab}	111.01±3.56^{ab}
N-0.2 mg/L n-TiO_2	61.75±3.33^{c}	99.07±1.31^{ab}	92.60±8.42^{c}	109.42±3.63^{ab}
N- 8 mg/L n-TiO_2	86.83±0.64^{ab}	106.32±2.71^{a}	110.28±0.91^{bc}	113.65±0.04^{ab}
N-16 mg/L n-TiO_2	78.65±4.50^{ab}	105.74±2.18^{a}	114.23±0.64^{bc}	114.42±0.34^{ab}
N-32 mg/L n-TiO_2	78.57±3.56^{ab}	87.37±2.85^{ab}	100.97±4.43^{c}	108.45±0.37^{ab}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different (P<0.05).

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

Table 4.7: Protein content (µg/ml) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited nitrogen treatments

Treatments	24h	48h	72h	96h
Control	43.65±2.11^{a}	75.45±0.59^{ab}	77.90±1.33^{ab}	78.82±0.12^{ab}
0.2 mg/L n-TiO_2	42.81±1.40^{a}	77.29±0.12^{a}	78.27±0.16^{ab}	77.47±0.34^{ab}
8 mg/L n-TiO_2	40.93±0.11^{ab}	76.82±0.18^{a}	78.82±0.64^{ab}	78.64±0.07^{ab}
16 mg/L n-TiO_2	41.34±1.05^{ab}	76.04±0.41^{a}	79.31±0.02^{ab}	79.15±0.30^{ab}
32 mg/L n-TiO_2	42.75±0.85^{a}	77.02±0.05^{a}	78.51±0.69^{ab}	79.31±0.21^{ab}
N-	42.38±0.12^{ab}	77.82±0.50^{a}	80.95±0.50^{a}	78.92±0.64^{ab}
N-0.2 mg/L n-TiO_2	43.03±0.07^{a}	76.59±0.28^{ab}	80.19±0.04^{a}	78.68±0.96^{ab}
N- 8 mg/L n-TiO_2	43.01±0.20^{a}	77.39±0.02^{a}	80.01±0.14^{a}	78.43±0.78^{ab}
N-16 mg/L n-TiO_2	43.83±0.69^{a}	77.23±0.20^{a}	79.72±0.31^{ab}	77.68±0.48^{ab}
N-32 mg/L n-TiO_2	44.63±0.21^{a}	76.53±0.52^{ab}	78.70±0.28^{ab}	80.11±0.11^{a}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different (P<0.05).

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

Table 4.8: Glutathione-s-transferase activity (µmol/mL/min) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited

Treatments	24h	48h	72h	96h
Control	0.0069 ± 0.000^{ab}	0.009 ± 0.0006^{abc}	0.0088 ± 0.0004^{ab}	0.0067 ± 0.0008^{bc}
0.2 mg/L n-TiO_2	0.0071 ± 0.001^{ab}	0.0094 ± 0.0004^{abc}	0.0081 ± 0.0004^{b}	0.0071 ± 0.0008^{b}
8 mg/L n-TiO_2	0.0081 ± 0.0004^{a}	0.0098 ± 0.0002^{abc}	0.0075 ± 0.0000^{bc}	0.0077 ± 0.0006^{ab}
16 mg/L n-TiO_2	0.0050 ± 0.0014^{bc}	0.0102 ± 0.0002^{ab}	0.0081 ± 0.0004^{b}	0.0085 ± 0.0006^{ab}
32 mg/L n-TiO_2	0.0077 ± 0.0004^{a}	0.0102 ± 0.0002^{ab}	0.0088 ± 0.0004^{ab}	0.0085 ± 0.0004^{ab}
N-	0.0077 ± 0.0002^{a}	0.0075 ± 0.0000^{c}	0.0071 ± 0.0002^{bc}	0.0069 ± 0.0006^{b}
N-0.2 mg/L n-TiO_2	0.0075 ± 0.0000^{a}	0.0075 ± 0.0000^{c}	0.0081 ± 0.0007^{b}	0.0065 ± 0.0006^{bc}
N- 8 mg/L n-TiO_2	0.0081 ± 0.0000^{a}	0.0079 ± 0.0002^{bc}	0.0073 ± 0.0002^{bc}	0.0071 ± 0.0004^{b}
N-16 mg/L n-TiO_2	0.0075 ± 0.0000^{a}	0.0088 ± 0.0000^{bc}	0.0092 ± 0.0002^{ab}	0.0081 ± 0.0004^{ab}

N-32 mg/L n-TiO_2	0.0077 ± 0.0006^{a}	0.0088 ± 0.0004^{bc}	0.0088 ± 0.0004^{ab}	0.0069 ± 0.0000^{b}

Values represent mean ± standard error, n = 3 per treatment group; Means along the same column with different superscripts are significantly different ($P<0.05$); n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

4.3.2 Lipid peroxidation

There was higher lipid peroxidation (MDA equivalent ($nmol.cm^{-3}$)) under limited nitrogen concentration (2.8×10^{-6} M) compared with the control nitrogen concentration (2.8×10^{-4} M) as at the final time of treatment (96h) (Table 4.9). Titanium dioxide nanoparticles treatments with limited nitrogen concentrations gave higher lipid peroxidation than titanium dioxide nanopartilces treatments with control nitrogen concentration. There was a significant difference ($P<0.05$) in lipid peroxidation between the control and limited nitrogen conditions, but there was no significant difference ($P>0.05$) in lipid peroxidation recorded between the control and the different titanium dioxide nanoparticle concentrations. There was also no significant ($P>0.05$) interaction between the nitrogen and titanium dioxide nanoparticle concentrations with regards to lipid peroxidation.

4.3.3 Peroxidase activity

Peroxidase activity (units/mg) showed a slight difference between the control nitrogen (2.8×10^{-4} M) and limited nitrogen condition (2.8×10^{-6} M) (Table 4.10), with the limited nitrogen condition recording higher peroxidase activity. With increasing titanium dioxide nanoparticle concentrations and increasing exposure time, there was higher peroxidase activity for both the control nitrogen and limited nitrogen conditions. There was a statistically significant difference ($P<0.05$) in peroxidase activity between the control and limited nitrogen conditions. There was no statistically significant difference in peroxidase activity recorded between the control and the different titanium dioxide nanoparticle concentrations, there was also no statistically significant interaction between

Table 4.9: Lipid peroxidation (MDA equivalent (nmol.cm^{-3})) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited

Treatments	**24h**	**48h**	**72h**	**96h**
Control	0.09±0.02^{ab}	0.03±0.00^{a}	0.02±0.00^{bc}	0.08±0.02^{ab}
0.2 mg/L n-TiO_2	0.09±0.02^{ab}	0.03±0.00^{a}	0.02±0.00^{bc}	0.06±0.00^{ab}
8 mg/L n-TiO_2	0.04±0.00^{bc}	0.03±0.00^{a}	0.04±0.01^{bc}	0.08±0.00^{ab}
16 mg/L n-TiO_2	0.05±0.01^{bc}	0.02±0.01^{a}	0.06±0.00^{b}	0.07±0.00^{ab}
32 mg/L n-TiO_2	0.06±0.00^{bc}	0.03±0.00^{a}	0.02±0.01^{bc}	0.07±0.00^{ab}
N-	0.02±0.00^{c}	0.04±0.00^{a}	0.18±0.04^{a}	0.12±0.01^{a}
N-0.2 mg/L n-TiO_2	0.09±0.01^{ab}	0.03±0.00^{a}	0.08±0.00^{b}	0.08±0.00^{ab}
N- 8 mg/L n-TiO_2	0.12±0.03^{a}	0.02±0.00^{a}	0.07±0.00^{b}	0.08±0.00^{ab}
N-16 mg/L n-TiO_2	0.09±0.01^{ab}	0.04±0.01^{a}	0.07±-0.00^{b}	0.09±0.01^{ab}
N-32 mg/L n-TiO_2	0.08±0.01^{abc}	0.04±0.00^{a}	0.07±0.00^{b}	0.12±0.02^{a}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different (P<0.05).

n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

Table 4.10: Peroxidase activity (units/mg) of *Chlorella vulgaris* under limited nitrogen, titanium dioxide nanoparticle and titanium dioxide nanoparticle concentrations with limited

Treatments	**24h**	**48h**	**72h**	**96h**
Control	0.0091±0.0000^{bc}	0.0103±0.0049^{ab}	0.0018±0.0000^{a}	0.0006±0.0006^{c}
0.2 mg/L n-TiO_2	0.0122±0.0006^{ab}	0.0049±0.0016^{bc}	0.0024±0.0006^{a}	0.0024±0.0006^{b}
8 mg/L n-TiO_2	0.0122±0.0022^{ab}	0.0055±0.0000^{bc}	0.0030±0.0006^{a}	0.0043±0.0006^{abc}
16 mg/L n-TiO_2	0.0118±0.0005^{ab}	0.0067±0.0006^{bc}	0.0036±0.0000^{a}	0.0049±0.0012^{abc}
32 mg/L n-TiO_2	0.0097±0.0012^{bc}	0.0067±0.0012^{bc}	0.0036±0.0000^{a}	0.0055±0.0011^{abc}
N-	0.0091±0.0011^{bc}	0.0018±0.0000^{d}	0.0018±0.0000^{a}	0.0024±0.0006^{b}
N-0.2 mg/L n-TiO_2	0.0097±0.0026^{bc}	0.0018±0.0000^{d}	0.0018±0.0000^{a}	0.0024±0.0006^{bc}
N- 8 mg/L n-TiO_2	0.0085±0.0006^{bc}	0.0018±0.0000^{d}	0.0018±0.0000^{a}	0.0012±0.0006^{bc}
N-16 mg/L n-TiO_2	0.0103±0.0006^{bc}	0.0018±0.0000^{d}	0.0018±0.0011^{a}	0.0036±0.0000^{abc}

N-32 mg/L n-TiO_2	0.0122 ± 0.0006^{ab}	0.0018 ± 0.0000^{d}	0.0030 ± 0.0006^{a}	0.0030 ± 0.0006^{ab}

Values represent mean ± standard error, n = 3 per treatment group.

Means along the same column with different superscripts are significantly different ($P<0.05$); n-TiO_2 (Titanium dioxide nanoparticle), N- (Limited nitrogen concentration)

the nitrogen and titanium dioxide nanoparticle concentrations with regards to peroxidase activity.

4.4 Correlations of Growth, Biomass Production, Biochemical Composition, and Antioxidant Enzymes Activity with Nitrogen/Titanium dioxide Nanoparticles Treatments

The PCA analysis showed that the first two components were responsible for 60.89% of the total variation (Fig. 3). Dry weight, Carbohydrate content, Cell counts, and Chlorophyll a content had a significantly negative correlation with nitrogen limitation. Lipid peroxidation had a significantly positive correlation with nitrogen limitation. While increasing titanium dioxide nanoparticle had no significant correlation with any of the parameters measured.

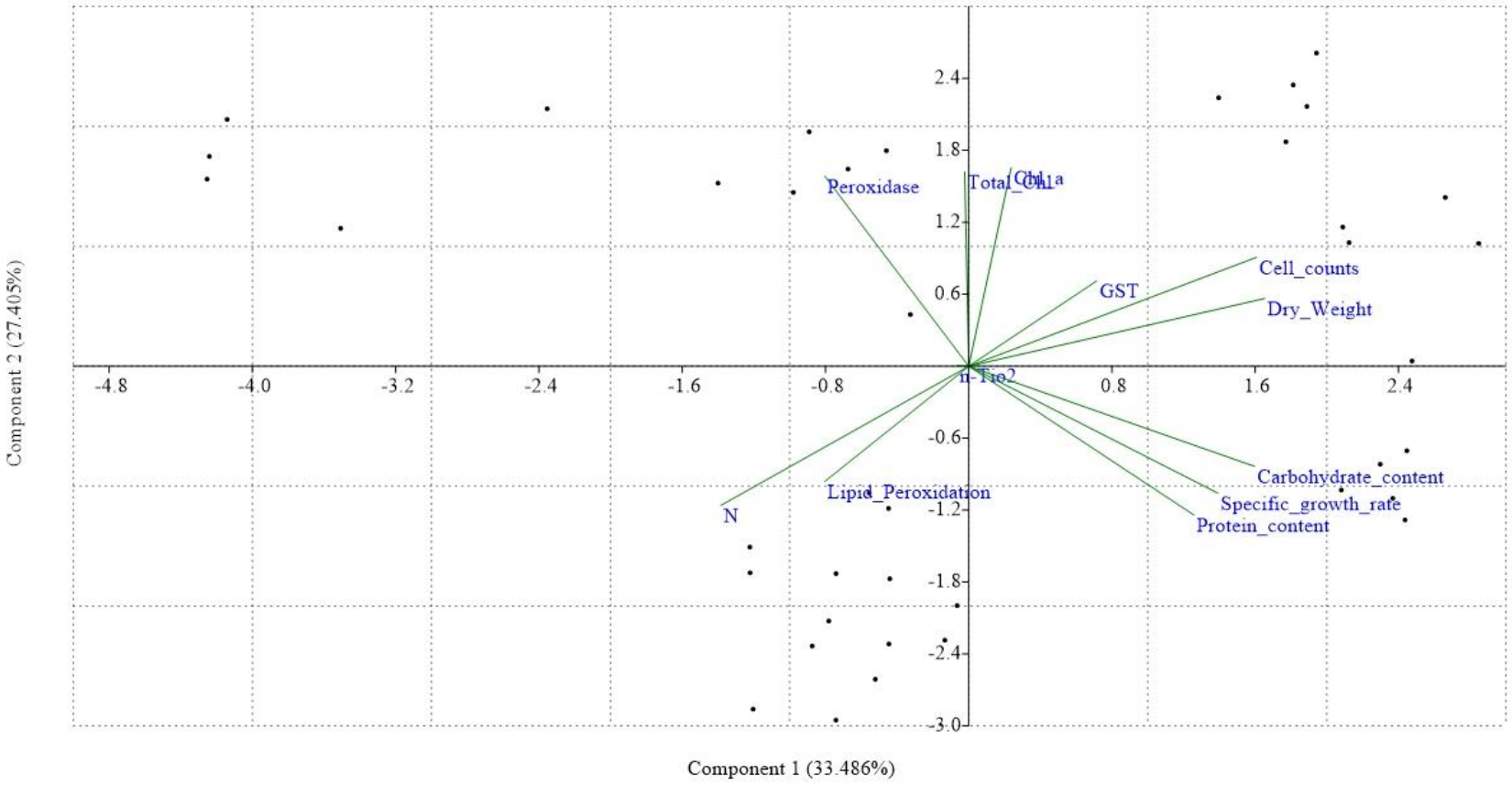

Fig. 3: PCA biplot of different parameters analyzed for *Chlorella vulgaris* after exposure to different nitrogen and titanium dioxide

nanoparticle concentrations showing the first and second components. N= nitrogen, DW= dry weight, n-TiO_2= titanium dioxide nanoparticle, GST= Glutathione-S-transferase

CHAPTER FIVE

5.0 DISCUSSION

Dry weight production of *Chlorella vulgaris* differed significantly ($P<0.05$) between the two nitrogen levels used for this study, with the control nitrogen level (2.8×10^{-4} M) giving a higher dry weight production than the limited nitrogen level (2.8×10^{-6} M) over the 96h study period, dry weight also showed a significantly ($P<0.05$) negative correlation with nitrogen limitation. This shows that a decrease in nitrogen levels leads to a decrease in dry weight of *Chlorella vulgaris*, which implies that *Chlorella vulgaris* needs an adequate supply of nitrogen which can be provided in different forms (Barsanti and Gualteiri., 2006), in this case provided as ammonium chloride to increase its dry weight because nitrogen accounts for about 7–10% of cell dry weight in algae (Hu, 2004). This is in agreement with results obtained for response of dry weight in algae to different levels of nitrogen (Nigam *et al*., 2011; Chia *et al*., 2015a). The decrease in the dry weight of *Chlorella vulgaris* by the titanium dioxide nanoparticle treatments could be due to the fact that they decreased the carbohydrate content of the algal cells, because the carbohydrate content accounted for most of the dry weight as observed from the significantly positive correlation between the two parameters. The significant interaction observed between nitrogen levels and titanium dioxide nanoparticle concentration in terms of dry weight in this study implies that the interaction between nitrogen levels and titanium dioxide nanoparticle was in an additive manner towards the dry weight of *Chlorella vulgaris*. This means the combined effect of both the limited nitrogen treatments and titanium dioxide nanoparticle is more than their individual effects

Total cell counts differed significantly ($P<0.05$) between the two nitrogen levels used for this study, with the control nitrogen (2.8×10^{-4} M) giving a higher cell count than the limited nitrogen level (2.8×10^{-6} M) over the 96h study period. Cell counts showed a significantly ($P<0.05$) negative correlation with nitrogen limitation, this shows that lower nitrogen level negatively affect cell count because nitrogen is an essential element needed for cell division to take place in algae. This agrees with the findings of Lai *et al*., 2011; Zachleder and Branyikova, (2014); Chia *et al*. (2015b), who stated that when microalgae are starved of nitrogen, cell divison ceases. Also when the nitrogen concentrations increase the substrate concentration of a series of physiological processes of the algae which in turn enhanced its assimilation and accelerated the growth of the algae (Lai *et al*., 2011) The different concentrations of titanium dioxide nanoparticles (0.2mg/L, 8mg/L, 16mg/L, 32mg/L) used did have a significant ($P<0.05$) effect on the cell counts of *Chlorella vulgaris.*, this could be because the metal affected the cell multiplication through cell division probably through binding of the metal to sulfhydryl groups that are important in regulating cell division. The significant interaction between nitrogen and titanium dioxide nanoparticle treatments which is in an additive manner, suggest that both treatments reduces cell division more than when either treatment occurs individually.

The specific growth rate decreased under limited nitrogen level (2.8×10^{-6} M), the different titanium dioxide nanoparticle treatments, and the combination of both. As growth rate is the increase in cell concentration per unit of time, this result shows that the treatments hindered the ability of the algae to increase its cell number during the period studied. The reason could be that the algal cells ability to multiply was hindered under these treatments which eventually affected their increase over time. Titanium dioxide

nanoparticles has been shown to coat algal cells (Hartmann *et al*., 2010; Clément *et al*., 2013) which could lead to shading at the individual cell level thereby leading to reduced light available to the cells. Consequently, low light condition led to poor cellular divisions. This result agrees with the findings of Aruoja *et al*. (2009); Hartmann *et al*. (2010); Sadiq *et al*. (2011), where they reported the inhibition of growth in algae by titanium dioxide nanoparticles, and Bono *et al*. (2013) who reported a decrease in algal growth under nitrogen limitation. However this results contrast with that of Kulacki and Cardinale (2012) who reported that titanium dioxide nanoparticles had little effect on algal growth rates.

The decrease in chlorophyll a and total chlorophyll production observed under limited nitrogen level and the different titanium dioxide nanoparticle concentrations, suggest a decrease in photosynthesis and eventually an impairment of the carbon fixation process of the alga. The interaction between nitrogen and titanium dioxide nanoparticle was in an additive manner, which means that the two treatments reduces the synthesis of chlorophyll a and total chlorophyll more than their individual effect. This can be further explained by the significant ($P<0.05$) negative correlation between nitrogen limitation and chlorophyll a production from the principal component analysis, which means that the *Chlorella vulgaris* cells were able to withstand the titanium dioxide nanoparticles treatments in terms of chlorophyll production as far as nitrogen concentration is not limiting. According to Hu, (2004); Ferreira *et al*. (2015) when algae are grown under nitrogen-limited conditions there is usually a decrease in the chlorophyll content of the cells, pigment concentration variation is associated with nutrient regulates the growth of algal cells and cell number (Lai *et al*., 2011) as observed in this results. This result agrees with other

literature which shows that algae biomass in the form of chlorophyll content decrease with decreasing nitrogen concentration (Chia *et al*., 2013). However the finding of this study contrast with the finding of Chen *et al*. (2012) who reported that the content of chlorophyll a in *Chlamydomonas reinhardtii* did not change under n-TiO_2 treatments, this could be because they used a different algal species in their study..

Microalgae vary their biochemical composition as a physiological response to environmental stress (Lai *et al*., 2011; Chia *et al*., 2015b). There was a decrease in carbohydrate production under limiting nitrogen concentration and titanium dioxide nanoparticle treatments. This indicates that nitrogen limitation and the titanium dioxide nanoparticle treatments led to a decrease in carbohydrate synthesis. Carbohydrates are synthesized in algae as the products of carbon fixation. The decrease in carbohydrate content observed could be because of the decrease in chlorophyll content as chlorophylls are the pigment responsible for the carbon fixation. Carbohydrates (primarily starch) are valuable component of the algal cell and carbohydrate dry weight in alga accounts for 20%-40% of the total cell mass (Hu, 2004). This is demonstrated by the significantly positive correlation between dry weight and carbohydrate content of the alga. The significantly positive correlation observed between carbohydrate content and dry weight shows that a major part of the algal dry weight was accounted for by the carbohydrate content. The significant interaction between nitrogen and titanium dioxide nanoparticle treatments with regards to carbohydrate synthesis indicates that the alga ability to synthesise carbohydrate under nitrogen limitation and titanium dioxide nanoparticle was more impeded than when treated with them individually. This result however contrast with that of Chia *et al*. (2015b) who reported an increase in carbohydrate content with increase

in nitrogen limitation, this could be because they recorded an increase in chlorophyll a per cell and chlorophyll a is the major pigment needed for carbon fixation to carbohydrates in *Chlorella vulgaris.*

Nitrogen is required by algae to manufacture amino acids, nucleic acids, chlorophyll, and other nitrogen-containing organic compounds. The protein content significantly ($P<0.05$) increased under limited nitrogen concentration. This could be because under the limited nitrogen the alga was stressed to the extent of up regulating the synthesis of different antioxidant enzymes such as GST and POD (assayed in this study), and even others to scavenge the presence of reactive oxygen species. This means that the alga were still able to synthesize these antioxidant enzymes which are proteins despite the limited nitrogen concentration at least for the duration of the duration of the study. This result contrast with the findings of Lai *et al*. (2011) who reported a decrease in protein content under nitrogen limitation.The protein content did not differ with the control under the titanium dioxide nanoparticle treatments and combination of different concentrations of n-TiO_2 with nitrogen. This implies that the titanium dioxide nanoparticles and the interaction titanium dioxide nanoparticle with nitrogen did not stress the alga significantly to the extent of producing more or less proteins most likely in the form of antioxidant enzymes as it seen that the activity of POD did not increase under these treatments.

The antioxidant enzymes are prominent biomarkers of defense against oxidative stress (El-Naggar and Sheikh, 2014) and they catalyze free radical quenching reactions in cells. Oxidative stress can be in form of increased production of reactive oxygen species (ROS) produced in cells when they are under stressed conditions. Antioxidant enzymes scavenge

for this ROS in algae cells under stress conditions. The mechanism of titanium dioxide nanoparticle phototoxicity under UV radiation as explained by Li *et al*. (2014) is by the excitation of an electron from the n-TiO_2, where the excited electron is promoted from the valence band to the conduction band. The transition results in an electron–hole pair that could react strongly with oxygen or water, and such an interaction will lead to reactive oxygen species (ROS) generation.

The findings of this study showed that the antioxidant enzymes glutathione-s-transferase (GST) activity was significantly increased when *Chlorella vulgaris* was subjected to limited nitrogen and n-TiO_2 treatments, while peroxidase (POD) activity was significantly increased when the alga was subjected to limited nitrogen treatment. The increase in the activity of GST and POD could be due to the fact that there was an increase in the production of ROS in the alga cells and the enzymes were trying to scavenge for these ROS. Furthermore GST has been shown to be responsible for the repair of macromolecules oxidized by ROS (Angelucci *et al*., 2005). The interaction of nitrogen and titanium dioxide nanoparticle did not increase the activity of GST and POD, this indicates that the alga were not significantly stressed to the extent of up-regulating the activity of these enzymes under these treatments.

Lipid peroxidation is also another indicator of oxidative stress in organisms where Malondialdehyde (MDA) is produced as its byproduct. Limited nitrogen has been shown

to increase lipid accumulation in algal species (Lin and Lin, 2011; Yilancioglu *et al*., 2014). The result of this study showed that limited nitrogen concentrations significantly increased the production of MDA equivalents. The increase in MDA levels may have resulted from lipid membrane damage and cell lysis. This can be due to the fact that the limited nitrogen treatment led to an increased production of lipids (Lin and Lin, 2011; Yilancioglu *et al*., 2014) and in turn more lipids were oxidised due to the stressed state of the algae. *Chlorella vulgaris* has been reported to produce more polyunsaturated fatty acids (PUFA) compared to other forms of fatty acids (Yusof *et al*., 2011) and PUFA has been shown to be the most sensitive to lipid peroxidation (Repetto *et al*., 2012). This result agrees with the findings of Yilancioglu *et al*. (2014) who reported an increase in lipid peroxidation in *Dunaliella salina* under limited nitrogen conditions. However the n-TiO_2 treatments and combined treatment of limited nitrogen with n-Tio_2 did not significantly influence the lipid peroxidation from this result. This means that these treatments did not elicit the increased production of lipids or the oxidation of the lipids present in the algae. According to Chen *et al*. (2012), lipid peroxidation was induced in *Chlamydomonas reinhardtii* under nano-TiO_2 exposure, but they found that the MDA contents reached its maximum value after 8h of exposure and then decreased. In contrast to the result of this study, the initial MDA content was measured after 24h of treatment and was not significantly increased by n-TiO_2 treatments.

The toxicity of nanomaterials like titanium dioxide nanoparticle according to Miller *et al*. (2012); Lai *et al*. (2014) are dramatically increased under ultraviolet (UV) light

(wavelength 10-400nm), this could be the reason why the activity of POD and lipid peroxidation were not increased as this study was conducted under white fluorescent light (wavelength 400-700nm).

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The toxicity of titanium dioxide nanoparticles in the presence of limited nitrogen concentration on *Chlorella vulgaris* was studied. It was found that limited nitrogen treatment significantly affected the dry weight, cell counts, specific growth rate, chlorophyll content, carbohydrate content, protein content, activity of glutathione-s-transferase, activity of peroxidase and lipid peroxidation of *Chlorella vulgaris,* while the titanium dioxide nanoparticles affected significantly the dry weight, cell counts, chlorophyll content, specific growth rate, carbohydrate content and glutathione-s-transferase activity. The combination of limited nitrogen treatment and titanium dioxide nanoparticles significantly affected the dry weight, cell counts, chlorophyll content, specific growth rate and carbohydrate content of *Chlorella vulgaris* negatively.

6.2 Conclusions

Limited nitrogen, titanium dioxide nanoparticles and the combination of both significantly decrease the growth and biomass (chlorophyll a (0.01 mg/L), total chlorophyll (0.012 mg/L), cell counts (114.44×10^4 cells/mL), dry weight (85.20 mg/L)) of *Chlorella vulgaris.*

Protein content of *Chlorella vulgaris* increases significantly under limited nitrogen (78.92 µg/ml), while carbohydrate content significantly decreases under limited nitrogen (111.01 µg/ml), titanium dioxide nanoparticles (121.94 µg/ml) and the combination of both (108.45 µg/ml).

The activity of glutathione-s-transferase increases significantly under nitrogen limitation (0.00069 µmol/mL/min) and titanium dioxide nanoparticles treatments (0.0085 µmol/mL/min), while peroxidase (0.0024 unit/mg) and lipid peroxidation (malondialdehyde equivalents) (0.12 $nmol.cm^{-3}$) significantly increase under nitrogen limitation.

6.3 Recommendations

The combined effect of titanium dioxide nanoparticle and other nutrient elements on *Chlorella vulgaris* and even other algae species should be studied to give a complete picture of how algae react to the presence of these treatments.

The occurrence and abundance of metal nanoparticles in the water bodies in the environment should be investigated.

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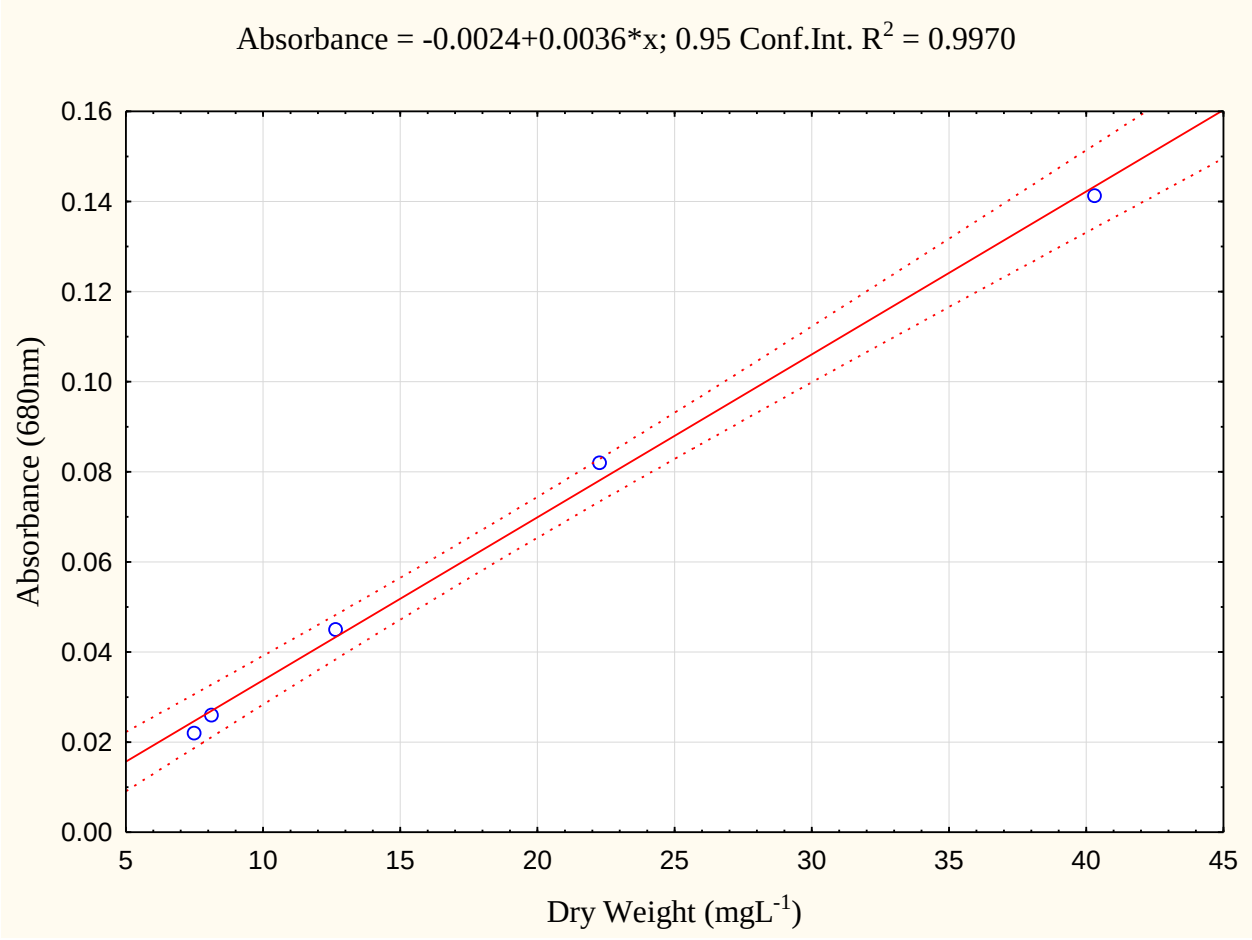
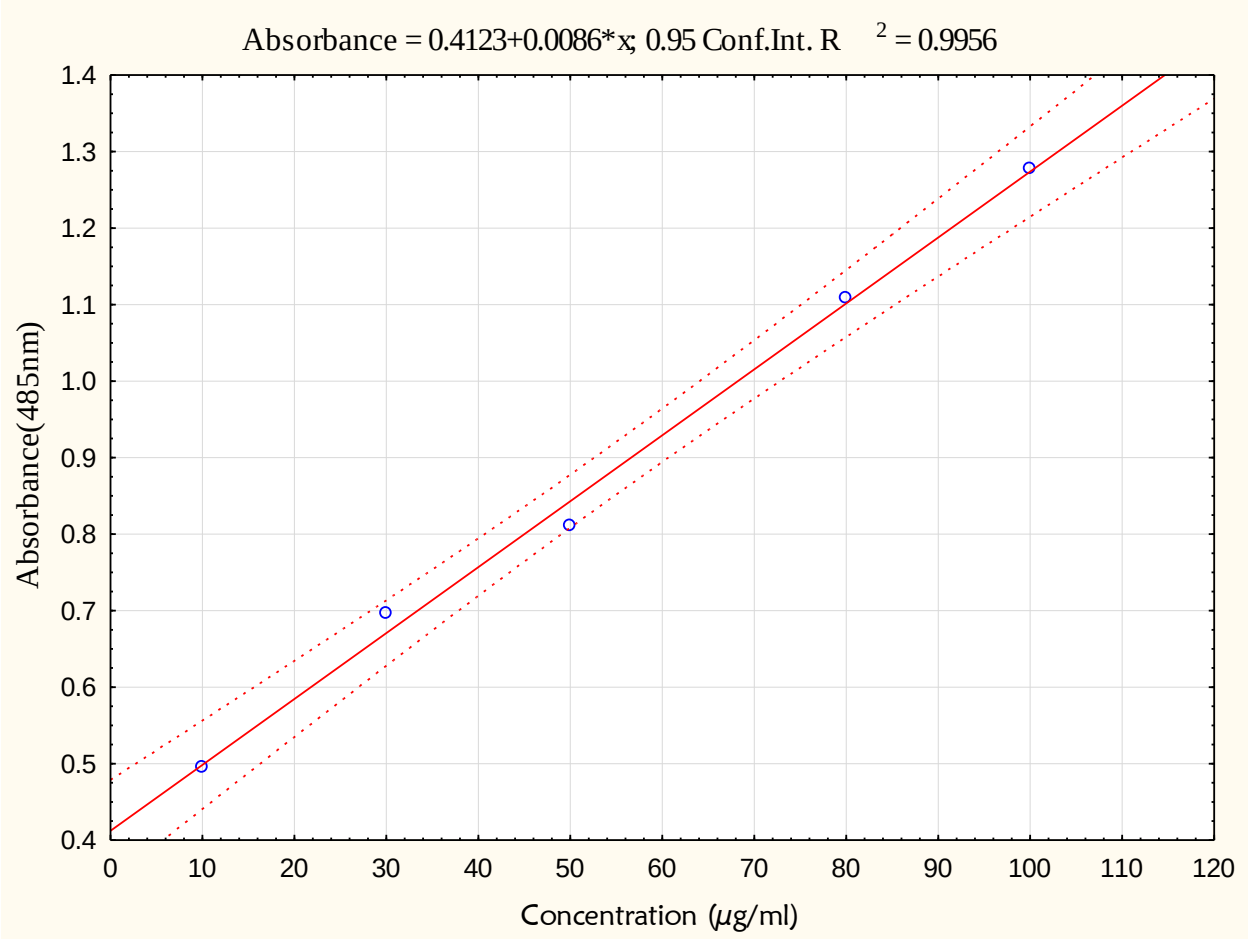
APPENDICES

Appendix I: Dry weight calibration curve

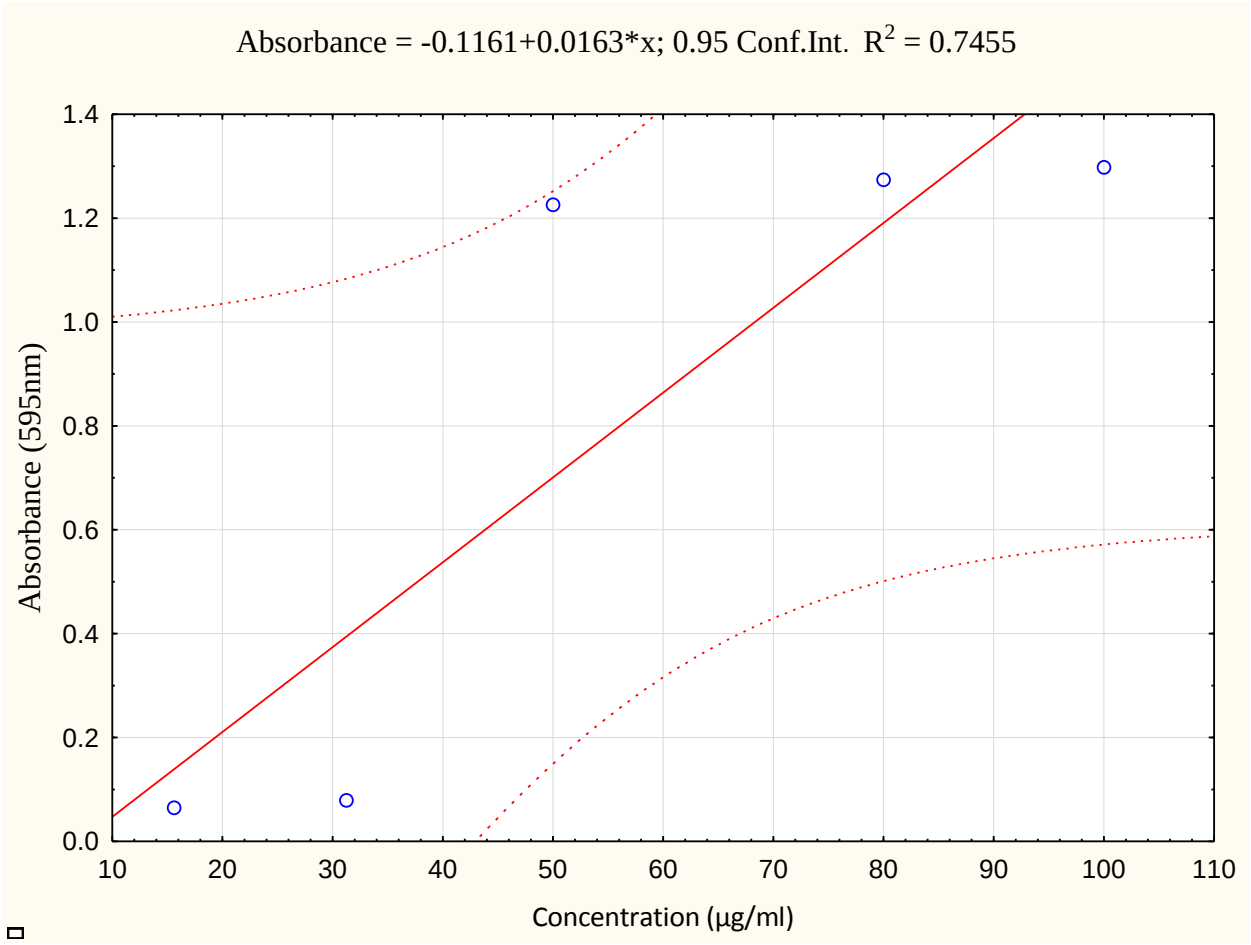


s

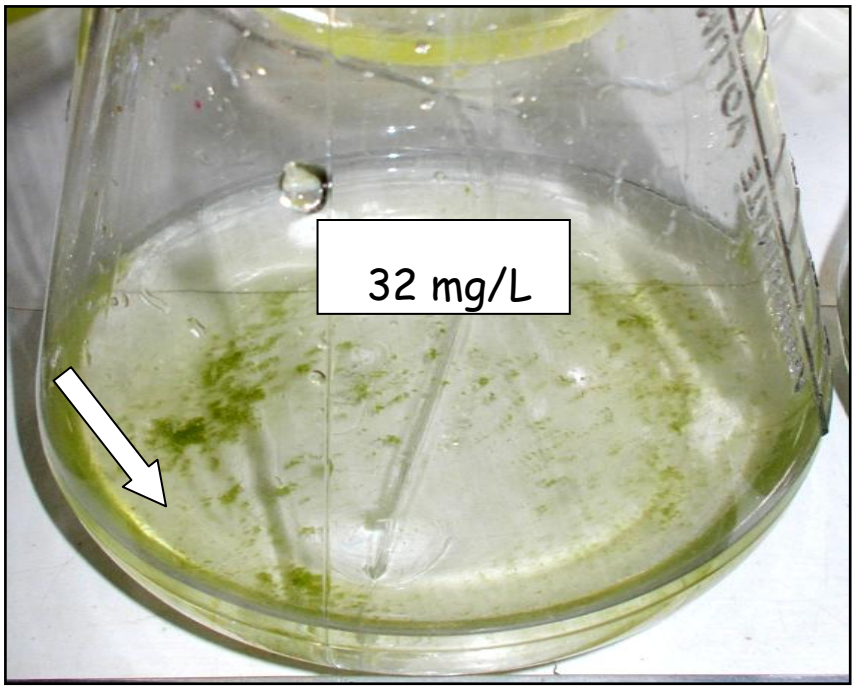
Appendix II: Carbohydrate calibration curve



Appendix III: Protein calibration curve



Appendix IV: Control culture showing no clumping of *Chlorella vulgaris* cells and 32mg/L titanium dioxide nanoparticle culture showing clumping of *Chlorella vulgaris* cells



Appendix V: Repeated Measures Analysis of Variance Tables

Dry weight

Effect	**DF**	**SS**	**MS**	**F**	**P**
Nitrogen	1	194000	194000	4379	0.000*
Titanium dioxide nanoparticles	4	3962	990	22	.000*
Nitrogen*Titanium dioxide nanoparticles	4	1006	251	6	.003*
TIME	3	25400	8464	914	0.000*
TIME*Nitrogen	3	2899	966	104	0.000*
TIME*Titanium dioxide nanoparticles	12	1129	94	10	.000*
TIME*Nitrogen*Titanium dioxide nanoparticles	12	434	36	4	.000*

Cell counts

Effect	**DF**	**SS**	**MS**	**F**	**P**
Nitrogen	1	1970000	1970000	6029	0.000*
Titanium dioxide nanoparticles	4	14300	3564	11	.000*
Nitrogen*Titanium dioxide nanoparticles	4	3417	854	3	0.066

TIME	3	96000	32000	54	0.000*
TIME*Nitrogen	3	67700	22600	38	.000*
TIME*Titanium dioxide nanoparticles	12	9851	821	1	0.2
TIME*Nitrogen*Titanium dioxide nanoparticles	12	12800	1064	2	0.07

Chl a

Effect	**DF**	**SS**	**MS**	**F**	**P**
Nitrogen	1	1.877	1.877	150.8	.000*
Titanium dioxide nanoparticles	4	1.208	0.302	24.3	.000*
Nitrogen*Titanium dioxide nanoparticles	4	0.207	0.052	4.2	.013*
TIME	3	5.276	1.759	107.8	0.000*
TIME*Nitrogen	3	5.626	1.875	114.9	0.000*
TIME*Titanium dioxide nanoparticles	12	2.262	0.189	11.6	.000*
TIME*Nitrogen*Titanium dioxide nanoparticles	12	1.981	0.165	10.1	.000*

Total Chl

Effect	DF	SS	MS	F	P
Nitrogen	1	2.21	2.21	65.1	.000*
Titanium dioxide nanoparticles	4	4.67	1.17	34.4	.000*
Nitrogen*Titanium dioxide nanoparticles	4	0.92	0.23	6.7	.001*
TIME	3	31.84	10.61	205.3	0.000*
TIME*Nitrogen	3	20.13	6.71	129.8	0.000*
TIME*Titanium dioxide nanoparticles	12	12.79	1.07	20.6	.000*
TIME*Nitrogen*Titanium dioxide nanoparticles	12	8.37	0.7	13.5	.000*

Specific growth rate

Effect	DF	SS	MS	F	P
Nitrogen	1	0.376	0.376	907.1	0.000*
Titanium dioxide nanoparticles	4	0.026	0.006	15.6	.000*
Nitrogen*Titanium dioxide nanoparticles	4	0.019	0.005	11.2	.000*
TIME	3	4.596	1.532	631.1	0.000*
TIME*Nitrogen	3	1.772	0.591	243.3	0.000*

TIME*Titanium dioxide nanoparticles	12	0.5	0.042	17.2	.000*
TIME*Nitrogen*Titanium dioxide nanoparticles	12	0.139	0.012	4.8	.000*

Carbohydrate

Effect	**DF**	**SS**	**MS**	**F**	**P**
Nitrogen	1	3159	3159	74.5	.000*
Titanium dioxide nanoparticles	4	976	244	5.8	.003*
Nitrogen*Titanium dioxide nanoparticles	4	1218	305	7.2	.001*
TIME	3	18400	6131	218.2	0.000*
TIME*Nitrogen	3	638	213	7.6	.000*
TIME*Titanium dioxide nanoparticles	12	642	54	1.9	0.052
TIME*Nitrogen*Titanium dioxide nanoparticles	12	904	75	2.7	.006*

Protein

Effect	**DF**	**SS**	**MS**	**F**	**P**
Nitrogen	1.0	18	18.0	11.0	.003*

Titanium dioxide nanoparticles	4.0	3	1.0	1.0	0.7
Nitrogen*Titanium dioxide nanoparticles	4.0	1	0.2	0.2	1.0
TIME	3.0	28300	9444.0	9359.0	0.000*
TIME*Nitrogen	3.0	7	2.0	2.0	0.1
TIME*Titanium dioxide nanoparticles	12.0	20	2.0	2.0	0.1
TIME*Nitrogen*Titanium dioxide nanoparticles	12.0	38	3.0	3.0	.002*

Glutathione-s-transferase activity

Effect	**DF**	**SS**	**MS**	**F**	**P**
Nitrogen	1	5.2E-06	5.2E-06	5.02	.037*
Titanium dioxide nanoparticles	4	1.2E-05	3.0E-06	2.95	.046*
Nitrogen*Titanium dioxide nanoparticles	4	5.5E-06	1.3E-06	1.32	0.295
TIME	3	0	0	29.92	.000*
TIME*Nitrogen	3	0	0	13.44	.000*
TIME*Titanium dioxide nanoparticles	12	0	0	3.9	.000*
TIME*Nitrogen*Titanium dioxide	12	0	0	1.48	0.157

nanoparticles

Lipid peroxidation

Effect	DF	SS	MS	F	P
Nitrogen	1	0.021	0.021	39.02	.000*
Titanium dioxide nanoparticles	4	0.003	0.001	1.25	0.323
Nitrogen*Titanium dioxide nanoparticles	4	0.001	0	0.37	0.828
TIME	3	0.047	0.016	43.02	.000*
TIME*Nitrogen	3	0.014	0.005	13.03	.000*
TIME*Titanium dioxide nanoparticles	12	0.018	0.001	4.13	.000*
TIME*Nitrogen*Titanium dioxide nanoparticles	12	0.037	0.003	8.62	.000*

Peroxidase activity

Effect	DF	SS	MS	F	P
Nitrogen	1	0.00014	.000	36.3	.000*
Titanium dioxide nanoparticles	4	2.5E-05	6.2E-06	1.7	0.199
Nitrogen*Titanium dioxide nanoparticles	4	1.1E-05	2.7E-06	0.7	0.593

TIME	3	0.001	0	104.5	0.000*
TIME*Nitrogen	3	0	0	7.2	.000*
TIME*Titanium dioxide nanoparticles	12	0	0	1.3	0.247
TIME*Nitrogen*Titanium dioxide nanoparticles	12	0	0	1.3	0.24