STUDY ON BIOCHEMICAL RESPONSES OF NUTRIENT MICROALGAE SPIRULINA PLATENSIS AND HAEMATOCOCCUS PLUVIALIS TO ZINC OXIDE NANOPARTICLES

SINOUVASSANE DJEARAMANE

DOCTOR OF PHILOSOPHY (SCIENCE)

LEE KONG CHIAN FACULTY OF ENGINEERING AND SCIENCE
UNIVERSITI TUNKU ABDUL RAHMAN
APRIL 2019

STUDY ON BIOCHEMICAL RESPONSES OF NUTRIENT MICROALGAE SPIRULINA PLATENSIS AND HAEMATOCOCCUS PLUVIALIS TO ZINC OXIDE NANOPARTICLES

By

SINOUVASSANE DJEARAMANE

A thesis submitted to Lee Kong Chian Faculty of Engineering and Science,
Universiti Tunku Abdul Rahman,
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy (Science)
April 2019

ABSTRACT

STUDY ON BIOCHEMICAL RESPONSES OF NUTRIENT MICROALGAE SPIRULINA PLATENSIS AND HAEMATOCOCCUS PLUVIALIS TO ZINC OXIDE NANOPARTICLES

Sinouvassane Djearamane

Zinc oxide nanoparticle (ZnO NP) is widely used in industrial and personal care products. The massive usage of this nanoparticle has created residues that can cause contamination to the environment. The presence of ZnO NPs in the aquatic environment could lead to accumulation of nanoparticles in aquatic organisms and cause cytotoxic effects. Thus, the present study has investigated the cellular accumulation and the cytotoxic effects of ZnO NPs in nutrient microalgae *Spirulina platensis* and *Haematococcus pluvialis*. The microalgal cells were exposed to the various concentrations of ZnO NPs (10 to 200 mg/L) from 6 to 96 h to explore the dose- and time-dependent cellular accumulation and the corresponding cytotoxic effects. Scanning electron microscopy -X-ray energy dispersive spectroscopy (SEM EDX) analysis evidenced the cellular accumulation of zinc in the algal biomass. Quantification of cell associated zinc by inductively coupled plasma optical emission spectroscopy (ICP OES) reported a substantial accumulation of zinc in both microalgae with higher zinc accumulation in *S. platensis*. Cytotoxicity

and oxidative stress assessment results revealed a typical concentration- and time-dependent increase in reactive oxygen species and lipid peroxidation levels and the corresponding decrease in cell viability, biomass and photosynthetic pigments on both microalgae. The study reported 13.97 mg/L of ZnO NPs as 96 h EC_{50} for *S. platensis* and 186.67 mg/L of ZnO NPs for *H. pluvialis*. The microscopic examinations of the algal cells that are interacting with ZnO NPs showed severe cell membrane and intracellular damages. Overall, the present study reported the accumulation of considerable amount of ZnO NPs in algal cells and the corresponding oxidative stress and cytotoxicity in both microalgae. Notably, 96 h EC_{50} results of the present study reported nearly 13 times higher sensitivity of *S. platensis* to ZnO NPs' toxicity compared to *H. pluvialis*. Hence, the study suggests that *S. platensis* could be potentially used as a bioindicator for identifying the contamination of ZnO NPs in the nutrient microalgae and also for testing the toxic effects of ZnO NPs on the aquatic organisms.

ACKNOWLEDGEMENT

First and foremost, my sincere appreciation and gratefulness to my supervisor, Assistant Professor Dr. Lee Poh Foong, for her continuous guidance, encouragement, patience and advice throughout the project. I would like to thank my co-supervisor, Professor. Dr. Lim Yang Mooi, for her guidance, support and advice, and to express my gratitude to Professor. Dr. Wong Ling Shing, INTI International University, for his opinions and feedback throughout the work. I have learned a lot from my supervisors and have broadened my perspective in analyzing the cellular accumulation and the cytotoxicity of ZnO NPs in microalgae.

This project was funded by Ministry of Education, Malaysia (Grant No. FRGS-1-2014-SG03-INTI-02-1), Universiti Tunku Abdul Rahman Research Fund (UTARRF) 2016, Vote No.: 6200/LH1 and UTARRF 2017, Vote No.: 6200/LJ3.
I thank UTAR, Faculty of Medicine and Health Sciences and Lee Kong Chian Faculty of Engineering and Science for providing me with laboratory instruments and good circumstances. Also thank the Central Unit for Advanced Research Imaging (CENTUARI), Faculty of Medicine, University Malaya, for the transmission electron microscopy used in this study.

APPROVAL SHEET

This dissertation/thesis entitled **"STUDY ON BIOCHEMICAL RESPONSES OF NUTRIENT MICROALGAE SPIRULINA PLATENSIS AND HAEMATOCOCCUS PLUVIALIS TO ZINC OXIDE NANOPARTICLES"** was prepared by SINOUVASSANE DJEARAMANE and submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy (Science) at Universiti Tunku Abdul Rahman.

Approved by:

_____ Date: ……

(Dr. LEE POH FOONG)

Assistant Professor/Supervisor

Department of Mechatronics and Biomedical Engineering

Lee Kong Chian Faculty of Engineering and Science

Universiti Tunku Abdul Rahman.

_____

(Prof. Dr. LIM YANG MOOI) Date: ……

Professor/Co-supervisor

Department of Pre-Clinical Sciences

Faculty of Medicine and Health Sciences

Universiti Tunku Abdul Rahman.

LEE KONG CHIAN FACULTY OF ENGINEERING AND SCIENCE
UNIVERSITI TUNKU ABDUL RAHMAN

Date: 23 April 2019

SUBMISSION OF THESIS

It is hereby certified that **SINOUVASSANE DJEARAMANE** (ID No: **16UED00156**) has completed this thesis entitled "STUDY ON BIOCHEMICAL RESPONSES OF NUTRIENT MICROALGAE SPIRULINA PLATENSIS AND HAEMATOCOCCUS PLUVIALIS TO ZINC OXIDE NANOPARTICLES" under the supervision of Dr. Lee Poh Foong (Supervisor) from the Department of Mechatronics and Biomedical Engineering, Lee Kong Chian Faculty of Engineering and Science and Prof. Dr. Lim Yang Mooi (Co-Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences.

I understand that the University will upload softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(SINOUVASSANE DJEARAMANE)

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

SINOUVASSANE DJEARAMANE

Date _____

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ag+	Silver ion
Ag NPs	Silver nanoparticles
BODIPY 581/591C11	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a-, 4a-diaza-s-indacene-3-undecanoic acid
chl-a	Chlorophyll-a
Cd+	Cadmium ion
CPI	Revised nanotechnology consumer products inventory
Cr	Chromium
Cu	Copper
d	Day
DNA	Deoxyribonucleic acid
DPA	Diphenylalanine
EC	Effective concentration
EDX	X-ray energy dispersive spectroscopy
EPS	Exoploymeric substance
FS	Ferrous sulphate
FTIR	Fourier transform infrared
H	Hour
HCl-ACE	Hydrochloric acid- acetone

H_2DCF-DA	2-7-didichlorodihydro fluorescein-diacetate
HL	High intensity light
H_2O_2	Hydrogen peroxide
ICP OES	Inductively coupled plasma optical emission spectroscopy
LPO	Lipid peroxidation
Mg^{2+}	Magnesium ion
µM	Micromole
M	Metre
MDA	Malondialdehyde
Min	Minute
mL	Millilitre
NaCl	Sodium chloride
NF	Norflurazon
NPs	Nanoparticles
nTiO2	Nano titanium dioxide
Pb	Lead
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
S	Second
SEM	Scanning electron microscopy
SEM-EDX	Scanning electron microscopy - X-ray energy dispersive spectroscopy

TEM	Transmission electron microscopy
UV	Ultra violet
Zn^{+}	Zinc ion
ZnO	Zinc oxide
ZnO NPs	Zinc oxide nanoparticles

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Background

Nano industry is one of the fastest growing industries in the history of mankind due to industrial revolution. The global socioeconomic value of nanotechnologies is progressively growing and currently nanosized particles have substantial influence on almost all industrial products and all areas of humanity (Piccinno et al., 2012). According to the European Commission (2013), nanomaterial is defined as a natural, or engineered material containing particles of at least one dimension between 1- 100 nm in an agglomerate or an aggregate or as an unbound state (Kassim et al., 2013). All nanoscale materials have higher reactivity and enhanced property due to increased relative surface area compared to their conventional 'bulk' (micro-size) materials (Bondarenko et al., 2013). Bulk materials constitute only few atoms at the surface level, whereas nanosized particles have most of the atoms at the surface or close to the surface that provides the unique physicochemical properties such as small size, high surface area to mass ratio and high reactivity to nanoparticles (NPs) (Khan et al., 2014; Kunhikrishnan et al., 2015) to utilize them in numerous commercial products (Casals et al., 2012). It is

projected that the annual manufacturing of nanoparticles incorporated products is likely to reach $3 trillion by the year 2020 (Roco et al., 2011).

Metallic nanoparticles have versatile applications in many industrial and consumer products (Miao et al., 2009). Revised Nanotechnology Consumer Products Inventory (CPI) released in 2013 has listed 1814 consumer products manufactured using NPs in 32 countries. About 762 (42%) listed products belong to health and fitness categories including personal care products such as toothbrushes, hairstyling products and skin care lotions which accounts for 39% of products (Vance and Marr, 2015). Metals and metal oxide nanoparticles contain the major nanomaterial composition publicized in the CPI. Titanium dioxide nanoparticles (TiO_2 NPs) and zinc oxide nanoparticles (ZnO NPs) are the largest produced nanomaterials worldwide. The annual manufacturing of silver nanoparticles (Ag NPs) represents only 2% of that of metal oxide NPs (Piccinno et al., 2012). However, Ag NPs are the most commonly publicized nanoparticles in the CPI as it is used in 438 products due to their renowned antimicrobial properties. Ag NPs and TiO_2 NPs are combined with other nanoparticles in 35 and 30 consumer products respectively. Ag NPs and TiO_2 NPs are paired with each other in 10 products including electronic and cosmetic products. TiO_2 NPs and ZnO NPs are paired in 10 products such as paints, sunscreens lotions and paints (Vance and Marr, 2015). Silver nanoparticles are used in various consumer products including textiles, laundry additives, home appliances, paints, personal care products, food supplements and also in food

storage containers (Maynard et al., 2006). Nano titanium dioxide ($nTiO_2$) is the next commonly used nanomaterial in various consumer products following Ag NPs. Titanium dioxide nanoparticles are widely used as white pigments in paints (including lacquers and varnishes), paper and plastics due to their unique physicochemical properties such as brightness, high refractive index and resistance to discoloration (Yu et al., 2014). Nearly 70% of all the $nTiO_2$ produced are used as pigments in the domestic products namely paints, glazes, enamels, plastics, paper and food products, and also in the cosmetic products including toothpastes, UV sunscreens, shampoos, deodorants, and shaving creams (Botelho et al., 2014). In addition, $nTiO_2$ is used as an antimicrobial agent, inorganic UV filter, and a catalyst for air and water purification (Janer et al., 2014). The maximum amount of $nTiO_2$ is found in common sunscreens and even in products intended for children such as sweets, candies or chewing gums (Weir et al., 2012). The global production of TiO_2 NPs and ZnO NPs for sunscreen products alone was estimated to be approximately 1000 tons during 2003/ 2004 (Borm et al., 2006). The next most commonly used metal oxide nanomaterial for a range of applications, following $nTiO_2$, is ZnO NPs. ZnO NP is a widely used metal oxide NP that has unique opto-electric properties due to its wurtzite crystal structure (Wang, 2004). ZnO NP has transparent conductivity that provides clear coatings on transparent surfaces, and its inherent property of piezoelectricity makes it particularly attractive for electronic sensor, solar voltaics and transducer applications (Ma et al., 2013). Further, ZnO NPs are widely used in ceramics, rubber, glass, cement, plastics, lubricants, pigments,

paints and textiles, this is owing to its large surface area, catalytic, antimicrobial and anticorrosion properties (Yung et al., 2014; Djearamane et al., 2016; Hou et al., 2018). It is used as a raw material for high-speed wear-resistant rubber products due to its advantages of anti-aging, anti-friction, low consumption and long service life (Klingshirn, 2007). Nano-zinc ceramics can be used as sanitary products for its antibacterial, deodorizing, and self-cleaning role (Moezzi et al., 2012). Its usage in paints or coatings further improves the protective capability of coatings, anti-ultraviolet radiation, anti-atmospheric damage, anti-degradation and discoloration (Keller et al., 2013). In addition, ZnO NPs have been used in automobile tail gas treatment as a highly reactive catalyst (Liu et al., 2014). Most importantly, the continuously growing market for ZnO NPs obsessed mainly by increasing demand for cosmetics and sunscreens. Statistically, the annual output of ZnO NPs has been reported between 550 and 5550 tons, and the yield is about 100 times higher than that of any other nano materials (Piccinno et al., 2012) because of its ultraviolet light absorption and reflective properties providing a long-term and broadband protection from UVA (320–400 nm) and UVB (290–320 nm). Moreover, the nanosize of ZnO makes it as a suitable sunscreen ingredient to provide a clear transparent appearance upon topical application compared to white residue left on the skin with normal-scale particles (Popov et al., 2005). Furthermore, the antibacterial property of this particle has extended its application in pharmaceutical and food industries (Dastjerdi and Montazer, 2010; Osmond and Mccall, 2010; Song et al., 2010). Antimicrobial property of ZnO NP against pathogenic bacteria

(*Escherichia Coli* and *Staphylococcus aureus*) recommended its application in food preservation field and also as a potent sanitizing agent for disinfecting and sterilizing food industry equipment and containers (Sabir et al., 2014). Recently, ZnO NPs have been used in numerous chemotherapeutic drugs such as doxorubicin, 5-flurouracil and doxorubicin as a carrier (Babu et al., 2017). Such widespread and expanding production and use of ZnO NPs increases the potential for their release into the environment. Environmental levels of ZnO NPs are expected to increase continually given the widespread application of these nanomaterials. Upon release into the aquatic environment, ZnO NPs might carry potential adverse effects on the ecosystem and human health (Ma et al., 2013).

There is an increasing evidence that the massive industrial usage and the subsequent release of NPs into the environment, render these nanomaterials more harmful to living organisms (Kumari et al., 2011; Kouhi et al., 2014). Nearly $US 1 trillion worth of products worldwide are expected to incorporate nanotechnology in key functional components by the year 2015, of which high end estimates suggest that 17% may end to soils, 21% to water and 2.5% to air, with balance entering landfill (Patil et al., 2016). Engineered nanomaterials in wastewater streams reach natural ecosystems by sludge disposal in terrestrial system and effluent discharge ending in aquifers and rivers and the reported level of ZnO NPs in Europe and United States sewage sludge are 13.6–57.0 and 17.4 – 57.7 mg/ kg (Kunhikrishnan et al., 2015). Due to the increasing volumes of NPs production and the growing likelihood

of industrial and environmental exposure to NPs, the legislative bodies of United States of America and European Union have recently focused their activities on assessing the impacts of nanomaterials on human and environmental health (Bondarenko et al., 2013; Patil et al., 2016).

Numerous studies have reported the toxicity of ZnO NPs on microalgae and recommended them as the suitable microorganism to study the influence of metallic oxide NPs in the environment (Franklin et al., 2007; Griffitt et al., 2008; Navarro et al., 2008; Battin et al., 2009; Manzo et al., 2013; Tang et al., 2013). Toxicity of ZnO NPs was reported to cause growth inhibition of freshwater microalga *Pseudokirchneriella subcapitata* (Franklin et al., 2007; Aruoja et al., 2009), cease the photosynthetic activity of cyanobacterium *Anabaena flos-aquae* and death of freshwater microalga *Euglena gracilis* (Brayner et al., 2010), and decrease the viable cells of freshwater microalga *Chlorella* sp. (Chen et al., 2012). Further, studies have also reported the toxic effects of ZnO NPs on marine phytoplankton *Dunaliella teriolecta, Thalassiosira pseudonana, Isochrysis galbana* and *Skeletonema marinoi* (Miller et al., 2010; Manzo et al., 2013). However, the cellular accumulation of metallic oxide NPs and their corresponding cytotoxic and oxidative stress effects on the microalgae are not yet reported in literatures. Hence our study was aimed to investigate the cellular accumulation and the corresponding cytotoxic and oxidative stress effects on microalgae.

Our study used the nutrient marine microalga *Spirulina platensis* and the nutrient freshwater microalga *Haematococcus pluvialis* to test the cellular accumulation and the corresponding cytotoxic effects due to the following reasons; (i) these nutrient microalgae are never been studied earlier for the cellular accumulation of metallic NPs and the corresponding oxidative stress and cytotoxic effects of metallic NPs, although a very little information is available in literatures regarding the growth inhibitory effects of ZnO NPs on *S. platensis* (Lone et al., 2013) and TiO_2 NPs on *S. platensis* and *H. pluvialis* (Comotto et al., 2014); (ii) profound sensitivity of both microalgae towards environmental stress (Comotto et al., 2014; Kavitha et al., 2015); (iii) to represent the toxic effects of ZnO NPs on a highly diverse collection of microorganisms live in a range of environments including all aquatic ecosystems, both freshwater and marine, and are also found in terrestrial habitats including on hard surfaces and snow since microalga *H. pluvialis* is a freshwater eukaryotic photosynthetic microbe and *S. platensis* is a marine prokaryotic oxygenic photosynthetic bacteria (cyanobacteria) (Kazamia 2015); (iv) most importantly to develop bio-indicator(s) to identify the contamination of ZnO NPs on these nutrient microalgae because *S. platensis* is one of the most popularly cultivated marine microalgae, has been widely used as a healthy food, forage and additives due to its rich nutrients including proteins, polyunsaturated fatty acids, polysaccharides, carotenoids and vitamins (Lanlan et al., 2015) and *H. pluvialis* is a fresh water microalga distributed in many habitats worldwide and it is considered as the best natural source of astaxanthin (antioxidant, anticancer, anti-

inflammatory) and the main producing organism of this commercial product (Honga et al., 2016; Matos et al., 2017), Lone et al., (2013) opined that occurrence of NPs in the aquatic environments due to several anthropogenic activities may cause detrimental effects in the nutritional quality of the nutrient microalgae through biochemical and physiological alterations. Hence, the additional information regarding the toxicity effects of ZnO NPs on the nutrient microalgae might bring insights to design methods to screen the environmental contamination of ZnO NPs in these nutrient microalgae. The outcomes of the study will be useful for the environmental impact assessment of ZnO NPs in aquatic system including the marine ecosystem, and also might bring insights to design methods for screening the contamination of ZnO NPs on these nutrient microalgae at the natural water sources. Otherwise, the intake of ZnO NPs contaminated microalgae food may cause health hazards to the consumers.

Hence the present study was aimed to explore the cellular accumulation of ZnO NPs and the corresponding cytotoxic effects in *S. platensis and H. pluvialis* using increasing concentrations of ZnO NPs over a period of 96 h to investigate the concentration- and time-dependent cellular accumulation and cytotoxic effects of ZnO NPs. The cytotoxic effects of 5 different concentrations of ZnO NPs from 10 to 200 mg/L were investigated from 6 to 96 h. To investigate the cellular accumulation of ZnO NPs in microalgal cells, SEM-X-ray energy dispersive spectroscopy (EDX) was employed to check the cellular accumulation of ZnO NPs

in the algal biomass and inductively coupled plasma optical emission spectroscopy (ICP OES) was used to quantify the amount of zinc accumulated in the algal cells. The cytotoxic effects of ZnO NPs on both microalgae were evaluated by quantifying the percentage of loss in viable cells, decrease in algal biomass and loss in photosynthetic pigments such as chlorophyll-a, carotenoids, phycocyanin and astaxanthin. The induction of oxidative stress by NPs' treatment was assessed by the measurement of reactive oxygen species (ROS) production and the subsequent lipid peroxidation (LPO) in algal cells treated with ZnO NPs. The surface attachment of NP agglomerates and the following extracellular modifications of algal cells were examined by optical and scanning electron microscopy (SEM). In addition, the intracellular modifications in algal cells by the toxicity of ZnO NPs were examined through transmission electron microscopy (TEM).

1.2 Objectives of the Study are to:

1. Determine the dose and time dependent cellular accumulation of zinc oxide nanoparticles in *Spirulina platensis* and *Haematococcus pluvialis*.

2. Investigate the dose and time dependent toxicity and oxidative stress effects of zinc oxide nanoparticles on *Spirulina platensis* and *Haematococcus pluvialis*.

CHAPTER 2

LITERATURE REVIEW

2.1 Background on Metallic Nanoparticles Toxicity on Aquatic Organisms

Excessive accumulation of metallic NPs by surface-water runoff into large aquatic reservoirs may signify the risk of toxicity for living aquatic organisms (Blaser, 2006). Nanoparticles have received much attention in recent years due to their distinctive properties in physical, chemical and biological processes and also because of their potential adverse effects on human health and ecosystem (Colvin, 2003; Moore, 2006; Wiesner et al., 2006; Patil et al., 2016). Nanopollution is the introduction of nanosized contaminants into the natural environment, which causes instability, harm, discomfort or disorder to the ecosystem. Nanopollution of water bodies occurs as a consequence of usage of manufactured metal and metal oxide nanoparticles (Hardman, 2006; Kouhi et al., 2014). Also, accidental spillages or intended discharge of nano industrial wastes into the aquatic systems results in the direct exposure of NPs to humans through inhalation of NPs contaminated water aerosols, dermal contact and direct ingestion of contaminated drinking water or the NPs adsorbed on vegetables or other foodstuffs posing potential health hazards (Daughton, 2004). After the entry of NPs into the aquatic environment, the

suspended particles would be taken up by the aquatic biota through direct uptake from the water phase and/or through food uptake and/or through cell wall, hence presence of NPs in the aquatic sediments can potentially cause toxic effects to the aquatic organisms (Baun et al., 2008). The toxic action of metal and metal oxide NPs can potentially comprise at least 3 distinct mechanisms (Brunner et al., 2006). Firstly, particles may release toxic metal ions into the exposure medium, for instance, ZnO NPs release zinc ions in the aqueous medium. Secondly, surface interactions of metal ions with the aqueous medium may produce toxic substances. Thirdly, particles may interact directly with and disturb the aquatic organisms, for example, cell wall damage, damage to photosynthetic system and release of the intracellular contents (Brunner et al., 2006). The extensive use of Ag NPs in industrial products has raised the alarms on the risks associated with Ag NPs releasing into the environment (Baker et al., 2014). The released Ag NPs into the aquatic environment possibly enters into the internal organs of aquatic organisms through skin or membranes of aquatic biota and exerting their toxicity effects on the aquatic organisms (Fröhlich and Roblegg, 2012). Besides being toxic to aquatic organisms, NPs are also reported to be toxic to mammalian cell lines (Hussain et al., 2005; Marin et al., 2015; Pandurangan and Kim, 2015). The significant toxicity of Ag NPs to the mammalian stem cells showed the potential of these particles to affect the reproductive system. There is also some other evidence that Ag NPs can bring detrimental effects to human health through ingestion or usage of medical devices (Carlson et al., 2008).

Most importantly, Ag NPs can concentrate over 0.1 mg/L in surface waters (Boxall et al., 2007). Former to the interest in NPs, silver ion (Ag^+) in aqueous medium was reported to be the most lethal form of silver (Ratte, 1999). The studies have reported that the dissolved Ag^+ in the environment are persistent and highly harmful to prokaryotes, various freshwater and marine invertebrates and fish (Fisher and Wang, 1998; Bianchini et al., 2002) (Table.2.1). Ag NPs have higher dissolution of Ag^+ in the aqueous solution than the bulk material due to the nanosized nature and larger surface area causing potential toxicity of nano-silver. Moreover, due to their capacity to get adsorbed into the biomolecules and react with biological receptors, Ag NPs can be internalized into the cells more rapidly (Reidy et al., 2013). A study by Griffitt et al., (2008) investigated the toxicity of Ag NPs on freshwater alga *Pseudokirchneriella subcapitata*, freshwater flea *Daphnia pulex* and Zebrafish *Danio rerio* and reported that Ag NPs induced toxicity with LD_{50} of 7.0 – 7.2 mg/mL in Zebrafish, 0.040 – 0.067 mg/mL in *Daphnia* and 0.19 mg/mL for *P. subcapitata* at 48 h of exposure to NPs. Consequently, a study by Gaiser et al., (2011) on *Daphnia magna* showed 100% mortality of *Daphnia* with 1 mg/mL and 43.3% for 0.1 mg/mL of Ag NPs during 96 h of exposure. Several studies have reported the toxicity of Ag NPs on the photosynthesis process of algal cells. Dewez and Oukarroum (2012a) investigated the toxic effects of Ag NPs on freshwater green alga *Chlamydomonas reinhardtii,* by exposing the algal cells to 1, 5 and 10 μmol/L of Ag NPs for 6 h under both light and dark

conditions and reported a stronger inhibition on electron transport activity of photosynthetic system under light exposure condition due to failure of photoprotective mechanisms against excessively absorbed light-energy by the photosynthetic apparatus. Similarly a study by Navarro et al., (2008) reported higher toxicity of Ag NPs on the photosynthetic reaction compared with Ag^{+} ($Ag\,NO_3$) on *Chlamydomonas reinhardtii* through the measurement of chlorophyll fluorescence emission. The treatment of Ag NPs on marine microalga *Dunaliella tertiolecta* and freshwater microalga *Chlorella vulgaris* at two different temperatures (25°C and 31°C) resulted in higher altering effect on photosynthetic electron transport to an increase in temperature (Oukarroum et al., 2012b).

Various studies have demonstrated the protective effects of aquatic organisms against the toxicity of Ag NPs. A study by Stevenson et al., (2013) on *C. reinhardtii* reported the production of extracellular dissolved organic carbon (DOC) compounds by algal cells as the protective effect against the toxicity of Ag NPs. Miao et al., (2009) reported a similar protective mechanism towards the toxicity of Ag NPs on marine alga *Thalassiosira weissflogii* through the secretion of polysaccharide-rich algal exudate exopolymeric substances (EPS) by the oxidative stress. The concentration of EPS secretion was significantly increased at an increasing concentration of Ag NPs, indicating the dose dependent defensive mechanism of the algal cells against toxicity. In addition, the same study reported

reduction in growth of algal cells as a result of fall in chlorophyll production and photosynthesis by the toxicity of Ag^+ released from Ag NPs. Chan and Chiu (2015) evaluated the chronic sub-lethal toxicity of Ag NPs on marine invertebrate larvae and reported growth retardation and decrease in larval settlement rate of *Balanus amphitrite*, *Crepidula onyx* and *Hydroides elegans,* and demonstrated the accumulation of Ag^+ in all three species of marine invertebrate larvae.

The toxicity of nano-silver on aquatic vertebrates has been reported by various studies, where the authors exposed zebrafish embryo to 10 - 20 mg/L of Ag NPs for 72 h and showed the penetration of particle aggregates into the skin and circulatory system (Yeo and Yoon, 2009), while the treatment of Ag NPs (5 – 100 mg/L) coated with bovine serum albumin (BSA) or potato starch resulted in deposition of Ag NPs in the cell nucleus, blood and also in the nervous system of zebrafish embryo at 72 h (Asharani et al., 2008). Further, the exposure of zebrafish to 0.4 - 4 mg/L Ag NPs for 2 - 36 d showed defects in fin regeneration and infiltration of Ag NPs into cellular organelles including cell nucleus (Yeo and Pak, 2008).

Following Ag NPs, the release of nTiO2 into the aquatic ecosystem causes damaging effects on the overall ecosystem (Aitken et al., 2009). The nanosized TiO_2 can produce free radicals and exert a strong oxidizing ability. Sunlight illuminated TiO_2 catalyzes DNA damage, both in vitro and in vivo, since exposure

to such nanoparticles is mainly through skin and inhalation (Srivastava et al., 2015). It was demonstrated that exposure to TiO_2 NPs affects the brain Murine microglial cells through production of superoxide radicals that caused substantial damage to DNA, resulting in additional environmental hazards (Long et al., 2006). Studies have also reported that the chronic inhalation of $nTiO_2$ is harmful and has been associated with a variety of pulmonary effects in rats (Aitken et al., 2009). Numerous studies have reported the toxic effects of titanium dioxide nanoparticles on aquatic organisms (Table 2.1). Battin et al., (2009) showed the exposure of planktonic and biofilm microorganisms to 5.3 mg/L $nTiO_2$ for 24 h resulted in cell membrane damage. Dabrunz et al., (2011) demonstrated the immobilization of *Daphnia* and a significant cytotoxicity with 72 h EC_{50} of 3.8 mg/L and 96 h EC_{50} of 0.73 mg/L of $nTiO_2$. Another study on *Daphnia magna* by Zhu et al., (2010) revealed a minimal toxicity of $nTiO_2$ in 48 h of exposure with EC_{50} >100 mg/L, however, high toxicity was reported at 72 h. Moreover, chronic exposure for 21 d resulted in reproductive defects, growth retardation and death of zebrafish with the accumulation of significant amount of nanoparticles. Thus, the results indicated that the long-term exposure of $nTiO_2$ on aquatic organisms might affect the growth of aquatic organisms, indicating the risk of $nTiO_2$ to the aquatic ecosystems. Further, several studies have investigated the effect of $nTiO_2$ on the aquatic vertebrates. Chen et al., (2011) reported a significant impairment in the swimming velocity of zebrafish embryo larvae, even to low dosage of $nTiO_2$ (0.1-1 mg/L) on day 10. A study by Federici et al., (2007) treated cold water fish

rainbow trout with TiO_2 NPs for 14 days and reported decrease in Na^+/K^+-ATPase activity in the gills, intestine and brain, and also demonstrated minor lipidosis and apoptotic bodies in the liver cells. The results have also showed erosions in the intestinal epithelium of the fish that was possibly due to the ingestion of $nTiO_2$ contaminated water. Linhua et al., (2009) reported the oxidative stress of $nTiO_2$ on freshwater fish *Cyprinus carpio* by analyzing the levels of antioxidant enzymes and lipid peroxidation (LPO) in gill, liver and brain cells of the fish. Furthermore, the pathological studies showed edema, thickening of gill lamellae and gill filaments, necrosis and apoptosis of liver cells when treated with $nTiO_2$ (100- 200 mg/L) for 20 d.

2.2 Toxicity Effects of Zinc Oxide Nanoparticles on Aquatic Organisms

The rising trend in the large-scale commercial production and use of ZnO NPs may result in an unintentional or intentional release of these particles from the industrial wastes into the aquatic environment (Baker et al., 2014; Wang et al., 2016). Notably, ZnO NPs has high risk of contaminating aquatic ecosystem posing great threat to the aquatic organisms (Gottschalk et al., 2009). The aquatic organisms including marine phytoplankton exhibit a wide range of sensitivity to dissolved zinc, though it is considered as an essential nutrient (Franklin et al., 2007; Miller et al., 2010). The toxicity of NPs can be influenced by various physico-chemical properties such as size, shape, coating and chemical composition (Morris et al.,

2007). The pH, ionic strength and solubility of the aqueous media determine the size and rate of aggregation of NPs and the release of metal ions from metal-based NPs that may also influence its toxicity (Wong et al., 2010; Quik et al., 2011). Upon releasing into the aquatic environment, it may lead to cellular exposure and uptake of NPs into the aquatic biota, which in turn stimulates distinctive cellular toxicological responses through lethal effects of the leached zinc as well as the cellular uptake of solid ZnO NPs and the subsequent intracellular leaching of zinc (Gunawan et al., 2013). The adverse effects of ZnO NPs evidently need to be assessed on various aquatic organisms as an integral part of environmental risk assessment (Hazeem et al., 2016) and also extremely important for sustainable development of nanotechnology (Kahru and Dubourguier, 2010) and there arises a need for better understanding and assessment of ecotoxicity of NPs to the key ecosystem organisms such as algae, plants, and fungi which are continuously being exposed to these nanomaterials (Srivastava et al., 2015). Previous toxicity studies using ZnO NPs have evidenced that these particles are toxic to algae (Xu et al., 2013), bacteria (Li et al., 2013), crustaceans (Blinova et al., 2010) and fish (Zhu et al., 2008). Algae are largely dispersed in fresh water and they are the primary producers in the food chain; so, consequently, any disturbance in their growth reflects a disturbance in the food chain of aquatic system (Auffan et al., 2011).

Alga is an organism that is sensitive to metallic contaminants when compared to fish and invertebrates (Zhou et al., 2014). Also, algae are used for bioremediation

of pollutants that contributes to the self-purification of polluted water (Ji et al., 2011). Therefore, they can potentially be used as a biosensor to monitor water quality and aquatic toxicity (Franklin et al., 2007). Investigating the toxicity of ZnO NPs on algae is of greater importance because the study results can potentially lead to strategies on evaluating the impacts ZnO NPs in the aquatic environment (Maynard et al., 2006). Of note, it is also important to understand and investigate the fate and behavior of ZnO NPs in the coastal waters and examine their biological effects on marine organisms as coastal waters and ocean are the ultimate sink of the NPs. Since marine microalgae are used as the food by the salt water organisms, they play a vital function as principal producers in the oceans. Consequently, the assessment on the adverse effects of ZnO NPs on marine microalgae is also important to predict the adverse effects of ZnO NPs on marine food web and its influence on the entire marine ecosystem (Manzo et al., 2013).

2.2.1 Biological Toxicity of ZnO NPs on Microorganisms

Numerous studies have confirmed the growth inhibitory effects of ZnO NPs on positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, and gram negative bacteria *Salmonella typhimurium, Escherichia coli* and *Klebsiella pneumonia* (Reddy et al., 2007; Wahab et al., 2010; Baek and An, 2011). Heinlaan et al., (2008) reported the growth inhibition of ZnO NPs on *Vibrio fischeri* with 30 min EC_{20} and EC_{50} of 0.9 and 1.9 mg/L respectively. Additionally, toxicological studies of ZnO

NPs showed growth inhibitory effects on *Campylobacter jejuni* (Xie et al., 2011), *Listeria monocytogenes* (Jin et al., 2009) and *Pseudomonas aeruginosa* (Feris et al., 2009). Notably, Jones et al., (2008) demonstrated greater growth inhibitory effect of smaller size ZnO NPs (8 nm, 50 – 70 nm) on bacteria than larger size particles (> 1 µm) and Applerot et al., (2009) reported a higher intracellular accumulation and the subsequent damage of *E. coli* by smaller ZnO NPs (7 nm) compared to larger particles (260 and 800 nm). Subsequently, Premanathan et al., (2011) reported the higher susceptibility of gram positive bacterium *S. aureus* compared to gram negative bacteria *P. aeroginosa* and *E. coli.* Besides bacteria, the studies have also reported toxic effects of ZnO NPs on fungi and protozoa. A study by Gondal et al., (2012) demonstrated a higher susceptibility of *Candida albicans* to ZnO NPs compared to *Aspergillus niger.* Another study on *C. albicans* by Lipovsky et al., (2011) reported 90% growth inhibition by ZnO NPs (1 mg/L) at 24 h and also demonstrated the size-based cytotoxic effects of ZnO NPs on *C. albicans* that the smaller particles (10 nm) caused higher toxicity than the larger particles (100 nm) of ZnO NPs. Further, Kasemets et al., (2009) demonstrated enhanced cytotoxicity of ZnO NPs compared to bulk ZnO on the yeast *Saccharomyces cerevisiae* with EC_{50} of 131 and 158 mg/L respectively at 24 h. Similarly, the protozoan *Tetrahymena thermophila* showed higher cytotoxicity to the treatment of ZnO NPs compared with bulk ZnO and $ZnSo_4$ with EC_{50} of 3.7, 3.9 and 4.9 mg/L of Zn at 4 h respectively (Mortimer et al., 2010).

2.2.2 Cytotoxicity Effects of ZnO NPs on Algae and Plants

Microalgae are the photosynthetic microorganisms that utilize solar light to convert the atmospheric carbon di-oxide into organic carbon. They are prokaryotic microalgae cyanobacteria or eukaryotic microalgae such as green microalgae, red microalgae and diatoms. Microalgae are the source of numerous valuable compounds such as carbohydrates, proteins, lipids, vitamins and pigments (Miazek et al., 2015). The chlorophylls, carotenoids and phycobilins are the microalgal pigments that harvest light in the process of photosynthesis. Chlorophylls are the primary photosynthetic pigments that contain tetrapyrrole ring with magnesium ion. Green microalgae contain chlorophyll content up to 6.7% and occur in various forms such as chlorophyll a, b and c. Chlorophyll a is the principal photosynthetic pigment of the microalgae that are majorly involved in light harvesting process and photosynthesis (Nakanishi and Deuchi, 2014). Carotenoids are the accessory photosynthetic pigments that are divided into non-oxygen containing carotene (β carotene) and oxygen containing xanthophylls such as lutein, astaxanthin and zeaxanthin (Takaichi, 2011). Phycobiliproteins are water soluble proteins that serve as accessory pigments that give blue color to the blue green microalgae; also known as cyanobacteria due to the presence of phycocyanin and allophycocyanin (Shukla et al., 2009; Ferrari et al., 2013). Human activity, industrial development and natural earth processes lead to release of several metals, for example, zinc, copper,

cadmium, chromium, mercury, nickel, lead, iron, lanthanum, etc., and metallic nanoparticles such as silver, titanium oxide, zinc oxide, cerium oxide, nickel oxide, lanthanum oxide, etc., that could induce stress to the growth and metabolism of microalgae (Klaine et al., 2008; Tangahu et al., 2011; Monteiro et al., 2012; Von Moos and Slaveykova, 2014). High concentration of heavy metals causes the inhibition of photosynthesis and enzymes, and thus causes block in cell division (Miazek et al., 2015). The accumulation of metal ions on the surface of algal cells involves two mechanisms; adsorption of metal ions on the functional groups of algal cell wall (hydroxyl, carboxyl, amino, sulfhydryl and phosphate) and subsequent internalization (Blaby-Haas and Merchant, 2012; Monteiro et al., 2012; Sun et al., 2015). Interaction of metal ions on algal cells can cause oxidative stress through production of reactive oxygen species (ROS) such as hydroxyl radical (OH), superoxide anion (O_2-), singlet oxygen (O*) and hydrogen peroxide (H_2O_2) that react with the biomolecules of algal cell lipids, proteins and nucleic acids resulting in cell membrane damage and degradation of intracellular organelles. (Perales-Vela et al., 2006; Monteiro et al., 2012; Cassier-Chauvat and Chauvat, 2014).

The toxicity of zinc ion (Zn^{2+}) in the aquatic medium best relates to the available levels of hydrated free Zn^{2+} or complexes of Zn^{2+} with inorganic substances (Wang et al., 2009). Previous studies have reported the higher toxic effects of ZnO NPs compared with $nTiO_2$ (Table. 2.1). Toxicity of ZnO NPs (6 d EC_{50} of 20 mg/L)

caused greater growth inhibition compared to $nTiO_2$ (6 d EC_{50} of 30 mg/L) on *Chlorella* sp. (Ji et al., 2011). Aruoja et al., (2009) reported the greater toxicity effects of ZnO NPs over TiO_2 NPs on *P. subcapitata* through higher decrease in chlorophyll fluorescence and the corresponding higher reduction in cell density with the treatment of ZnO NPs. A study by Heinlaan et al., (2008) demonstrated a higher cytotoxic effects of ZnO NPs to *D. magna* and *T. platyurus* even at low concentrations of 3.2 mg/L for *D. magna* and 0.18 mg/L for *T. platyurus*, while TiO_2 NPs were not toxic even at higher concentration (20 g/L). Subsequently, Miller et al., (2010) reported that the toxicity of ZnO NPs significantly caused growth inhibition of marine phytoplankton diatoms, chlorophytes, and prymnesiophytes, while $nTiO_2$ produced no quantifiable cytotoxic effects. ZnO NPs have been proven to be more toxic than bulk ZnO and zinc chloride ($ZnCl_2$). The toxicity study on marine alga *D. tertiolecta* by Manzo et al., (2013) reported more toxicity for 100 nm ZnO NPs with 96 h EC_{50} of 2.42 mg/ L than 200 nm bulk ZnO with 96 h EC_{50} of 4.45 mg/L. Likewise, Ji et al.,(2011) reported the size-dependent cytotoxicity of ZnO NPs on *Chlorella* sp. with the EC_{30} values of 20 mg/L and 100 mg/L for 20 nm and 100 nm ZnO NPs respectively after 6 days of exposure and confirmed the higher toxic effects of smaller size ZnO NPs. The possible reason behind the higher toxicity of smaller size nanoparticles is possibly due to the increased number of surface oxygen vacancies in nanosized particles with decreasing particle size. These higher oxygen vacancies can produce more number of electron pairs with oxygen molecules and hydroxyl

ions to generate excessive reactive oxygen species (Brayner, 2008). Besides the size dependent toxicity, studies have also reported the concentration- and time-dependent toxicity of ZnO NPs. A study by Schiavo et al., (2016) showed that the cytotoxic effects of ZnO NPS on green microalga *Dunaliella salina* were increased with increase in the concentrations of ZnO NPs. The cell viability of *D. salina* decreased to below 50% when treated with ≥ 25 mg/L of ZnO NPs. But no significant toxicity effect was reported at 0 - 5 mg/L of ZnO NPs. In addition, Zhang et al., (2016a) reported EC_{50} values of 7.0, 6.4, 4.4 and 3.6 mg/L of ZnO NPs for 24, 48, 72 and 96 h on the marine microalga *Skeletonema costatum*. Ji et al., (2011) reported a time-dependent cytotoxicity of ZnO NPs on *Chlorella* sp. with the percentage of cell viability 75%, 70% and 60% on day 2, 4 and 6 respectively. Further, the cytotoxicity of ZnO NPs caused a significant growth inhibition of marine microalgae *Skeletonema costatum* and *Thalassiosia pseudonana* with 96 h EC_{50} of 2.36 and 4.56 mg/L respectively (Wong et al., 2010). The TEM examination of ZnO NPs treated cyanobacterium *Anabaena flos-aquae* showed the accumulation of nanoparticle aggregates around the cell wall (Brayner et al., 2010). The toxicity effects of ZnO NPs have also reported to affect the growth and seed germination rates in plants. Treatment of ZnO NPs halted root elongation and inhibited seed germination in ryegrass *Daucus carota, Brassica campestris and Lolium perenne* (Lin and Xing, 2007) and ZnO NPs were also found to affect root elongation rate in garden cress and caused genotoxicity to broad bean (Manzo et al., 2011). In addition, Du et al., (2011)

demonstrated the uptake of zinc and biomass reduction in wheat upon treating with ZnO NPs.

Table 2.1: Summary of Silver, Titanium dioxide and Zinc Oxide Nanoparticles' Toxicity on Aquatic organisms

NPs	Size (nm)	Aquatic Organism(s)	Major Effects	Reference
Silver	20-30	Freshwater flea: *Daphnia pulex*	Ag NPs caused lesser cytotoxicity than its counterpart Ag^{+} on all three organisms.	(Griffitt et al., 2008)
		Freshwater algae: *Pseudokirchneriella subcapitata*		
		Water flea: *Ceriodaphnia dubia*		
Silver	25	Freshwater green alga: *Chlamydomonas Reinhardtii*	Ag NPs caused greater adverse effect on photosynthetic system compared to its counterpart $AgNO_3$.	(Navarro et al., 2008)
Silver	50	*Chlamydomonas Reinhardtii*	Deterioration of photosynthetic II reaction center.	(Dewez and Oukarroum, 2012)
Silver	40	*Chlamydomonas Reinhardtii*	More toxicity of Ag NPs to the early stages of growth than the later stages. Production of dissolved organic carbon (DOC) in response to Ag NPs toxicity.	(Stevenson et al., 2013)
Silver	50	Marine green algae: *Dunaliella tertiolecta* And freshwater microalga *Chlorella vulgaris*	Deterioration of photosynthetic reaction centre in both microalgae. *D. tertiolecta* showed more toxicity than *C. vulgaris.* Higher toxicity was observed at 31°C than 25°C.	(Oukarroum et al., 2012b)

Silver	75	Larvae of marine invertebrate: *Balanus amphitrite, Crepidula onyx* and *Hydroides elegans*	Severe inhibition in development and growth of larvae with reduced larval settlement rate.	(Chan and Chiu, 2015)
Silver	60-70	Marine algae: *Thalassiosira Weissflogii*	Ag^+ released from Ag NPs caused reduction in cell growth, photosynthesis and chlorophyll production. Secretion of algal exudate called exopolymeric substances (EPS) for Ag^+ detoxification.	(Miao et al., 2009)
Silver	14-20	Free living soil roundworm: *Caenorhabditis Elegans*	Reduced reproduction with infiltration of Ag NPs in uterine area and increased oxidative stress.	(Roh et al., 2009)
Silver	10-20	Zebrafish	Infiltration of Ag NPS into intracellular organelles including nucleus.	(Yeo and Pak, 2008)
Silver	20-30	Zebrafish embryo	Aggregates of NPs into the skin and circulatory system.	(Yeo and Yoon, 2009)
Silver	5-20	Zebrafish embryo	Deposition of Ag NPs in cell nucleus, blood and nervous system.	(Asharani et al., 2008)
Zinc oxide	30	Freshwater Microalga: *Pseudokirchneriella Subcapitata*	Nano-ZnO exhibited growth inhibition. No difference in growth inhibition among nano-ZnO, bulk ZnO & $ZnCl_2$.	(Franklin et al., 2007)
Zinc oxide	100	Marine algae: *Dunaliella tertiolecta*	Nano-ZnO exerted more toxicity on growth rate of *D. tertiolecta* bulk ZnO.	(Manzo et al., 2013)
Zinc oxide	30-100	Cyanobacterium *Anabaena flos-aquae* and green microalga *Euglena gracilis*	Decreased photosynthetic activity in *A. flos-aquae* and cell death in *Euglena gracilis*. Production of algal exudates by *A. flos-aquae* was reported as the protective response.	(Brayner et al., 2010)
Titanium dioxide	20	Planktonic and biofilm	Significant damage of cell membranes with substantial	(Battin et al., 2009)

		microorganisms in natural water	impact of light on the membrane damage.	
Titanium dioxide	21	*Daphnia magna*	Minimal cytotoxicity in 48 h and high toxicity at 72 h of exposure in *Daphnia*. Chronic exposure for 21 d, resulted in mortality and reproductive defects.	(Zhu, Chang, & Chen, 2010)
Titanium dioxide	100	*Daphnia magna*	Immobilization of *Daphnia* at the nominal concentration of 72 h EC_{50} 3.8 mg/L and 96 h EC_{50} 0.73 mg/L. Nanosized TiO_2 was significantly more toxic than non-nanosized TiO_2 (200 nm).	(Dabrunz et al., 2011)
Titanium dioxide	ND	Freshwater fish: *Cyprinus carpio*	Decrease in antioxidant enzymes level as the indication of greater production of reactive oxygen species and increase in lipid peroxidation level in liver cells. Swelling of gills with necrosis and apoptosis of liver cells at 20 d.	(Linhua et al., 2009)
Titanium dioxide	ND	Zebrafish embryos	Significant impairment in the swimming capacity of fish embryo.	(Chen et al., 2011)
Titanium dioxide	ND	Cold water fish rainbow trout: Oncorhynchus mykiss	Inhibited Na^+/K^+-ATPase activity in the gills, intestine and brain. Caused lipidosis and apoptotic bodies in liver cells.	(Federici et al., 2007)
Zinc oxide	50-70	Freshwater microalga *Pseudokirchneriella subcapitata*	Nanosized ZnO particles caused higher toxicity compared to $nTiO_2$ by greater loss in chlorophyll fluorescence emission and cell density.	(Aruoja et al., 2009)
Titanium dioxide	25-70			
Zinc oxide	ND	Green alga: *Chlorella* sp.	Nano-ZnO particles showed greater growth inhibition of algal cells than $nTiO_2$.	(Ji et al., 2011)
Titanium dioxide	5-10 Anatase			
Zinc oxide & Titanium dioxide	ND	Marine phytoplankton:	ZnO NPs significantly suppressed the growth rate of marine phytoplanktons. While	(Miller et al., 2010)

		Diatoms, chlorophytes, and prymnesiophytes	TiO_2 NPs caused no quantifiable toxic effects.	
Zinc oxide & Titanium dioxide	ND	Crustaceans: *Daphnia magna* and *Thamnocephalus platyurus*	Suspension of nanoTiO_2 was not toxic even at higher concentration (20 g/L). Nano-ZnO suspension was toxic even at low concentrations from 3.12 mg/L for *D. magna* and 0.18 mg/L for *T. platyurus* at 48 h.	(Heinlaan et al., 2008)

2.2.3 Cytotoxicity of ZnO NPs on Invertebrates and Vertebrates

The experiments conducted in fresh water flea *Daphnia magna* showed size-dependent toxicity with 48 h EC_{50} of 1.02 mg/L for 30 nm sized ZnO NPs and 1.1 mg/L for 80 - 100 nm sized ZnO NPs respectively (Bacchetta et al., 2016). Similarly, the treatment of nanosized ZnO particles to the oyster *Crassostrea gigas* resulted in infiltration of ZnO NPs in gills and digestive glands and also caused oxidative stress (Trevisan et al., 2014). Montes et al., (2012) reported the accumulation of zinc in coastal marine mussel *Mytilus galloprovincialis* when treated with 1 - 10 mg/L of ZnO NPs at day 4. Another study on *M. galloprovincialis* found the accumulation of zinc in somatic and gonadal tissues with the subsequent decrease in shell growth rate and increase in cell mortality due to the chronic exposure to ZnO NPs (2 mg/L) for a period of 12 weeks (Hanna et al., 2013). Furthermore, the research studies have also investigated the sensitivity

of crustaceans to ZnO NPs. Ates et al., (2013) reported a size-based toxicity effects of ZnO NPs (100 mg/L) on brime shrimp *Artema salina* with 26% mortality for 10 – 30 nm size particles and 18% mortality for 200 nm size particles. Exposure of marine copepod *Tigriopus japonicus* to ZnO NPs resulted in the accumulation of NP aggregates to the exoskeleton and subsequently caused mechanical injury (Wong et al., 2010). The toxicity of ZnO NPs caused growth inhibition on crustacean *Daphnia magna* with 48 h EC_{50} of 3.2 mg/L (Heinlaan et al., 2008) and in *zebrafish* with 96 h EC_{50} 4.92 mg/L (Xiong et al., 2011). The earlier studies have also reported the adverse effects of ZnO NPs on animal and human cells. ZnO NPs can also cause toxic damage to mammalian cells and the smaller size of these particles enables them to get internalized into the cells and cause cellular inflammatory response by inducing oxidative stress (Magdolenova et al., 2014; Golbamaki et al., 2015). A study in mice displayed increased blood viscosity and severe toxic changes such as kidney damage, anemia and liver damage (Wang et al., 2008). Deng et al., (2009) reported a dose dependent toxic effects of ZnO NPs on mouse neural stem cells, for instance, ZnO NPs caused loss in cell viability of 38.2, 71.8 and 86.8% at 24 h for 12, 18 and 24 mg/L respectively with 24 h EC_{50} of 10.8 - 13.2 mg/L. Another study exposed chicken red blood cells to ZnO NPs (50 mg/L) and reported 40% loss in viable cells in 24 h and also showed a concentration and time dependent release of lactate dehydrogenase enzyme due to plasma membrane damage and the subsequent hemolysis (Babu et al., 2017). Furthermore, Guan et al.,(2012) showed a dose dependent cytotoxicity of ZnO NPs on human

cell lines with the percentage of cell viability 52.63, 41.12 and 36.7% on human hepatocytes, and 38.74, 19.43 and 15.21% on human embryonic kidney cells at the concentrations 50, 75 and 100 mg/L for 24 h. Chen et al., (2014) demonstrated a concentration dependent cytotoxicity of ZnO NPs on the endothelial cells of human umbilical vein with the highest loss in cell viability of 40% at 480 µM (42 mg/L) for 24 h exposure and another dose dependent cytotoxicity of ZnO NPs on monocytes was reported by Andersson-Willman et al., (2012) with a percentage of cell death of 23.6, 79.9 and 91.2% at 10, 50 and 100 mg/L, respectively for 24 h exposure.

2.3 Sensitivity of *S. platensis* Towards Heavy Metals and Metallic Oxide Nanoparticles

Spirulina platensis is a prokaryotic cyanobacterium, which is also known as marine microalga as it grows in high salt concentrations ranging from 8.5 to 200 g/L and in alkaline pH (9.5 to 11). It is a photosynthetic filamentous cyanobacterium with a diameter of 10 µm and length of 10 to 100 µm. It is a multicellular cylindrical trichomes in a helix along the entire length of the filaments (Moraes et al., 2013). The harvested biomass of *S. platensis* has been used as a source of food for centuries by the native people around Chad lake in Africa and Texcoco lake in Mexico (Vonshak, 1997). Therefore, it is an economically important type of alga ever since it has been commercially produced for over 30 years for usage as fish food, vitamin supplements, food dyes, aquaculture, pharmaceuticals and nutraceuticals (Ciferri

and Tiboni, 1985; Abdulqader et al., 2000). *S. platensis* is a recommended nutritional supplement as it contains rich source of nutrients such as phycocyanin, chlorophyll, vitamin E, omega 6 fatty acids , numerous minerals and antioxidant carotenoids (Gershwin and Belay, 2007). It is 60 - 70% protein by weight (including all essential amino acids) with the available energy 2.5 - 3.29 kcal/gram and phosphorus content of 41.0% and contains up to 10 times more beta-carotene than carrots per unit mass (Belay et al., 1993). It is widely cultivated around the world and available as tablet, capsule flake and powder form. Researchers have discovered that *Spirulina* products have numerous medical benefits because of its nutritional qualities and proven to treat many health problems including diabetes, arthritis, anaemia and cancer and also been proven to be a lipid lowering agent and modulator for immunological function (Ruan et al., 1988). *S. platensis* provides adequate iron in anemic pregnant women. Also, it is shown to minimize HIV-1 replication in human T-cell lines and the algal extracts exhibited antiviral activities against various pathogenic viruses such as hepatitis, herpes simplex virus, cytomegalovirus, measles virus and influenza A virus (Hayashi and Hayashi, 1996). *Spirulina* biomass can be used as a matrix for pharmaceuticals containing zinc for human and veterinary use (Zinicovscaia et al., 2013). Carotenoids of *Spirulina* are responsible for producing pigmentation of egg yolk in the poultry industry to produce carotenoid rich eggs. In addition, it is also used as a biofertilizer (Lorenz, 1999; Hoseini et al., 2013). Interestingly, cyanobacteria are the effective accumulators of trace metals, especially *S. platensis* can effectively accumulate

trace metals and more effectively zinc upto 96% of exposed zinc (Dazhi et al., 2003, Al-Dhabi, 2013).

A treatment of heavy metal lead on *S. platensis* resulted in the loss of biomass and photosynthetic pigments, and the addition of tellurium to *S. platensis* culture resulted in the uptake of metal ions into the peptides of *Spirulina* cells and also caused enhanced production of phycocyanin due to the oxidative stress induced by the metal uptake (Arunakumara et al., 2008). Nalimova et al., (2005) confirmed the lethal actions of copper and zinc on *S. platensis*, while Choudhary et al., (2007) reported the toxic effects of heavy metals such as lead, copper, and zinc to *S. platensis* with growth inhibition and increased oxidative stress. The treatment of copper (Cu II) and sodium chloride (NaCl) resulted in the reduction of cell growth and chlorophyll-a (chl-a) content in *Spirulina* sp. through the induction of oxidative stress (Deniz et al., 2011). The study also recommended the investigation of chlorophyll-a as a useful tool to determine the physiological alterations in plant cells that are subjected to heavy metals stress which results in the degradation of chlorophyll as a result of oxidative stress.

Moreover, the treatment of chromium caused substantial reduction in growth and chl-a content of *S. platensis* due to the inhibition in cell division, in addition to deterioration of carbohydrate and protein content of *S. platensis* (Shilpi et al., 2014). A study by Lone et al., (2013) investigated the toxicity of copper oxide NPs

(50 nm, 10 ppm) and ZnO NPs (50 nm, 10 ppm) at day 10 by measuring the algal biomass and photosynthetic pigments (chlorophylls and carotenoid). The results showed growth inhibition, reduction in photosynthetic pigments and protein synthesis of *S. platensis* due to the treatment with ZnO NPs, whereas no inhibitory effects were observed with copper oxide NPs. The study inferred that the toxic effects of nano-zinc were related to binding of zinc ions to proteins of algal cell wall that resulted in growth inhibition. A recent study by Comotto et al., (2014)) evaluated the toxic effects of TiO_2 NPs (100 mg/L) at day 15. The results showed that the pure anatase TiO_2 NPs lead to a significant growth inhibition of S. *platensis* with the accumulation of antioxidant compounds.

2.4 Sensitivity of *H. pluvialis* to Environmental Stress

Haematococcus pluvialis is an eukaryotic, unicellular, motile, biflagellate, green, fresh water algae (Kang et al., 2005) with size of 20-50 µm in diameter and 8-12 µm long (Shah et al., 2016). *H. pluvialis* occurs in fresh water bodies like natural and artificial ponds and pools (Czygan, 1970, Burchardt et al., 2006). It has been found across various environmental and climate conditions like in freshwater with snow, black water or rocks on the seashore (Chekanov et al., 2014; Klochkova et al., 2013). *H. pluvialis* exists in two forms as green motile vegetative form in environmentally favourable conditions and also as red immotile cystic form under unfavourable conditions (Harker et al., 1996; Nagaraj et al., 2012). The general

composition of *H. pluvialis* alga meal consists of carotenoids (>1.75%), astaxanthin (>1.5%), fatty acids (7 - 25%), proteins (20 - 30%), carbohydrates (30 - 40%), and minerals. *H. pluvialis* alga meal has been used in diets of farmed salmon, trout, sea bream, prawns and ornamental fish (Dore and Cysewski, 2003). Astaxanthin is the most important carotenoid pigment contained in *H. pluvialis* which has approximately 10 times of antioxidant effect than other carotenoids such as zeaxanthin, lutein, canthaxanthin, and β-carotene, and 38 fold of beta carotene (Tripathi et al., 1999) and widely used as colorant in fish and poultry farms to enhance the color of cultured salmon, trout ornamental fish and egg yolk sac respectively (Sarada et al., 2002; Göksan and Ak, 2006). Astaxanthin is also serves as a vitamin source in poultry industry, natural preservative, food additive for food industry, superior antioxidant to vitamin E and β carotene in the cosmetic industry, and an antiaging agent as a precursor of vitamin A. The antioxidant property of astaxanthin is believed to have protection against UV light photooxidation (Sarada et al., 2002) and also it is known as anticancer agent through singlet oxygen quenching and an immunomodulatory in pharmaceutical industry. In addition, it is proven to have anti-inflammatory action in cardiovascular disease, age-related degenerative diseases, and enhance immune response (Park and Lee, 2001; Cifuentes et al., 2003; Nagaraj et al., 2012). However, *H. pluvialis* cells are very sensitive to environmental stress and they undergo morphological alterations under various environmental conditions (Hata et al., 2001; Imamoglu et al., 2007).

On the contrary to *S. platensis*, so far only few studies have been conducted on *H. pluvialis* regarding the toxicity effects of metals and metallic nanoparticles. A study by Li et al., (2008) reported the sensitivity of *H. pluvialis* to the treatment of ferrous sulphate with an increase in astaxanthin level relative to the total carotenoids at 72 h. Recently, Comotto et al., (2014) demonstrated the sensitivity of *H. Pluvialis* towards titanium dioxide NPs (100 mg/L) which caused reduction in biomass on day 9 with considerable increase in extra cellular phenolic compounds as a protective mechanism. In addition, the sensitivity of *H. pluvialis* to other environmental conditions also have been reported in the literatures. A noticeable decrease in chlorophyll content and increase in total carotenoids content were reported by Kavitha et al., (2015) when exposed to UV light for 15 min and a study by Cifuentes et al., (2003) showed reduction in chlorophyll level and induced carotenogenesis with increased production of astaxanthin on day 11 in high salt and high intensity light conditions.

A critical analysis of the existing literatures reports that the toxicity effects of ZnO NPs have been tested broadly on the wide range of aquatic organisms including algae, bacteria, crustaceans and fish. However, suggests that there is a need for the detailed assessment of ZnO NPs toxicity effects on the nutrient microalgae *S. platensis* and *H. pluvialis* since very basic information only is available in the literatures which are considered insufficient to design the methods for screening the contamination of ZnO NPs in the nutrient microalgae as the previous studies have

not reported the accumulative capacity and sensitivity of these two microalgae towards ZnO NPs. Hence, the present study has investigated the detailed dose- and time-dependent cellular accumulation and the corresponding cytotoxicity of ZnO NPs on *S. platensis* and *H. pluvialis.* The results of this study might help in identifying the bio-indicator to test the toxicity effects of ZnO NPs in the aquatic environment and also in developing the methods to screen the presence of ZnO NPs in the nutrient microalgae to prevent the ZnO NPs associated health hazards to the consumers.

CHAPTER 3

MATERIALS & METHODS

3.1 Primary Characterization of ZnO NPs

Nano-zinc oxide powder with the specified particle size of >100 nm was procured from Sigma-Aldrich. The scanning electron microscope (Scanning Electron Microscope, S-3400N, HITACHI, Japan) operated at 15 kV voltage, 10 mA probe current, 2.5 X 10^{-4} Pa vacuum, under high definition scanning mode (2560 X 1920 Pixels) with a working distance of 5-6 mm was used to determine the shape and size of ZnO NPs in the present study. The chemical composition of the nanomaterial was investigated through X-ray energy dispersive spectroscopy (EDX) (Scanning Electron Microscope, S-3400N, HITACHI, Japan), operated at 15 kV voltage, 10 mA probe current, 2.5 X 10^{-4} Pa vacuum under high definition scanning mode (2560 X 1920 Pixels) with a working distance of 5-6 mm. X-ray diffractometer (Lab X, SHIMADZU, XRD-6000, Japan), operated at an angle of 2θ with 40 volts and 30 mA current was applied to confirm the crystalline structure and size of the nanomaterial.

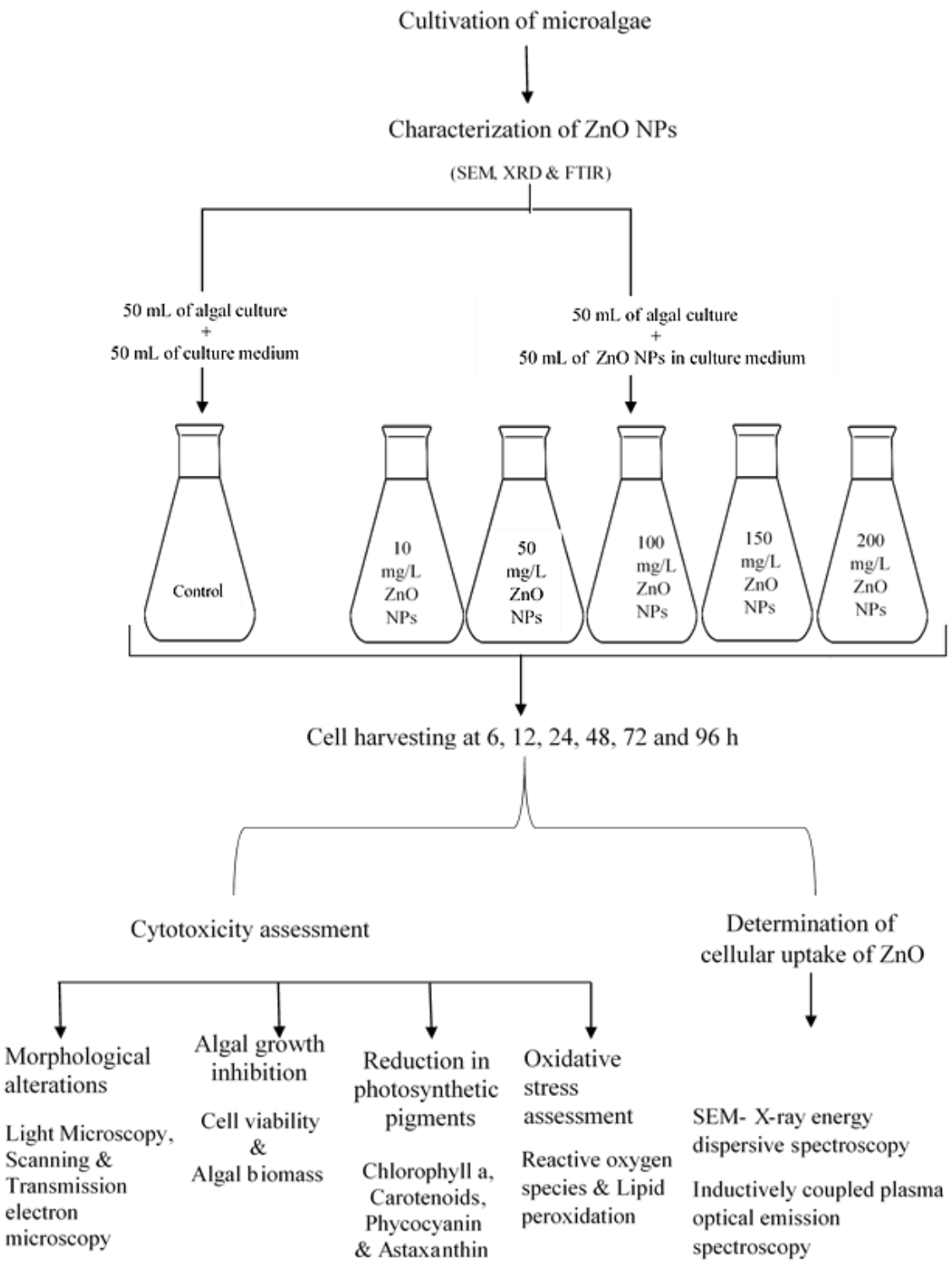

Figure 3.1: Experimental workflow of the study

Further, Fourier transformed infrared (FTIR, Nicoler IS 10, United States), operated at 120 Volts, 15 A current and line frequency of 50 Hz was used to confirm the functional groups present in the ZnO NPs.

3.2 Cultivation of Microalgae

The marine microalga *S. platensis* and the fresh water microalga *H. pluvialis* stock cultures were procured from UTEX1926 (University of Texas Culture Collection, Austin, TX, USA). *S. platensis* was grown in 500 mL-Erlenmeyer flasks by adding 2 ml of stock culture *in* 100 mL of *Spirulina* medium. *Spirulina* medium contains the following nutrients (g/ L): $NaHCO_3$, 13.61; Na_2CO_3, 4.03; NaCl, 1.00; K_2SO_4, 1.00; $NaNO_3$, 2.50; K_2HPO_4,0.50; $MgSO_4.7H_2O$, 0.20; $CaCl_2.2H_2O$, 0.04. All nutrients were dissolved in distilled water containing (per liter): 6 mL of metal solution (750 mg Na_2EDTA, 97 mg $FeCl_3.6H_2O$, 41 mg $MnCl_2.4H_2O$, 5.0 mg $ZnCl_2$, 2 mg $CoCl_2.6H2O$, 4.0 mg $Na_2MoO_4.2H_2O$) and 1 mL of micronutrient solution (50.0 mg Na_2EDTA, 618 mg H_3BO_3, 19.6 mg $CuSO_4.5H_2O$, 44.0 mg $ZnSO_4.7H_2O$, 20.0 mg $CoCl_2.6H2O$, 12.6 mg $MnCl_2.4H_2O$, 12.6 mg $Na_2MoO_4.2H_2O$ (Cruz-Martínez et al., 2015). Similarly, *H. pluvialis* was cultivated in Basal bold medium (BBM) by the addition of 2 ml of stock culture into 100 ml of BBM which is composed of $NaNO_3$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.025; $MgSO_4 \cdot 7H_2O$, 0.075; $NaHCO_3$, 0.0126; K_2HPO_4, 0.075; KH_2PO_4, 0.175; NaCl, 0.025;

$Na_2EDTA \cdot 2H_2O$, 0.05; KOH, 0.031; FeSO4·7H2O, 0.005; $FeCl_3 \cdot 6H2O$, 0.00315; H_3BO_3, 0.01142; $ZnSO_4 \cdot 7H_2O$,0.00882; $MnCl_2 \cdot 4H_2O$, 0.00144; MoO_3, 0.00071; $Na_2MoO_4 \cdot 2H2O$, 0.000006; $CuSO_4 \cdot 5H_2O$, 0.00157; $Co(NO_3)_2 \cdot 6H_2O$, 0.00049; $CoCl_2 \cdot 6H_2O$. 0.00001 (Menezes et al., 2016). The algal cultures were maintained in Erlenmeyer flasks under around 1200 lux illumination using white cool fluorescent lamp with 16 h light and 8 h dark condition at room temperature (21-23°C).

3.3 Exposure of Microalgae to ZnO NPs

A 200 ml stock solution of 400 mg/L ZnO NPs was prepared by adding 80 mg of ZnO NPs powder in 200 ml of the respective culture medium (*Spirulina* medium and BBM. The stock solution was sonicated for 30 min at 40 kHz to prepare the homogenous solution of nanoparticles. The five testing concentrations of nanoparticles were prepared at 10, 50, 100, 150 and 200 mg/L by diluting the stock solution with the respective culture medium. *S. platensis* cells from 5^{th} day culture and *H. pluvialis* cells from 4^{th} day culture, with an initial cell density of 1 x 10^5 cells/ mL, were subjected to the exposure with 10, 50, 100, 150 and 200 mg/L of ZnO NPs. 50 ml of algal cultures were mixed with 50 ml of the respective concentrations of ZnO NPs in 250 ml Erlenmeyer flask for a period of 96 h along with the control that was devoid of NPs. The ZnO NPs treated algal cells together with control cells were subjected for the assessment of cellular accumulation of

ZnO NPs and the corresponding cytotoxicity with oxidative stress at 6, 12, 24, 48, 72 and 96 h respectively (Fig. 3.1).

3.4 Investigation of Cellular Accumulation of ZnO NPS in Algal Cells

3.4.1 SEM EDX Analysis of ZnO NPs Treated Algal Biomass

SEM EDX examination was carried out to identify the cellular accumulation of ZnO NPs in the biomass of *S. platensis* and *H. pluvialis*. An about 5 ml of NPs treated cell suspension was centrifuged at 5000 rpm for 10 min and the pelleted algal biomass was washed two times in 1X PBS to remove unbound NPs from the biomass. Then the pelleted algal biomass was freeze-dried overnight to remove the moisture and the freeze-dried algal biomass was glued on sample holders by carbon adhesive tabs and subjected for SEM EDX analysis (S-3400N, Scanning Electron Microscope, HITACHI, Japan), operated at 15 kV voltage, 10 mA probe current, 2.5 X 10^{-4} Pa vacuum, under high definition scanning mode (2560 X 1920 Pixels) with a working distance of 5-6 mm.

3.4.2 Determination of Cellular Accumulation of Zinc in Algal Cells

The quantification of zinc accumulation in the algal cells was performed using inductively coupled plasma optical emission spectroscopy (ICP OES, 5300 DV

Perkin Elmer Optima, USA), operated at voltage 1.3 kV, electrospray chamber temperature of 90°C, plasma gas flow rate of 15 L/min, Zn measurement at 213.857 λ with axial plasma viewing position for the test durations 6, 12, 24, 48, 72 and 96 h. The nanoparticles treated algal cells were pelleted at 5000 rpm for 10 min and the sedimented cells were washed twice with 1X PBS to remove the loosely bound NPs. Then the algal cells were acid treated with concentrated nitric acid and the zinc content in the algal cells was analyzed using ICP OES. The external calibration curve was prepared for zinc with the concentrations ranging from 0.5 to 10.0 mg/L of zinc solution.

3.5 Cytotoxicity Assessment

3.5.1 Determination of Cell Viability

The algal cells treated with ZnO NPs along with the control cells were loaded in Neubauer cell counting chamber (Marienfeld, Germany). The number of cells with intact cell membrane and without any alteration in shape and size were counted as the viable cells. The percentage of loss in viable cells due to the treatment of nanoparticles was determined with respect to the control cells using Equation 1.

$I\% = (\mu C - \mu T) / \mu C \times 100$. (Eq. 1)

Where,

I% = percentage of loss in viable cells

μC = mean value of viable cells in control

μT = mean value of viable cells in treatment

The effective concentrations, EC_{10} (amount of NPs required to kill 10 % of the algal cells) and EC_{50} (amount of NPs required to kill 10 % of the algal cells) were determined using EPA Probit Analysis Program (version 1.5) with 95% confidence interval using cell viability data.

3.5.2 Quantification of Biomass

The loss in the biomass of *S. platensis* and *H. pluvialis* by the treatment of nanoparticles was estimated using spectrophotometer (Genesys 20, United Kingdom) at 560 nm and 680 nm respectively, with the respective culture medium as blank. An additional control called as particle-only (cell-free) control was included in this analysis as a reference to derive the actual absorbance by the algal biomass at the specific wavelengths, 560 nm for *S. platensis* and 680 nm for *H. pluvialis* (Gunawan et al., 2013). The percentage of decrease in biomass was calculated with respect to the control.

I% = (μC - μT) / μC x 100. (Eq. 2)

Where,

I% = percentage of loss in biomass

μC = mean value of biomass in control

μT = mean value of biomass in treatment

3.5.3 Measurement of Photosynthetic Pigments

To quantify the percentage of reduction in photosynthetic pigments, a 3 ml of nanoparticles treated and also 3 ml of control cell suspensions were subjected for centrifugation at 5000 rpm for 10 min. The pelleted cells were washed in 1X PBS for two times to remove the unbound nanoparticles from the algal cells. Subsequently, the pigments from the algal cells were extracted in 100% methanol at 65°C for 60 min or until the cell debris were almost colorless (Tocquin et al., 2012). The extracted pigments in the methanol were measured by spectrophotometer (Genesys 20, United Kingdom) at 470, 653 and 666 nm. The quantity of chlorophyll-a and carotenoids was calculated using the following equations of Lichtenthaler and Wellburn (1985) (Deniz et al., 2011).

Chlorophyll a (Ca) = 15.65 x A666 - 7.34 x A653 (Eq. 3)

Chlorophyll b (Cb) = 27.05 x A653 - 11.21 x A666 (Eq. 4)

$$\text{Carotenoids} = \frac{1000 \times A470 - 2.86 \times Ca - 129.2 \times Cb}{245} \quad \text{(Eq. 5)}$$

*A- absorbance at the specific wavelength (nm).

For the estimation of phycocyanin content, glass pearls were added to the washed pelleted cells and sonicated for 1 h at 40 kHz in the ultrasonic water bath to extract

the pigment. The absorbance of the pigment present in the supernatant was measured at 652 and 620 nm by spectrophotometer. Then the phycocyanin content (mg/mL) was determined using Eq. 6 (Moraes et al., 2011).

$$\text{Phycocyanin (mg/mL)}: \frac{A_{620} - 0.474A_{652}}{5.34} \quad \text{(Eq. 6)}$$

*A- absorbance at the specific wavelength (nm).

To quantify astaxanthin, 3ml of the algal suspensions from the control and treatments were pelleted and washed twice in 1X PBS and then the cell pellets were treated with 4 N of hydrochloric acid at 70 °C for 2 min and cooled. Further, the washed cell pellets were subjected to acetone extraction for 1 h and the resultant supernatant was used for quantifying the astaxanthin. The whole extraction process was performed in the dim light. The quantity of Astaxanthin was determined at 480 nm using an extinction coefficient of 2500 at 1% level by the method of Davies (Davies, 1976). The percentage of decrease in the photosynthetic pigments was determined comparing with control.

3.5.4 Morphological Examination of Algal Cells Treated with ZnO NPs

The optical and scanning electron microscopic examinations were performed to study the surface interaction of NPs on algal cells and the consequent morphological modifications in the treated cells. About 10 µl of algal cell

suspension, after each exposure period, was loaded onto a glass slide and sealed with cover glass and examined under phase contrast microscope (Nikon, Eclipse, TS 100, Japan). For SEM study, a 5 ml of cell suspension was centrifuged at 5000 rpm for 10 min and the resultant cell pellets were washed in 1X PBS for 2 times to remove the unbound NPs from the algal cells. The cell pellets were subjected for overnight freeze-drying in order to remove the moisture (Dmytryk et al. 2014) and then the freeze-dried algal cells were glued on sample holders by carbon adhesive tabs and subsequently subjected for sputtered coating (Sputter Coater SC7620, HITACHI, Japan). The sputtered specimens were studied through SEM (S-3400N, Scanning Electron Microscope, HITACHI, Japan).

3.5.5 Microscopic Examination of Ultra-Thin Sections of Algal Cells Treated with ZnO NPs

The intracellular deformities in cellular organelles were examined by transmission electron microscopy. The microalgal cells exposed to nano-zinc oxide particles were centrifuged for 10 min at 50000 rpm and the pelleted cells were pre-fixed in 4% glutaraldehyde diluted in PBS. The post-fixation was carried out in 1% osmium tetra oxide and dehydrated in a graded series of ethanol (50–99.9%). Infiltration and embedding was done with epoxy resin and then polymerized at 60°C at 72 h. The polymerized samples were cut into semi-thin sections and then into ultra-thin sections. The ultrathin sections were attached onto the copper grid and examined

under transmission electron microscope (TEM Libra 120, ZEISS, Germany) for trans-sectional studies of algal cells.

3.6 Oxidative Stress Assessment

3.6.1 Detection of Intracellular ROS in Algal Cells Treated with ZnO NPs

The production of intracellular reactive oxygen species (ROS) was investigated using fluorescent molecular probe 2',7'-dichlorodihydro fluorescein diacetate (H_2DCF-DA) (Invitrogen, Molecular Probes Inc., U.S.A.). H_2DCF-DA is a membrane permeable fluorescent dye, upon entering into the cells gets oxidized into a green fluorescent compound dichlorofluorescein in the presence of cellular esterases and ROS. For ROS measurement, a 0.5 mL of particle-exposed and untreated algal cell suspensions were washed twice in 1X PBS to remove the unbound NPs. Then 5 µL of 1mM dye was added to 1.0 ml of washed cell suspension to provide the final working concentration of 5 µM dye and incubated in dark for 30 min at room temperature (21- 23°C). Then the stained cells were washed with 1X PBS to remove the unreacted dye and then the washed cells were re-suspended in 1X PBS. The green fluorescence emission from the algal cells was quantified using fluorescent microplate reader (TECAN, Infinite M200PRO, Switzerland) at an excitation / emission wavelength of 485/ 530. The percentage increase in ROS production was determined by comparing with control. The

fluorescent microscopic examination (Nikon ECLIPSE 90i, United Kingdom) of H_2DCF-DA stained algal cells was carried out to further confirm the intracellular production of ROS in the algal cells that were exposed to ZnO NPs. In addition, the experiment included both negative control which contained the algal cells only and positive control which contained the algal cells treated with 5 % (v/v) hydrogen peroxide for 20 min.

3.6.2 Determination of Lipid Peroxidation in Algal Cells Treated with ZnO NPs

The level of LPO was assessed through the measurement of Malondialdehyde (MDA), which is a well-known biomarker of LPO using BODIPY 581/591C11 (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a-,4a-diaza-s-indacene-3-undecanoic acid-Invitrogen Molecular Probe, USA). BODIPY is a fluorescent fatty acid analog with fluorescent properties, which emits green fluorescence after the oxidation induced by the excessive production intracellular ROS. After 6, 12, 24, 48, 72 and 96 h of treatment with NPs, 0.5 mL of particle-exposed and untreated algal cell suspensions were centrifuged at 5000 rpm for 10 min to remove the unbound NPs. The cells were washed twice with 1X PBS, followed immediately by addition of 5 µL of 1mM dye to 1.0 ml of washed cell suspension to reach 5 µM of dye and incubated in dark for 30 min at room temperature (21- 23°C). Then the stained cells were washed with 1X PBS to remove the unreacted dye and re-suspended in 1X PBS. The green fluorescence emitted from the algal cells was

quantified by florescent microplate reader (TECAN, Infinite M200PRO, Switzerland) at excitation/emission wavelength of 485/530. Increase in lipid peroxidation level was determined by comparing with control. The experiment included both negative control which contained the algal cells only and positive control which contained the algal cells treated with 5 mM hydrogen peroxide for 30 min.

3.7 Statistical Analysis

The experiments were performed in triplicates (n=3) and the calculated results are presented as mean ± standard deviation. Shapiro-Wilk test was used to test the normal distribution of the data. Then, One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons (SPSS version 22) was carried out for analyzing the significant level. The level of significance was accepted at p value < 0.05. A significant difference at $p < 0.05$ between the control (0 mg/L of ZnO NPs) and the tested concentrations (10, 50, 100, 150 and 200 mg/L of ZnO NPs) at the specific time period (6, 12, 24, 48, 72 and 96 h) are denoted with the symbol ***.**

CHAPTER 4

RESULTS

4.1 Characterization of ZnO NPs

The scanning electron microscopic micrograph of nano-zinc oxide powder showed irregularly distributed spherically-shaped particles in an agglomerated form (Fig. 4.1A). The size of the nanoparticles is ranging from 39.7 – 49.6 nm in diameter with an average size of 44.6 nm. The X ray energy dispersive spectroscopy spectrum of nano-ZnO powder (Fig. 4.1B) presented a peak absorbance corresponded to zinc and oxygen molecules which confirmed that the purchased nano- ZnO powder containing zinc and oxygen molecules. Additionally, the X-ray diffractometer (XRD) spectrum of nano-zinc oxide powder (Fig. 4.1C) displays the strongest diffraction peaks at 31.7º, 34.36 ° and 36.19° and also the diffraction peaks at 47.05º, 56.09º, 62.38º, 65.90º, 67.45º, and 68.60º. All diffraction peaks of nano-zinc oxide powder corresponded to the characteristic hexagonal wurtzite crystalline structure of ZnO NPs (Ramesh et al., 2015). In addition, the XRD spectrum of ZnO NPs was used to further confirm the size of ZnO NPs using Scherrer's equation and the size was estimated to be at the range of 40 - 47 nm with a mean particle size of 43 nm.

Scherrer's equation: Particle Size = $(K \times \lambda)/ (d \cos\theta)$ (Eq.7)

K is a dimensionless shape factor (0.9), λ is the X-ray wavelength for Cu Kα radiation (1.5406 Å), d is line broadening at half of the maximum intensity (FWHM) in radian and θ is Bragg angle in degree.

The FTIR spectrum of ZnO NPs (Fig. 4.2) was used to further investigate the chemical composition of nano-zinc oxide powder. FTIR spectrum shows peak absorbance at 1636.59, and 671.88 cm^{-1}. A peak shown at 1634.00 represented Zn-O stretching (Kumar and Rani, 2013) and the stretching of ZnO NPs were found around 400–800 cm^{-1} (Ramesh et al., 2015). Thus, the FTIR spectrum of ZnO NPs demonstrated the presence of zinc and oxygen in the nanopowder used in this study.

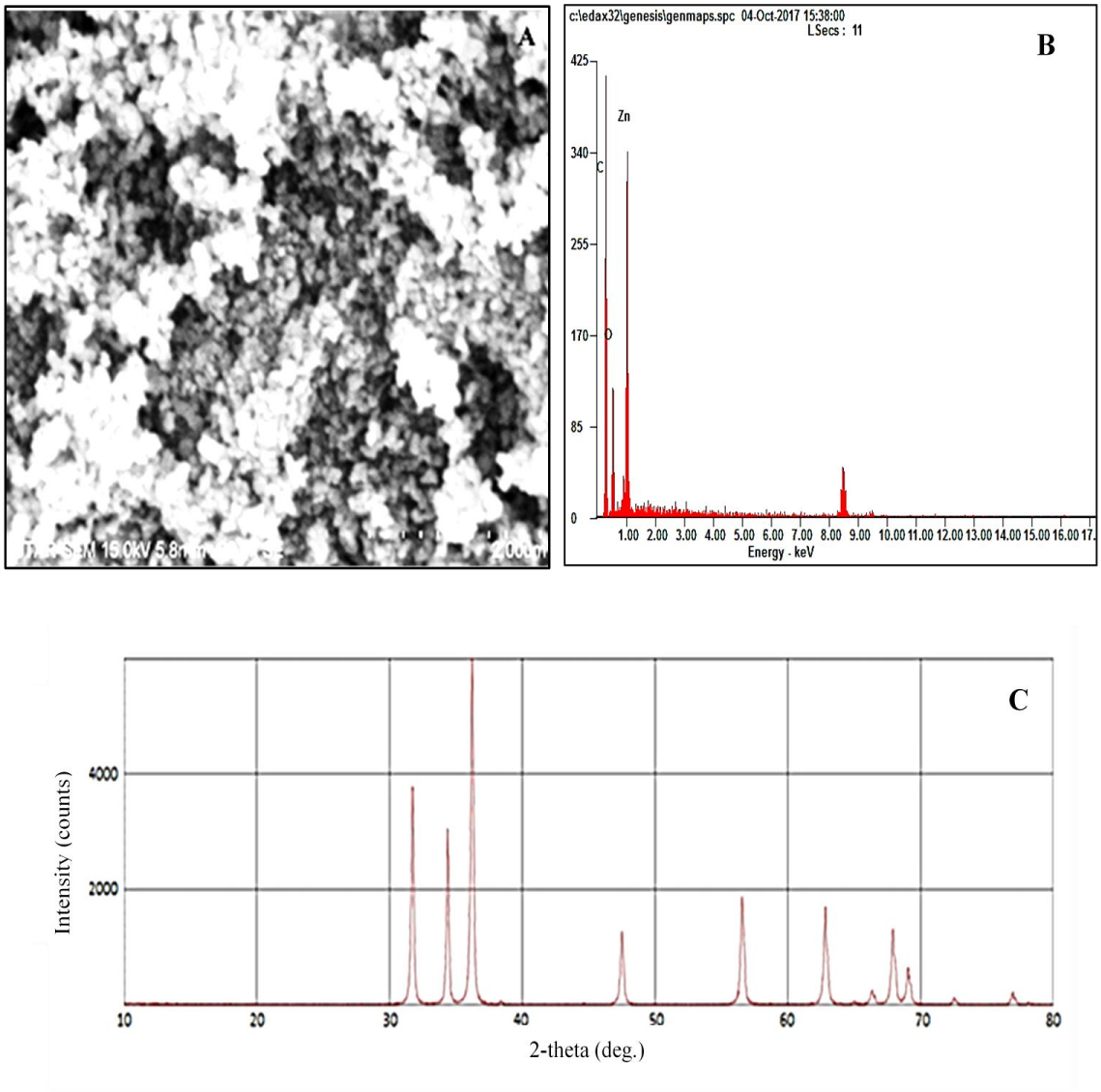

Figure 4.1: Scanning electron microscopy image (A), X ray energy dispersive spectroscopy spectrum (B) and X-ray diffractometer spectrum (C) of ZnO NPs.

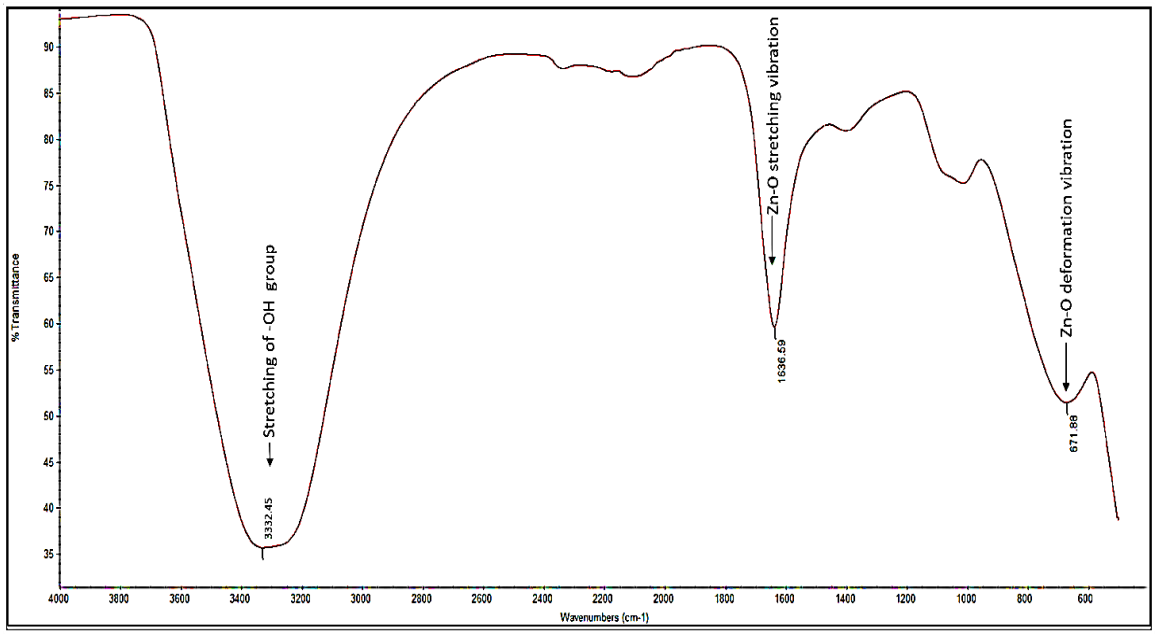

Figure 4.2: FTIR spectrum of zinc oxide nanoparticles powder

4.2 Cellular Accumulation of ZnO NPs in Algal Cells

4.2.1 Qualitative Analysis of Zinc Accumulation in Algal Cells

The SEM image of *S. platensis* shown in Fig. 4.3A displays non-aggregated filamentous cells with no observable peak for zinc in the EDX spectrum of *S. platensis* biomass (Fig. 4.3B). Similarly, the SEM micrograph of *H. pluvialis* shows smooth spherical non-aggregated cells (Fig. 4.4A) with no observable peak for zinc in the EDX spectrum of *H. pluvialis* biomass (Fig. 4.4B).

Whereas, the SEM pictures of microalgae *S. platensis* and *H. pluvialis* treated with zinc oxide nanomaterial show the surface accumulation of nanosized particles and the resultant algal cell aggregation in *S. platensis* biomass (Fig.4.3C) and *H. pluvialis* biomass (Fig. 4.4C). The EDX spectral peaks characteristic for zinc from the algal biomass treated with ZnO NPs confirmed the surface of accumulation of Zn in the biomass *S. platensis* (Fig. 4.3D) and *H. pluvialis* (Fig. 4.4D).

4.2.2 Quantitative Analysis of Cellular Accumulation of Zinc in Algal Cells

The results exhibited a dose and time dependent zinc accumulation in both algal cells. Both microalgae *S. platensis* and *H. pluvialis* accumulated significant ($p < 0.05$) amount of zinc with the treatment of 50 - 200 mg/L of ZnO NPs, but not with 10 mg/L of ZnO NPs at 6 h. The significant accumulation of zinc in both algal cells was noticed from 12 h onwards even at the treatment of 10 mg/L of ZnO NPs. The maximum accumulation of zinc was reported at 96 h with the resultant values of 16.67 ± 0.67, 32.33 ± 3.1, 43.2± 3.1, 46.93 ± 4.4 and 52.74 ± 3.99 pg/cell of zinc in *S. platensis* (Fig. 4.5), and 6.27 ± 0.34, 9.97 ± 0.8, 13.3± 0.9, 15.36 ± 1.2 and 18.36 ± 1.38 pg/cell of zinc in *H. pluvialis* (Fig. 4.6) at 10, 50, 100, 150 and 200 mg/L of ZnO NPs, respectively.

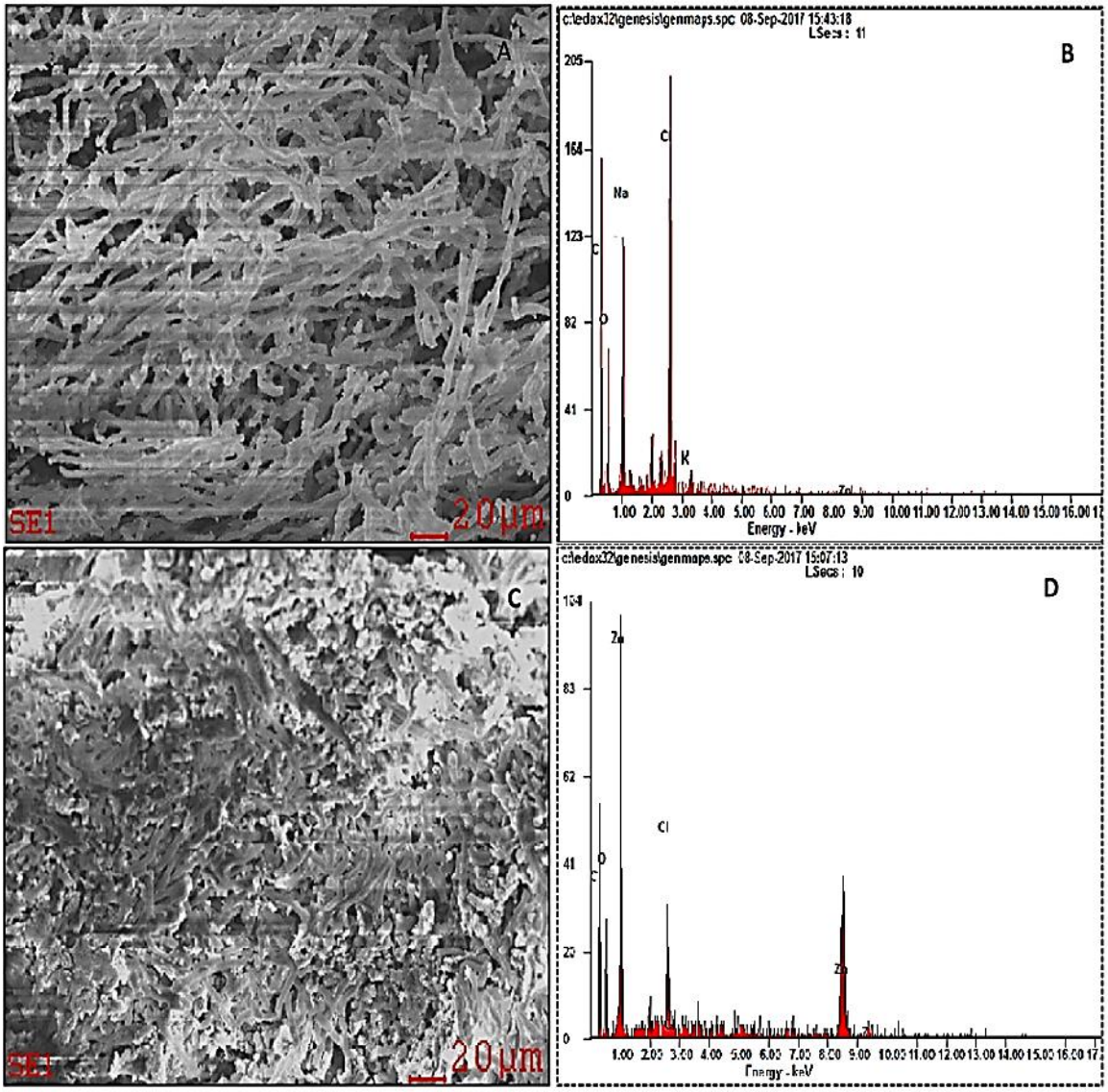

Figure 4.3: Scanning electron microscopic micrograph of *S. platensis* biomass (A) with EDX spectrum (B). Scanning electron microscopic micrograph of *S. platensis* biomass exposed to 200 mg/L of ZnO NPs at 96 h (C) with EDX spectrum (D).

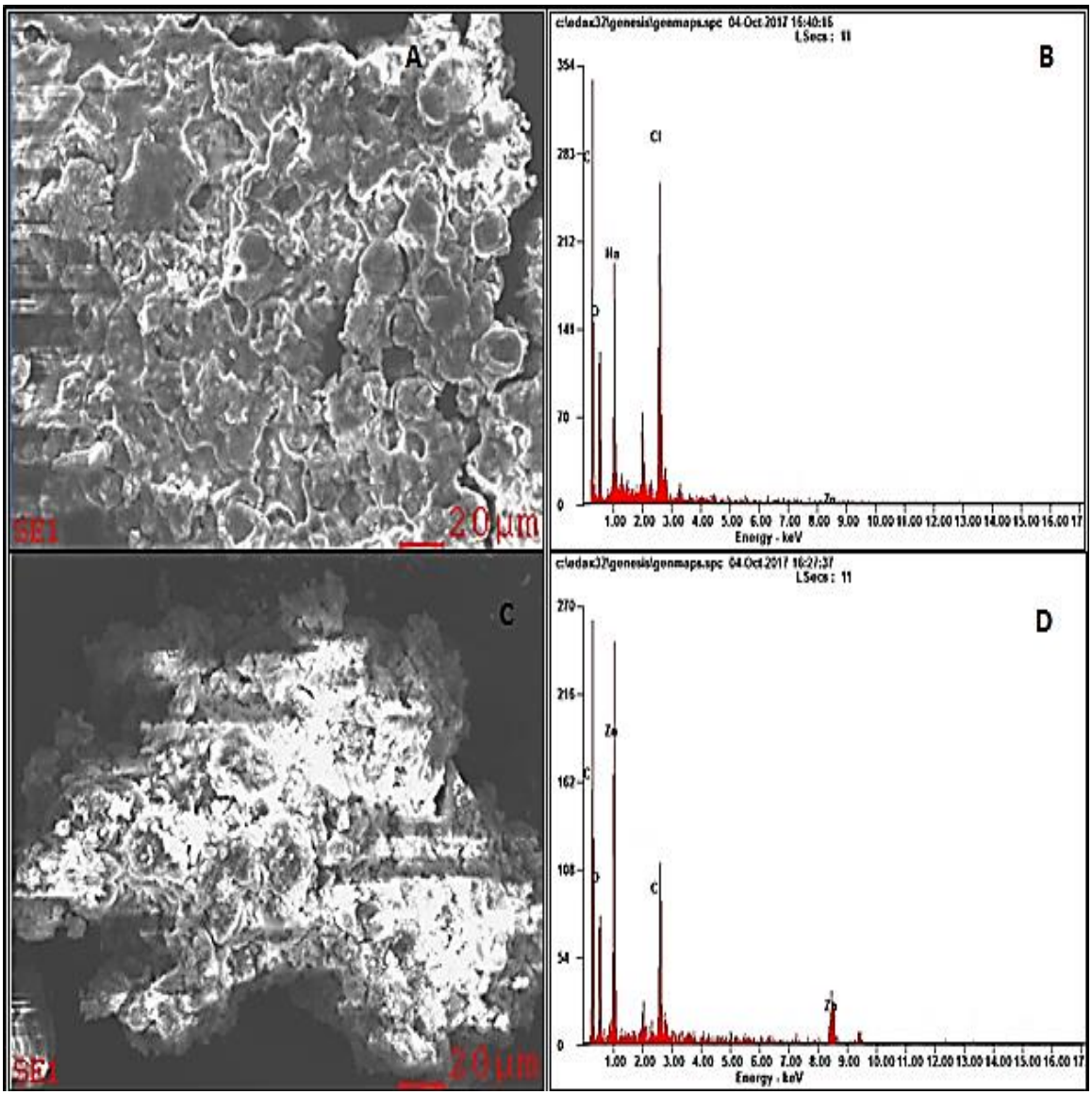

Figure 4.4: Scanning electron microscopic micrograph of *H. pluvialis* biomass (A) with EDX spectrum (B). Scanning electron microscopic micrograph of *H. pluvialis* biomass exposed to 200 mg/L of ZnO NPs at 96 h (C) with EDX spectrum (D).

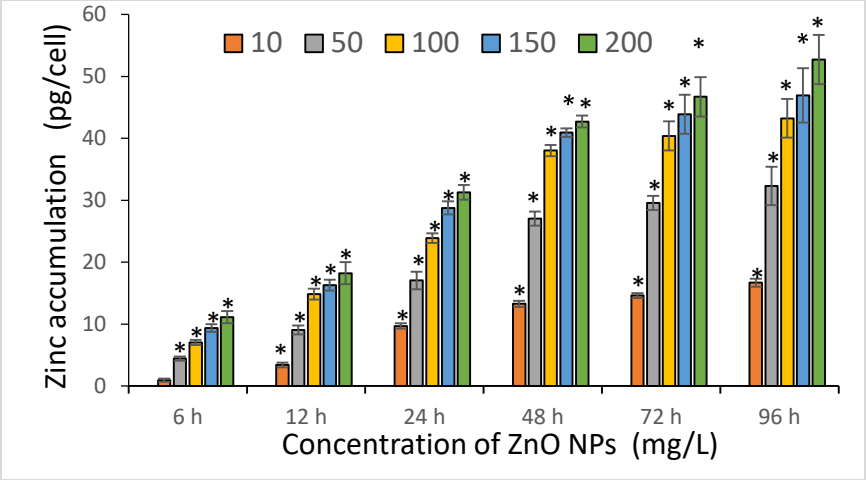

Figure 4.5: Zinc accumulation in S. *platensis* upon treatment with ZnO NPs.

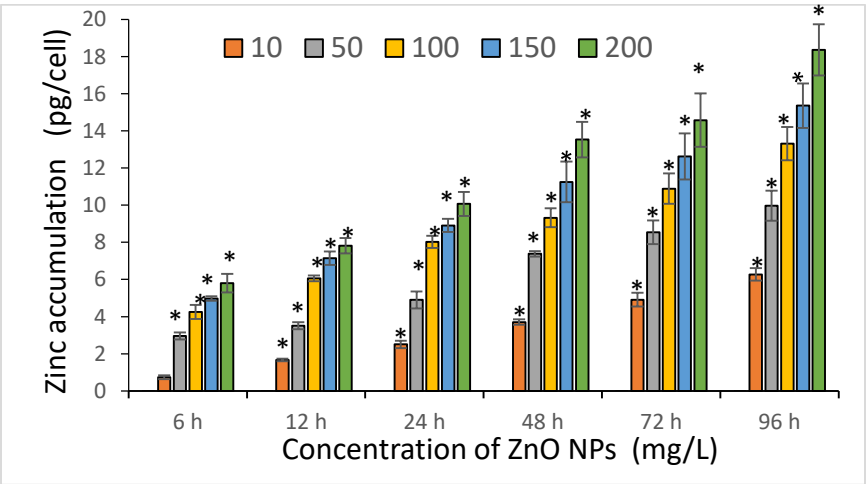

Figure 4.6: Zinc accumulation in H. *pluvialis* upon treatment with ZnO NPs.

4.3 Cytotoxic Assessment of ZnO NPs on Microalgae

4.3.1 Interaction of ZnO NPs with Microalgae in Erlenmeyer Flask

Fig. 4.7A shows the Erlenmeyer flask of *S. platensis* devoid of NPs that showed vigorous cell growth with no cell aggregation. In contrary, *S. platensis* interacted with ZnO NPs showed large clumps of flocculated algal cells with presumably visible growth inhibition (Fig. 4.7B). Similarly, Fig. 4.8A shows the Erlenmeyer flask with *H. pluvialis* without ZnO NPs that showed uniformly distributed cells with no aggregation. Whereas, Fig. 4.8B displays aggregation of algal cells with reduced cell growth. However, *S. platensis* treated with ZnO NPs showed remarkably larger cell aggregates or flocculates and visibly higher growth inhibition compared to *H. pluvialis*.

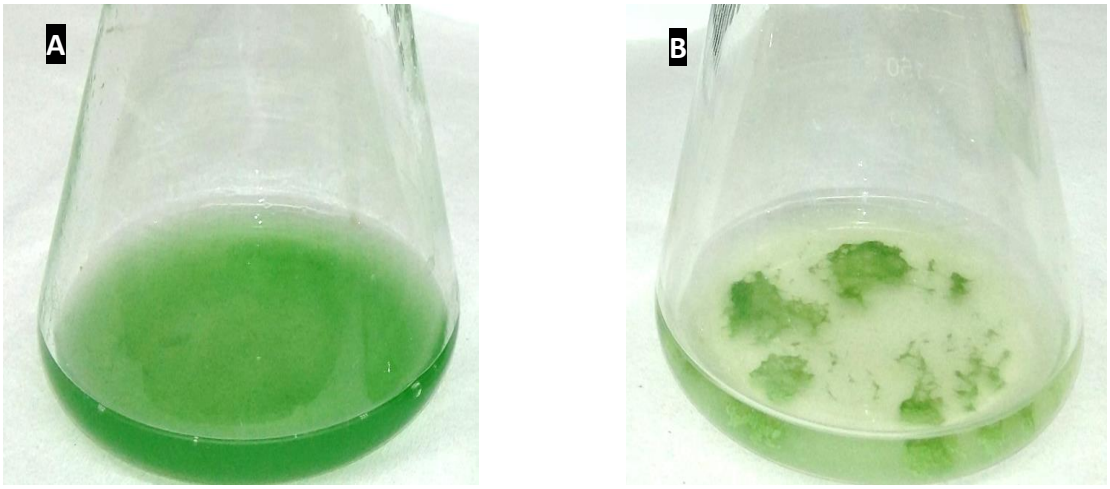

Figure 4.7: Erlenmeyer flask with *S. platensis*(A) and *S. platensis* treated with 200 mg/L ZnO NPs at 96 h (B).

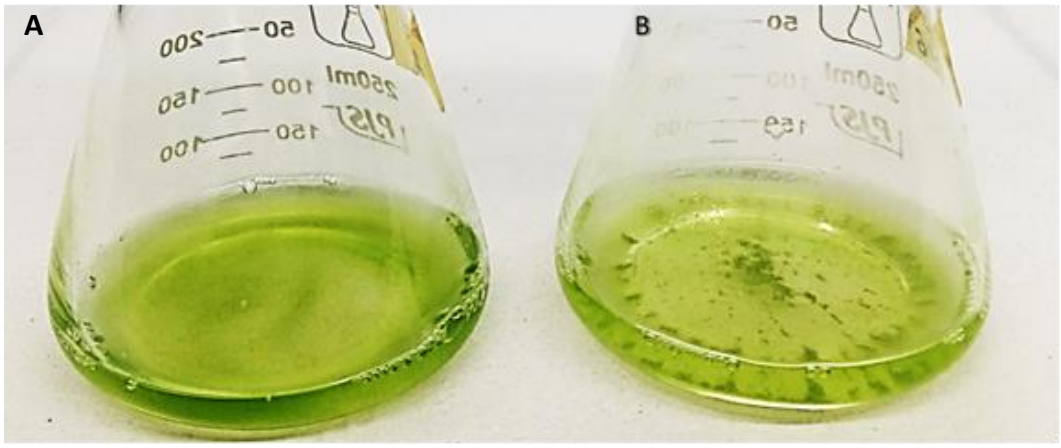

Figure 4.8: Erlenmeyer flask of *H. pluvialis* (A) and *H. pluvialis* treated with 200 mg/L ZnO NPs at 96 h (B).

4.3.2 Loss of Cell Viability

The results showed the potential toxicity of ZnO NPs on both microalgae through substantial loss in cell viability. The treatment of ZnO NPs caused significant ($p < 0.05$) loss in cell viability of *S. platensis* at the concentration ≥ 100 mg/L at 6 h and at ≥ 50 mg/L at 12 h. A significant ($p < 0.05$) loss in viable cells of *S. platensis* was observed with the treatment of all the tested concentrations of ZnO NPs (10 - 200 mg/L) from 24 to 96 h. The maximum cell death occurred at 96 h with a reported cell death of 44.3 ± 4.0, 69.7 ± 2.1, 83.8 ± 0.9, 86.7 ± 1.2 and 87.3 ± 1.0% at 10, 50, 100, 150, 200 mg/L of ZnO NPs respectively compared to control (Fig. 4.09). Whereas, the treatment of ZnO NPs caused significant ($p < 0.05$) loss in cell viability of *H. pluvialis* at the concentration ≥ 150 mg/L at 6 h and at ≥ 100 mg/L at 12 h. ZnO NPs treatment did not cause significant ($p < 0.05$) loss in cell viability until 48 h at 10 mg/L of ZnO NPs on *H. pluvialis* but caused a significant ($p < 0.05$) loss in cell viability at 50 to 200 mg/L of ZnO NPs from 24 h. From 72 h of treatment time, all the tested concentrations of ZnO NPs (10 - 200 mg/L) caused significant ($p < 0.05$) loss in cell viability of *H. pluvialis*. The maximum loss in cell viability of *H. pluvialis* was reported at 96 h with the resultant values of 20.0 ± 2.6, 34.6 ± 3.9, 41.7 ± 3.8, 46.4 ± 5.3 and 52.8 ± 5.1% at 10, 50, 100, 150, 200 mg/L of ZnO NPs respectively as compared to control (Fig. 4.10). Both the test organisms showed a characteristic dose- and time-dependent loss in cell viability upon treating

with nanoscale zinc oxide particles. However, the results showed nearly two times higher loss in cell viability of *S. platensis* compared to *H. pluvialis,* indicating the higher sensitivity of *S. platensis* towards the toxic effects of ZnO NPs.

4.3.3 Effective Concentration

The EC_{10} and EC_{50} values for ZnO NPs particles on algal growth inhibition are presented in Table 4.1. The present study demonstrated a substantially higher sensitivity of *S. platensis* towards the toxicity of nanoscale zinc oxide particles with 1.29 and 31.56 mg/L as the 72 h EC_{10} and EC_{50} values, and 0.83 and 13.97 mg/L as the 96 h EC_{10} and EC_{50} values, respectively. Whereas, *H. pluvialis* showed much lower sensitivity to ZnO NPs compared to *S. platensis* with 6.8 and 241.21 mg/L as 72 h EC_{10} and EC_{50} values, and 2.37 and 186.67 mg/L as the 96 h EC_{10} and EC_{50} values respectively. The present study showed that *S. platensis* requires nearly 13 times lower concentration of ZnO NPs to cause 50 % loss in cell viability at 96 h compared to *H. pluvialis* that confirmed the greater sensitive of *S. platensis* towards ZnO NPs toxicity.

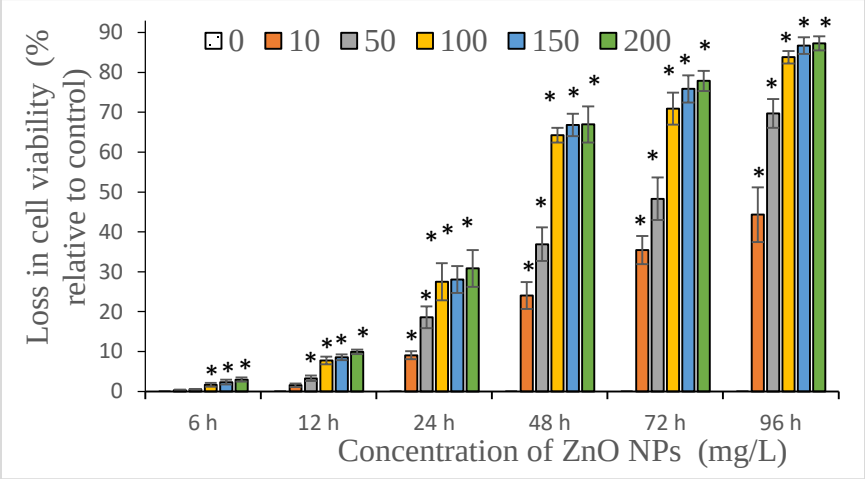

Figure 4.09: Percentage of loss in cell viability of *S. platensis* upon treatment with ZnO NPs.

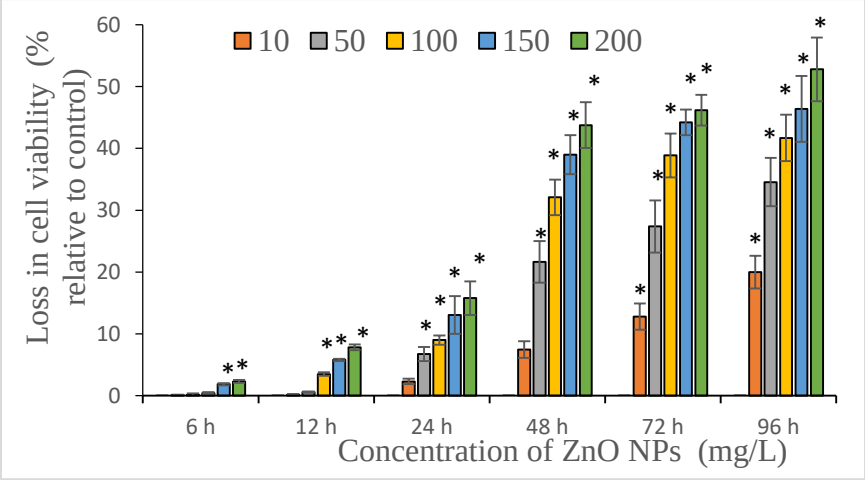

Figure 4.10: Percentage of loss in cell viability of *H. pluvialis* upon treatment with ZnO NPs.

Table 4.1: **EC_{10}** and **EC_{50} values of ZnO NPs on S. *platensis* and *H. pluvialis* from 48 to 96 h**.

Test organism	Effective Concentration	48 h	72 h	96 h
S. platensis	EC_{10} (mg/L)	3.65	1.29	0.83
H. pluvialis	EC_{10} (mg/L)	14.99	6.8	2.37
S. platensis	EC_{50} (mg/L)	67.87	31.56	13.97
H. pluvialis	EC_{50} (mg/L)	296.51	241.21	186.67

4.3.4 Reduction in Algal Biomass

The treatment of ZnO NPs resulted in loss of algal biomass of both microalgae *S. platensis* and *H. pluvialis* that followed the same trend with loss in cell viability. Results showed a significant ($p < 0.05\%$) decrease in algal biomass of *S. platensis* from 24 to 96 h among all the tested concentrations of ZnO NPs. *S. platensis* treated with NPs for 96 h was reported to have highest loss in biomass with 31.2 ± 2.7, 56.5 ± 2.9, 71.1 ± 1.2, 74 ± 0.97 and 76.1 ± 1.7% at 10, 50, 100, 150 and 200 mg/L respectively (Fig. 4.11). At the same time, a significant reduction in biomass of *H. pluvialis* was reported from 72 to 96 h for all the tested concentrations of ZnO NPs. The highest reduction in biomass of *H. pluvialis* was reported at 96 h with the reported values of 17.1 ± 3.0, 31.69 ± 4.0, 38.7 ± 0.7, 44.4 ± 2.2 and 49.4 ± 3.7% at 10, 50, 100, 150 and 200 mg/L respectively (Fig.4.12). Similar to loss in cell

viability, results exhibited a typical dose- and time-dependent loss in biomass of *S. platensis* and *H. pluvialis* upon interaction with ZnO NPs. In addition, results showed that the treatment of ZnO NPs caused comparatively more loss in biomass of *S. platensis* compared to *H. pluvialis*.

4.3.5 Loss in Photosynthetic Pigments

The growth inhibitory effects of ZnO NPs on microalgae were further established by investigating the loss in photosynthetic pigments namely chlorophyll-a (chl-a), carotenoids and phycocyanin in *S. platensis,* and chl-a, carotenoids and astaxanthin in *H. pluvialis*. Both the organisms showed precisely a same trend on the loss in chl-a with a significant ($p < 0.05$) loss from 6 h at 50 - 200 mg/L of ZnO NPs. Meanwhile, all the tested concentrations of ZnO NPs triggered a significant loss in chl-a from 24 h on both microalgae. The maximum loss in chl-a content was noticed at 96 h and reported to be 62.8 ± 4.4, 75.0 ± 3.5, 86.1± 2.7, 88.1 ± 3.5 and 92.5 ± 2.2 % in *S. platensis* (Fig. 4.13), and 27.1 ± 2.8, 47.7 ± 4.4, 54.6 ± 3.1, 58.4 ± 2.8, and 63.3 ± 2.4 % in *H. pluvialis* (Fig. 4.14) for 10, 50, 100, 150 and 200 mg/L of ZnO NPs respectively. In agreement with loss in cell viability and biomass, the loss of chl-a in *S. platensis* was nearly two folds higher than the loss of chl-a from *H. pluvialis.*

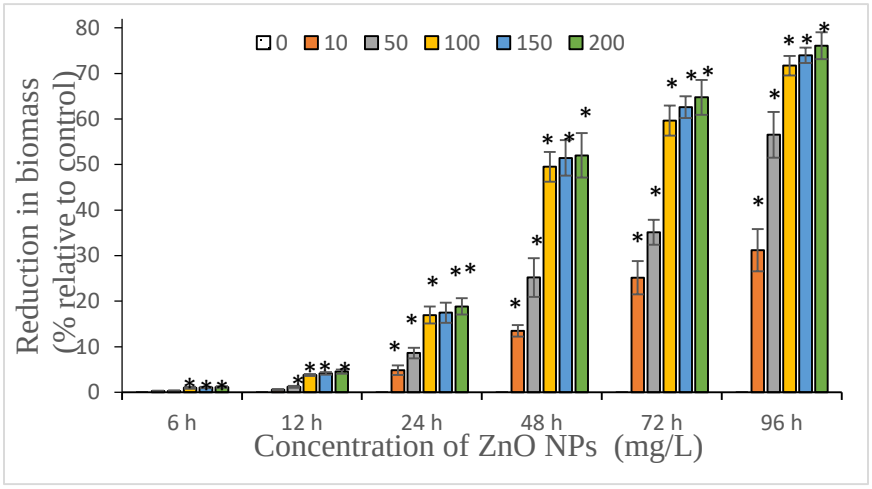

Figure 4.11: Percentage of loss in biomass of *S. platensis* upon treatment with ZnO NPs.

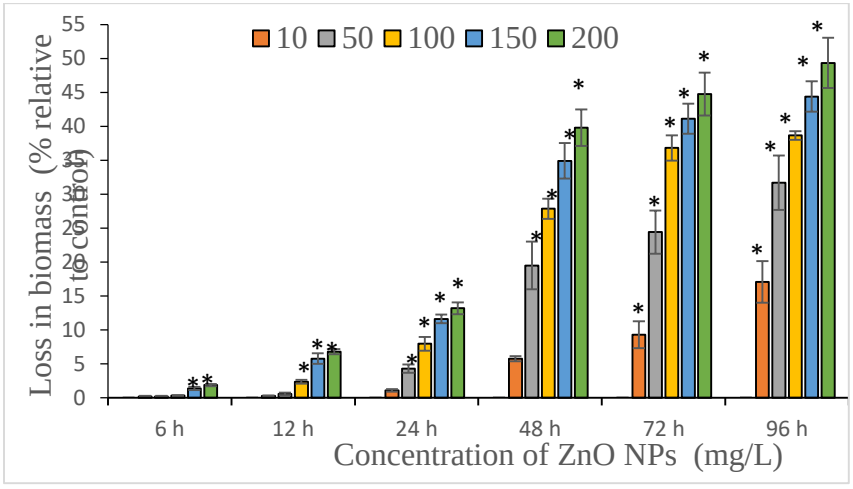

Figure 4.12: Percentage of loss in biomass of *H. pluvialis* upon treatment with ZnO NPs.

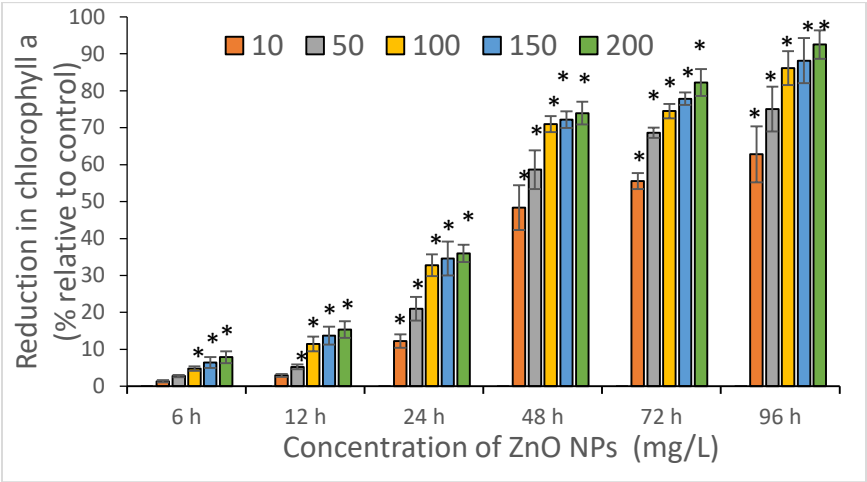

Figure 4.13: Percentage of loss in chlorophyll-a of *S. platensis* upon treatment with ZnO NPs.

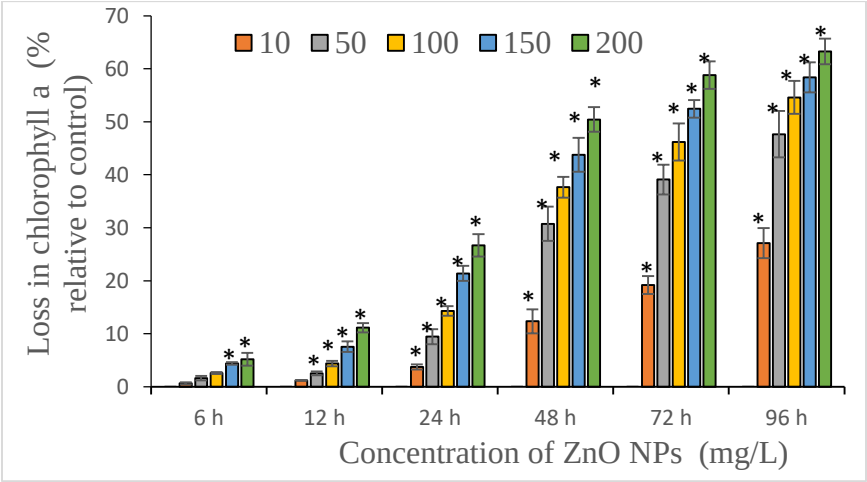

Figure 4.14: Percentage of loss in chlorophyll-a of *H. pluvialis* upon treatment with ZnO NPs.

Furthermore, the treatment of ZnO NPs caused significant loss ($p < 0.05$) in carotenoids of *S. platensis* from 24 to 96 h for all the tested concentrations of ZnO NPs with maximum reduction in carotenoids content at 96 h with the reported values of 56.1 ± 1.3, 64.1 ± 1.5, 70.3± 2.4, 75.9 ± 1.9, and 76.2 ± 2.2% for 10, 50, 100, 150 and 200 mg/L of ZnO NPs respectively (Fig. 4.15). In contrast, ZnO NPs caused comparatively lesser reduction in carotenoids of *H. pluvialis* and showed significant loss in carotenoids for all the tested concentrations of ZnO NPs from 48 h and the maximum drop in carotenoids was reported at 96 h with the resultant values of 16.3 ± 2.1, 28.1 ± 4.4, 36.7± 3.9, 39.9 ± 3.3 and 43.4 ± 3.6 % for 10, 50, 100, 150 and 200 mg/L of ZnO NPs respectively (Fig. 4.16).

Interestingly *S. platensis* contains phycocyanin that gives blue color to *Spirulina* cells and also acts as secondary photosynthetic pigment in harvesting light energy for photosynthesis. The toxicity of ZnO NPs on the phycocyanin content of *S. platensis* exhibited a mild rise in phycocyanin content for all tested concentrations of ZnO NPs at 6 h and also for 10 and 50 mg/L of ZnO NPs at 12 h. However, loss in phycocyanin content was noticed from 100 to 200 mg/L of ZnO NPs at 12 h. A significant ($p < 0.05$) decrease in phycocyanin for all tested concentrations of ZnO NPs was observed from 24 to 96 h (Fig. 4.17).

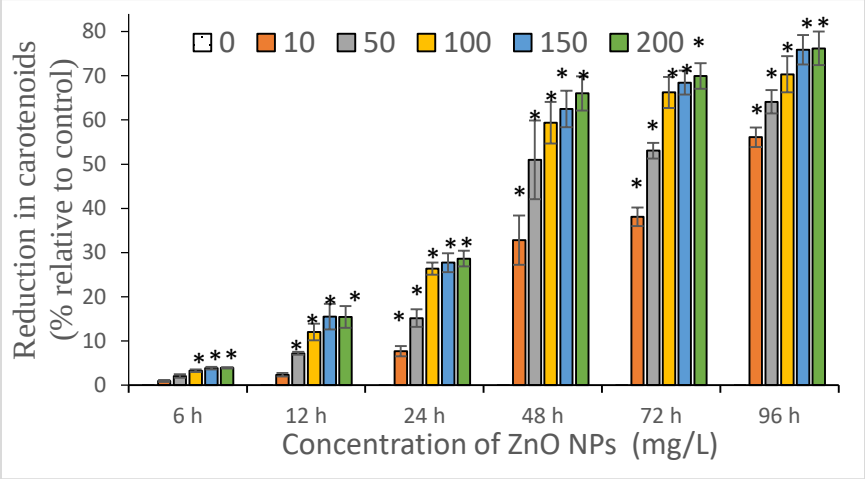

Figure 4.15: Percentage of loss in carotenoids of *S. platensis* upon treatment with ZnO NPs.

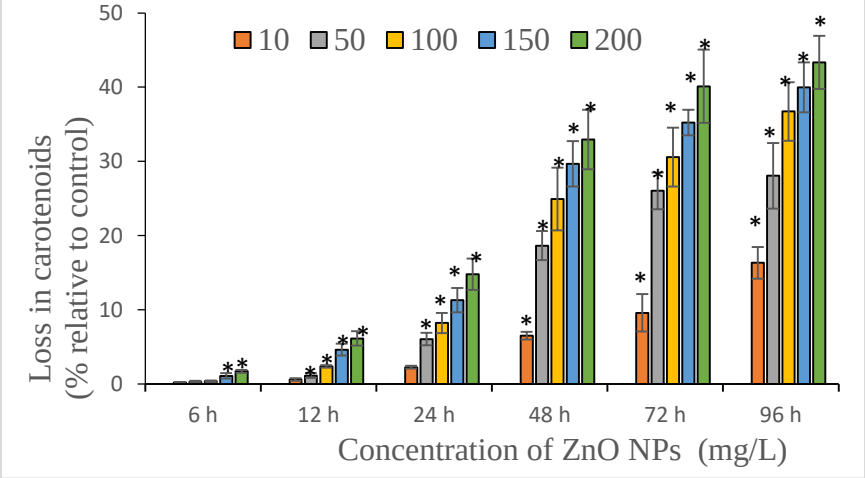

Figure 4.16: Percentage of loss in carotenoids of *H. pluvialis* upon treatment with ZnO NPs.

A maximum fall in phycocyanin pigment was reported at 96 h with the measured values of 47.4 ± 2.0, 61.8 ± 2.2, 68.5± 2.7, 72.4 ± 1.8 and 74.1 ± 3.4% at 10, 50, 100 ,150 and 200 mg/L of ZnO NPs respectively (Fig. 4.17). Similar to phycocyanin present in *S. platensis*, the astaxanthin content of *H. pluvialis* was decreased upon the treatment with ZnO NPs. Exactly similar to the trend of carotenoids, a significant ($p < 0.05$) reduction in astaxanthin content was reported from 48 h for all the tested concentrations of ZnO NPs with a maximum reduction at 96 h of treatment with the reported values of 17.5 ± 1.9, 27.0 ± 1.2, 34.1± 1.9, 39.9 ± 2.2 and 47.9 ± 3.1% at 10, 50, 100 ,150 and 200 mg/L of ZnO NPs, respectively (Fig. 4.18). The percentage decrease in all the photosynthetic pigments for an exposure duration from 6 to 96 h to 10, 50, 100, 150 and 200 mg/L of ZnO NPs resulted in a progressive decrease in pigment content as the treatment duration increased except for phycocyanin of *S. platensis* which showed mild rise in pigment content until 12 h of treatment time. The time and dose dependent decrease in photosynthetic pigments confirmed the dose and time dependent growth inhibitory effect of nanoscale zinc oxide nanoparticles. The results reported that the toxicity of ZnO NPs showed substantially greater reduction in the photosynthetic pigments of *S. platensis* over *H. pluvialis*.

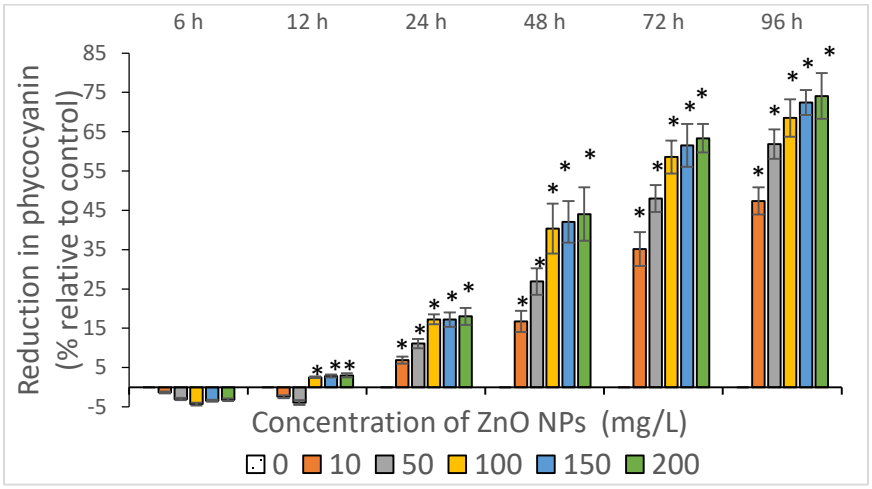

Figure 4.17: Percentage of loss in phycocyanin content of *S. platensis* upon treatment with ZnO NPs.

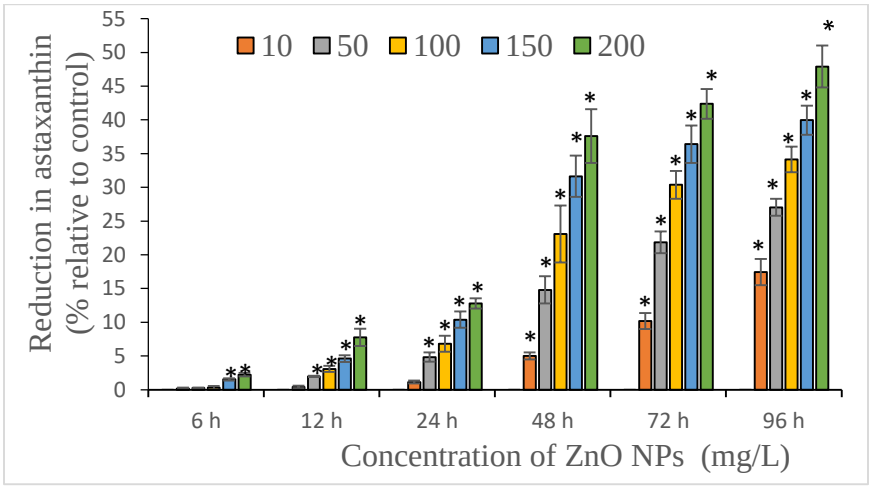

Figure 4.18: Percentage of loss in astaxanthin content of *H. pluvialis* upon treatment with ZnO NPs.

4.3.6 Morphological Examination of Algal Cells Treated with ZnO NPs

Fig. 4.19 shows the light microscopic images of *S. platensis* cells before and after treatment with ZnO NPs. Fig. 4.19A displays the filamentous *S. platensis* cells with healthy uncompromised cell membrane before treating with ZnO NPs. While, *S. platensis* cells treated with ZnO NPs showed entrapment of algal cells with aggregates of nano-zinc oxide particles (Fig. 4.191B), aggregation or flocculation of algal cells (Fig. 4.19C), rupture of *Spirulina* filament (Fig. 4.19D) and distortion of cells (Fig. 4.19E). Similarly, the scanning electron microscopic image of untreated control cells of *S. platensis* displayed smooth filamentous cells with healthy intact cell membrane (Fig. 4.20A). On the other hand, the exposure of ZnO NPs on *Spirulina* cells resulted in the aggregation and adsorption of ZnONP agglomerates on the surface of algal cells (Fig.4. 20B), rupture of cell membrane and fragmentation of trichrome (Fig. 4.20C), aggregation of algal cells (Fig. 4.20D), distortion of algal cells (Fig. 4.20E) and aggregation of distorted cells (Fig. 4.20F). *S. platensis* used in the present study showed a linear shape in the culture (4.20A). The linear morphology of *S. platensis* has been commonly recognized as the one of the morphologies of *S. platensis* (Wang and Zhao, 2005).

Likewise, the light microscopic images of *H. pluvialis* cells without ZnO NPs treatment showed spherical shaped membrane intact cells with many motile cells

(Fig. 4.21A). However, the treatment of ZnO NPs on *H. pluvialis* showed adsorption and aggregation of nanoparticle agglomerates on algal cells (Fig. 4.21B), algal cell aggregation, degradation and bleaching of cells by loss of chlorophyll through cell wall rupture (Fig. 4.21C), aggregation of algal cells with many ghost cells (Fig. 4.21D), and clustering of distorted cells (Fig. 4.21E). Similarly scanning electron microscopic images of *H. pluvialis* revealed smooth spherical cells with intact cell membrane for control cells (Fig. 4.22A). On contrary, ZnO NPs treated *H. pluvialis* cells showed entrapment of algal cells with nanoparticle agglomerates (Fig. 4.22B), algal cell aggregation (Fig. 4.22C), cell distortion cells with altered cell membrane (Fig. 4.22D), cell membrane rupture and the subsequent cell rupture (Fig. 4.22E) and aggregates of distorted cells (Fig. 4.22F)**.**

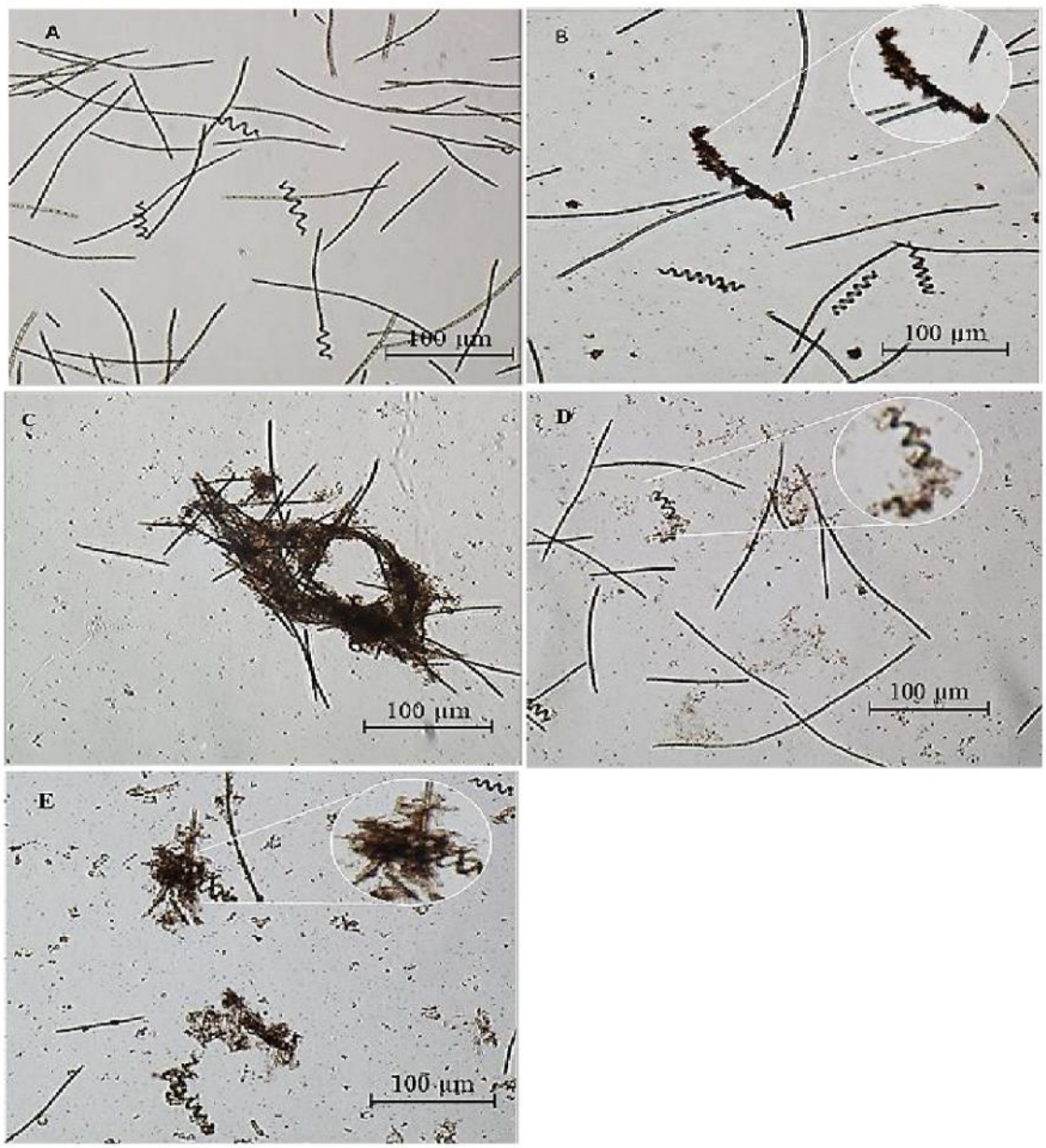

Figure 4.19: Light microscopic picture (10x) of control cells of *S. platensis* (A), treatment of ZnO NPs on *S. platensis* show adsorption of nanoparticle agglomerates (B), aggregation of algal cells (C), rupture of *Spirulina* filament (D) and cell distortion (E) at 96 h with 200 mg/L of ZnO NPs.

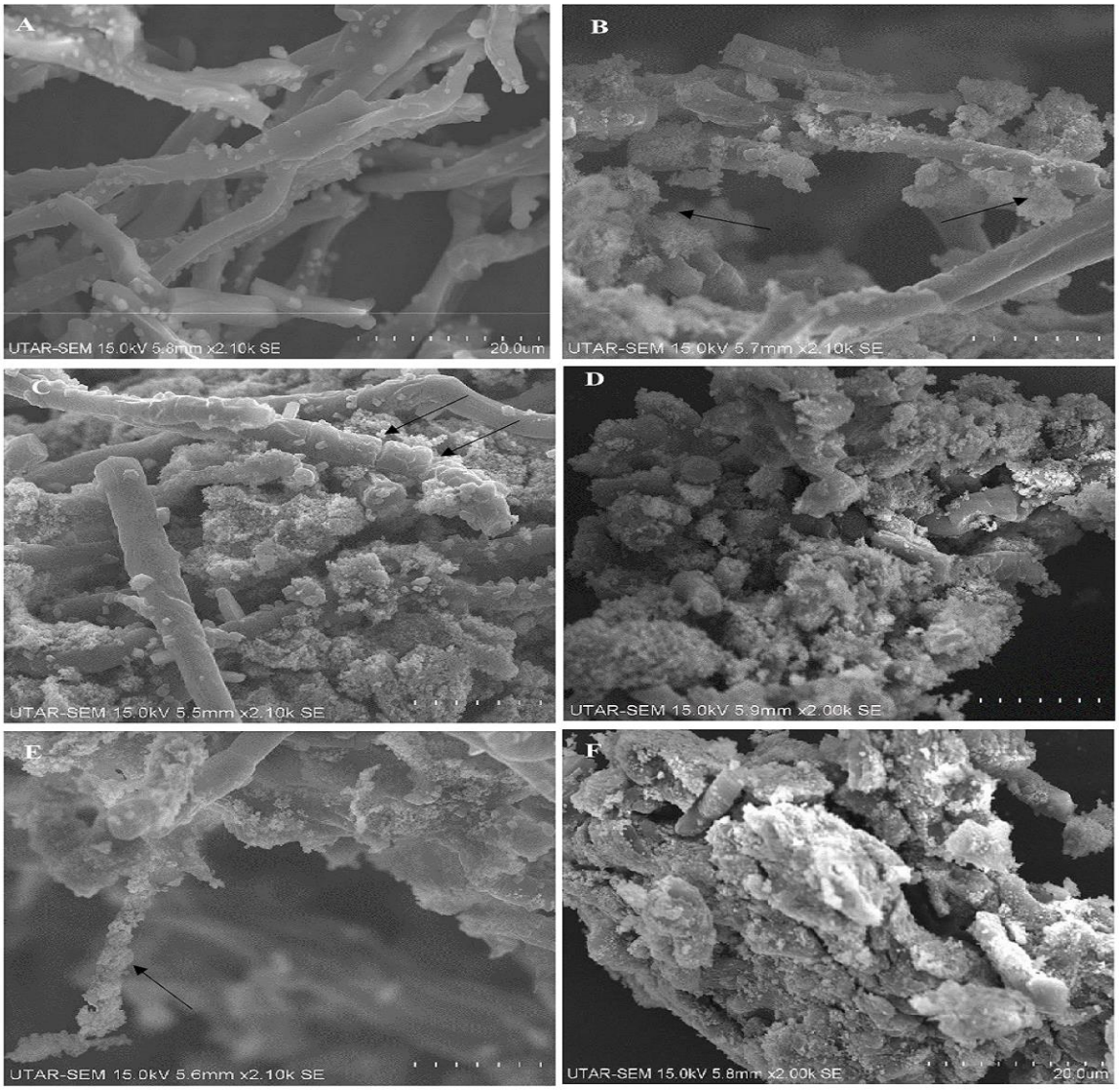

Figure 4.20: Scanning electron microscopic micrograph of control cells of *S. platensis* (A) and treatment of ZnO NPs on *S. platensis* display adsorption of NPs agglomerates on algal cells (B), rupture of the cell membrane and fragmentation of algal filament (C), algal cell aggregation (D), cell distortion (E) and aggregates of distorted cells (F) with 200 mg/L of ZnO NPs at 96 h. Scale bar - 20 µm.

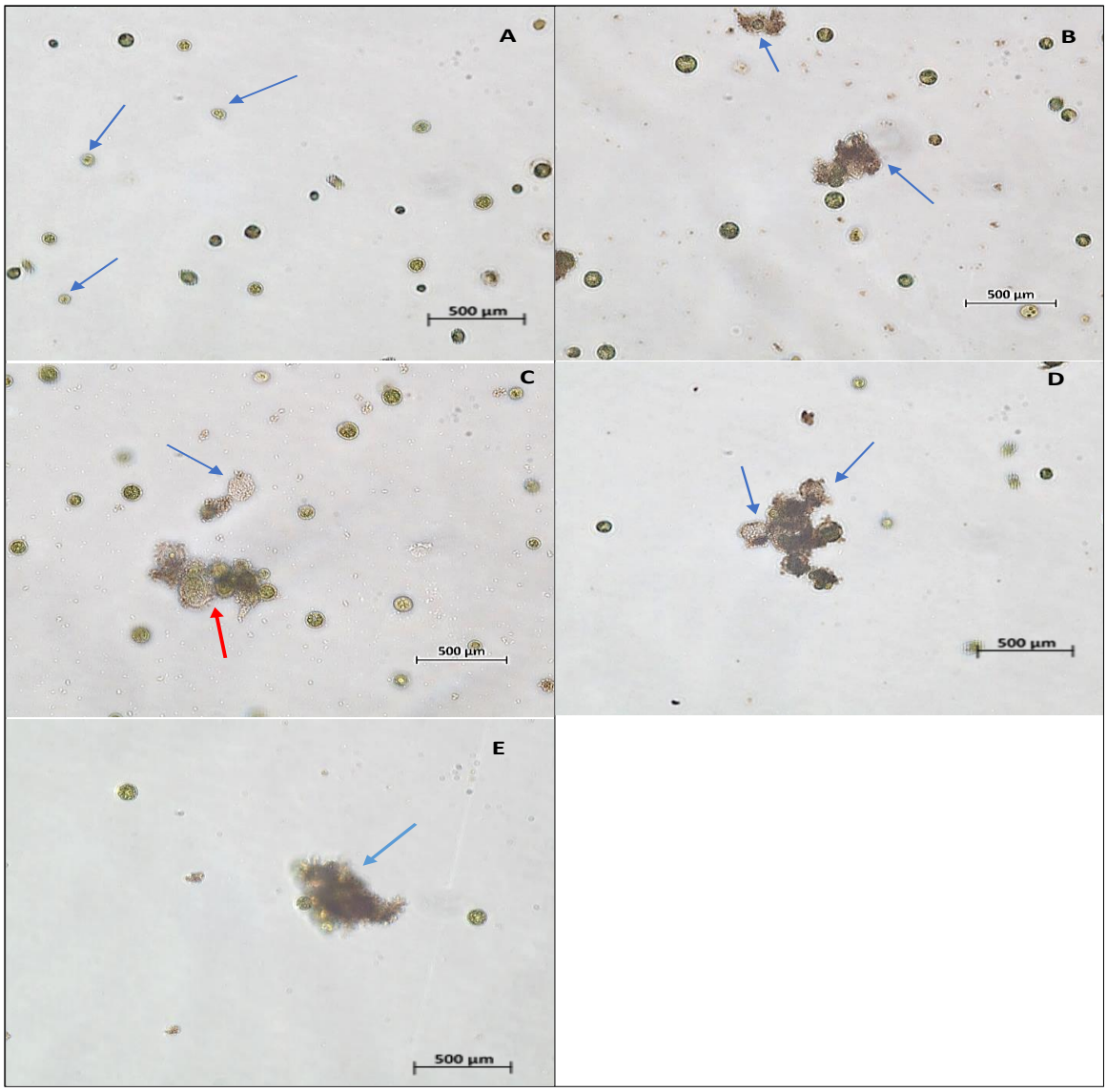

Figure 4.21: Light microscopic picture (10x) of control cells of *H. pluvialis* show cell membrane intact cells along with motile cells (blue arrow) (A) and the treatment of ZnO NPs on *H. pluvialis* resulted in wrapping of algal cells with nanoparticles (B), algal cell aggregation, degraded cell (red arrow) and bleached ghost cell (blue arrow) (C), aggregation of algal cells with bleached ghost cells (D) and aggregates of distorted cells with 200 mg/L of ZnO NPs at 96 h (E).

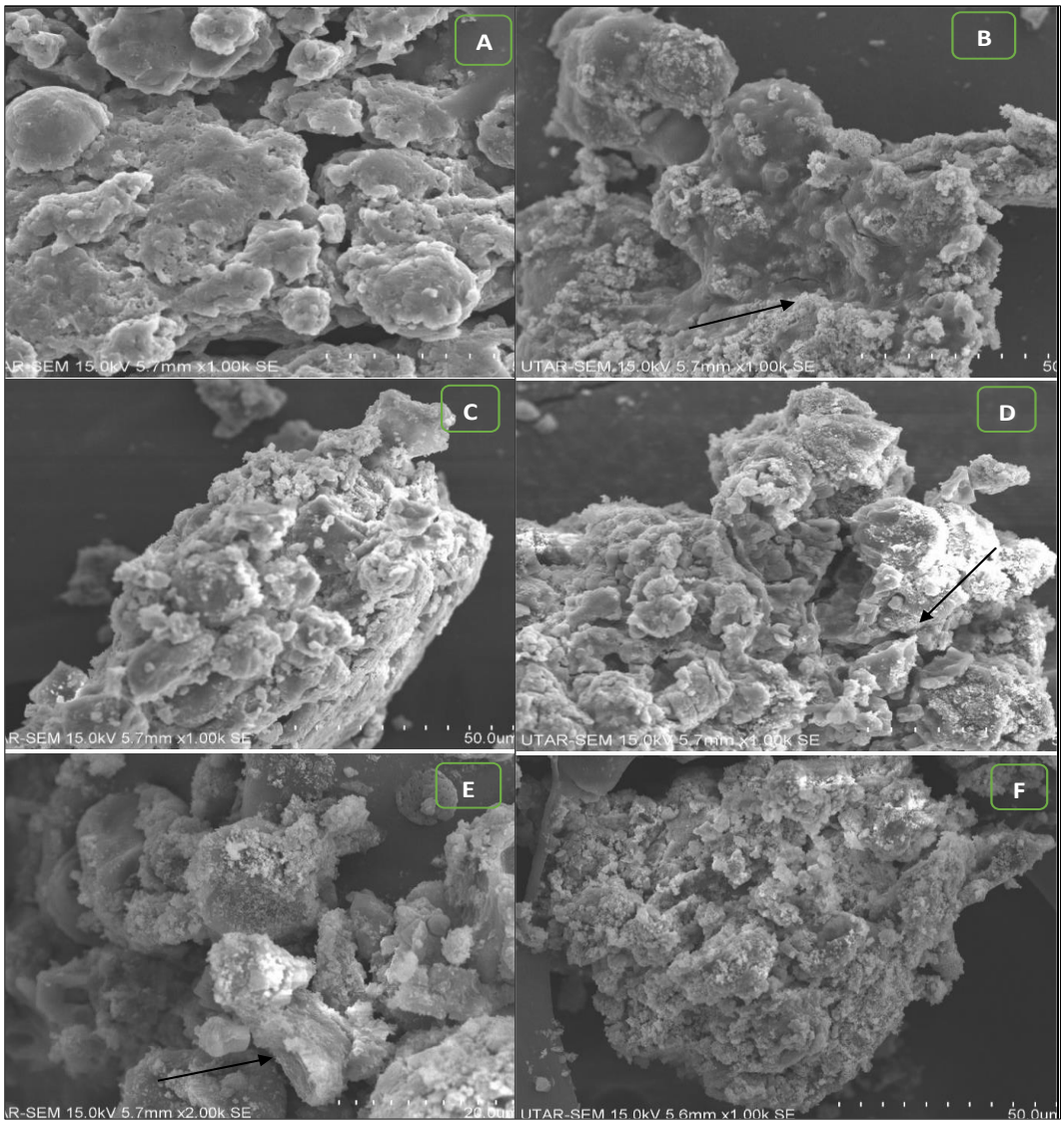

Figure 4.22: Scanning electron microscopic micrograph of control cells *H. pluvialis* (A) and the treatment of ZnO NPs on *H. pluvialis* display the adsorption of nanoparticle agglomerates on the algal cells shown by arrow head (B), aggregates of algal cells (C), distorted cells with altered cell membrane, black arrow head shows wrinkled cells (D), cell membrane rupture and cell rupture, black arrow head shows broken cell (E) and aggregation of distorted cells (F) upon treating with 200 mg/L NPs at 96 h. Scale bar - 50 µm.

4.3.7 TEM Examination of Algal Cells Treated with ZnO NPs

TEM examination was performed to study the ultra-structural damage of algal cells due to the treatment of ZnO NPs. TEM micrographs of control cells of *S. platensis* (Fig. 4.23A and C) display a smooth healthy cell membrane and huge number of packed thylakoid lamellae along the long axis of *Spirulina* filament. The presence of round shaped photosynthetic pigment phycobilisomes was also shown in the thylakoids. In contrast, the treatment of nanoscale ZnO particles on *S. platensis* resulted in destruction of cell membrane, rupture of trichrome, damage and lysis of packed thylakoid lamellae and phycobilisomes (Fig. 4.23B and D).

Similarly, TEM picture from the control cell of *H. pluvialis* shown in Fig. 4.24A revealed a thick cell wall that surrounding the vegetative cell and highly developed chloroplasts with densely packed thylakoids in the cytoplasm. Fig. 4.24C shows the control cell displaying lipid vesicles in the cytoplasm with accumulated astaxanthin inside the vesicles. Whereas, the cells treated with ZnO NPs showed complete degradation of thick cell wall along with loss of smooth layers (Fig. 4.24B). ZnO NPs treatment resulted in plasmolysis with leakage of cytoplasmic contents, deformation of lipid vesicles and degradation cytoplasmic organelles (Fig. 4.24D).

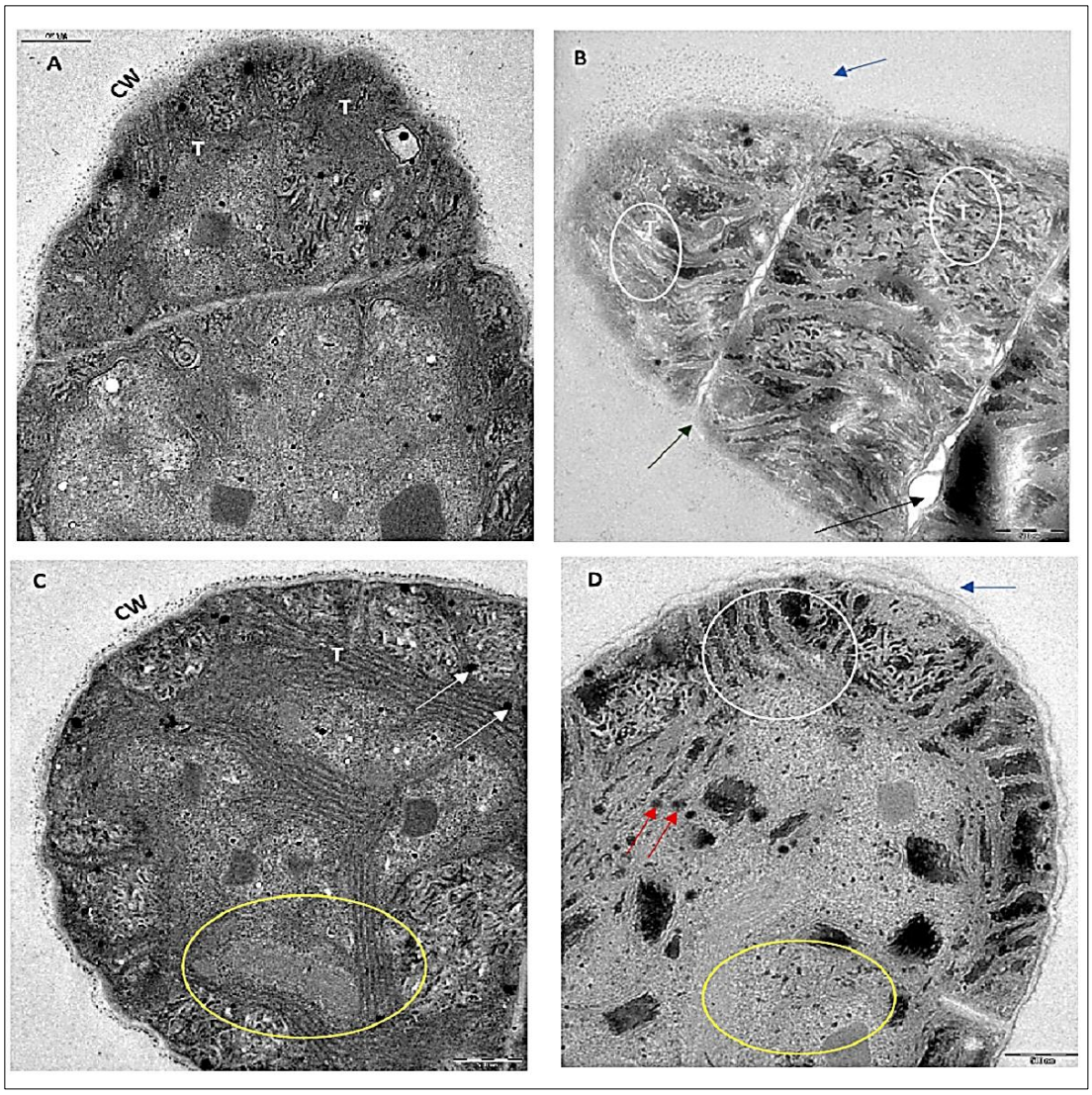

Figure 4.23: TEM images of *S. platensis* cells. The longitudinal (A) and transverse section (C) of *S. platensis* show a dense complete cell wall (CW), tightly arranged thylakoid lamellae (T) and the photosynthetic pigment phycobilisomes (white arrowhead). The longitudinal (B) and transverse section (D) of *S. platensis* exposed to ZnO NPs (200 mg/L) for 96 h resulted in the rupture of cell wall shown by electron transparent hallow around the cell (blue arrowhead), cell wall breakage (green arrowhead), rupture of *Spirulina* filament (black arrowhead), destruction of thylakoids (white circle) and destruction of phycobilisomes (red arrowhead). The area under yellow circle in (D) indicates the scattered electron transparent cytoplasm due to the destruction of intra-cytoplasmic organelles against the electron dense cytoplasm of control cells shown in the area under yellow circle in (C). Scale bar - 500 nm.

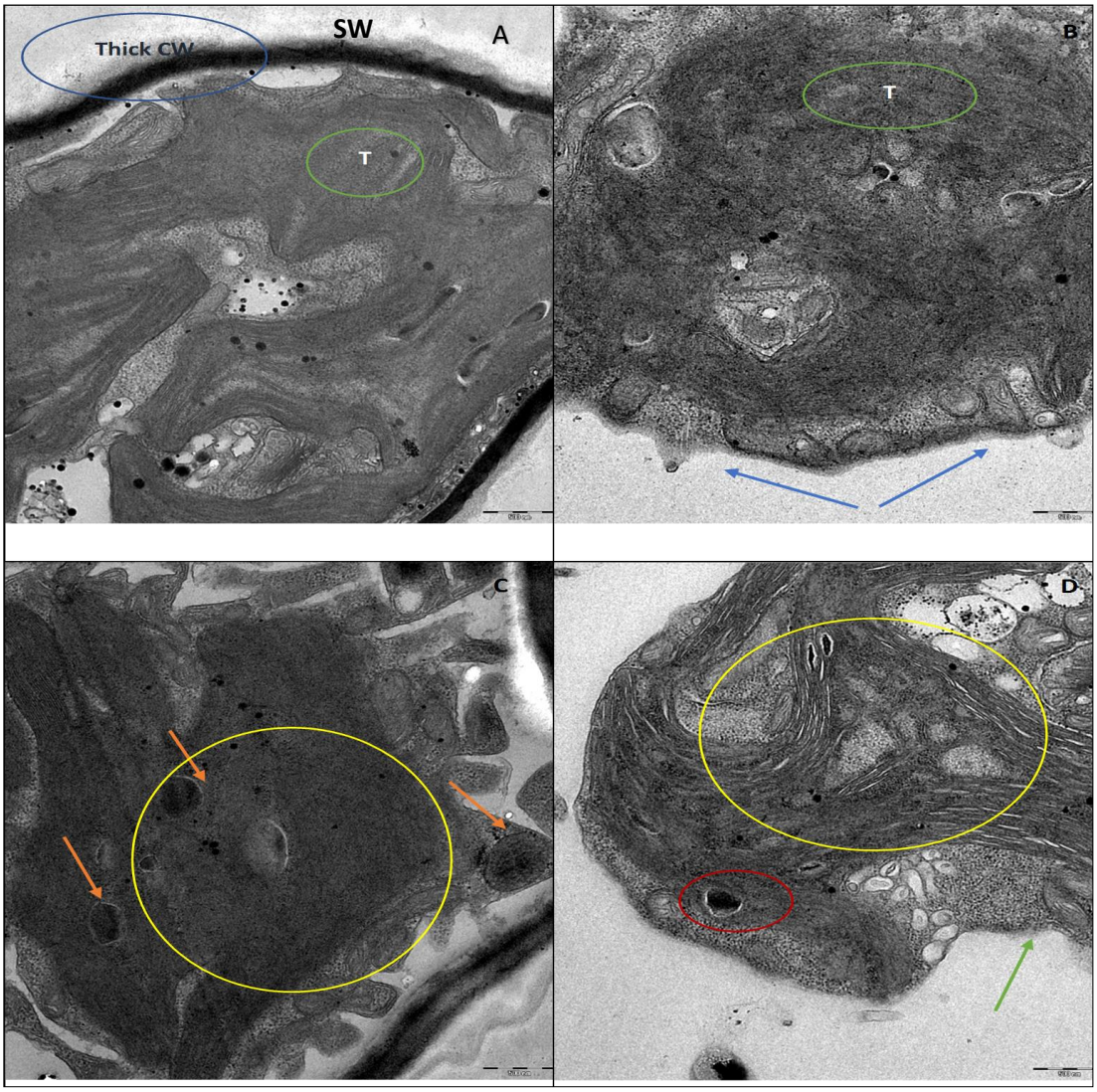

Figure 4.24: TEM pictures of *H. pluvialis*. TEM micrographs of *H. pluvialis* without ZnO NPs treatment are shown in (A, C). TEM picture of control cell in (A) display thick cell wall (CW) and smooth secondary wall (SW), densely packed thylakoids (T), and control cell in (C) shows astaxanthin containing lipid vesicles (orange arrowhead in C). TEM pictures of *H. pluvialis* exposed to ZnO NPs (200 mg/L) for 96 h are presented in (B, D). Fig. B displays the complete loss of secondary smooth wall and thick cell wall (blue arrowhead) and destruction of thylakoids (green circle). Test cell treated with ZnO NPs (D) displayed plasmolysis with leakage of intracellular contents (green arrowhead), shrunken and deformed astaxanthin containing lipid vesicles (red circle in D). The area under yellow circle in (D) displays the scattered electron transparent cytoplasm resulted from the destruction of intracytoplasmic organelles against the electron dense cytoplasm of control cell shown in the area under yellow circle in (C). Scale bar - 500 nm.

4.4 Oxidative Stress Assessment

4.4.1 Determination of Reactive Oxygen Species

The toxic effects of ZnO NPs on microalgae *S. platensis* and *H. pluvialis* induced a significant ($p < 0.05$) increase in in vivo production of reactive oxygen species for all the tested concentrations of ZnO NPs (10 to 200 mg/L) from 6 h of exposure. A maximum ROS production was noticed at 96 h on both microalgae with enhanced production of ROS in *S. platensis* cells with the reported values of 944.7 ± 118.4, 1494.5 ± 171.9, 2260 ± 91.9, 2438 ± 163.9 and 2597.4 ± 122.4 % (Fig. 4.25), compared to ROS production of 451.5 ± 38.6, 674.1 ± 48.3, 836.9 ± 14, 894.9 ± 54 and 973 ± 75.2 % from *H. pluvialis* cells (Fig. 4.26) for 10, 50, 100 ,150 and 200 mg/L of ZnO NPs, respectively. The exposure of nano-zinc oxide material on both microalgae *S. platensis* and *H. pluvialis* demonstrated a characteristic dose- and time-dependent increase in ROS production. However, ZnO NPs treatment induced nearly 2.0 – 2.5 times higher production of ROS from *S. platensis* compared to *H. pluvialis*.

4.4.2 Determination of Lipid Peroxidation Level

Increase in lipid peroxidation level by the treatment of ZnO NPs showed a similar pattern with ROS production for both microalgae *S. platensis* and *H. pluvialis*.

Results showed a significant ($p < 0.05\%$) increase in lipid peroxidation level in *S. platensis* from 12 to 96 h for all the tested concentrations of ZnO NPs. *S. platensis* treated with NPs for 96 h showed the highest increase in lipid peroxidation level with 147.7 ± 21, 286.5 ± 11, 437.2 ± 24.8, 492 ± 25.7 and 506.8 ± 27% for 10, 50, 100, 150 and 200 mg/L respectively (Fig. 4.27). But the significant ($p < 0.05\%$) increase in lipid peroxidation level in *H. pluvialis* was reported from 24 h for all the tested concentrations of ZnO NPs. The highest level of lipid peroxidation was reported to be 109.5 ± 13.7, 214.9 ± 19, 293.1 ± 26.1, 312.1 ± 29.2 and 323.9 ± 21.6% at 96 h for 10, 50, 100, 150 and 200 mg/L, respectively (Fig. 4.28). Similar to ROS production, results exhibited a distinctive dose- and time-dependent increase in lipid peroxidation level by the treatment of nanoscale zinc oxide particles on both microalgae with the higher level of lipid peroxidation in *S. platensis* compared to *H. pluvialis.*

The fluorescent microscopic examination of H_2DCF-DA dye stained control cells showed red fluorescence. Whereas, the test cells treated with ZnO NPs displayed green fluorescence due to the cleavage of the fluorescent probe by the presence of excessive intracellular ROS that were produced through the oxidative stress resulted from the treatment of zinc oxide nanomaterial (Fig. 4.29).

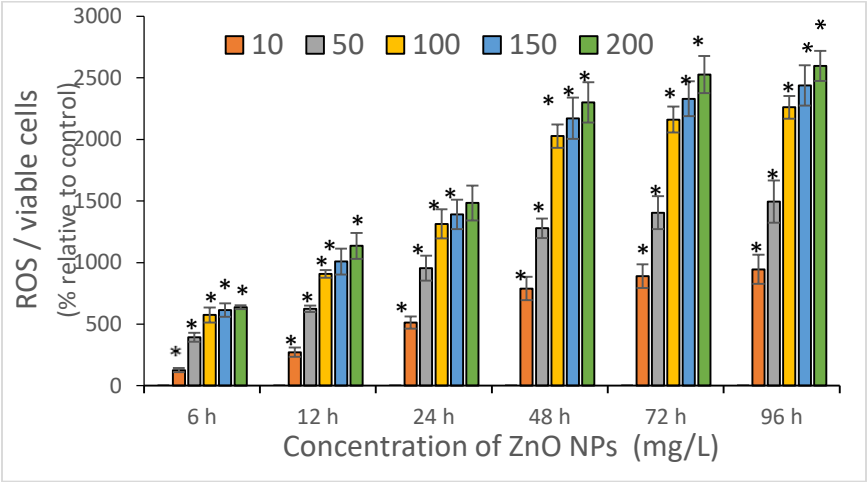

Figure 4.25: Percentage increase in reactive oxygen species from *S. platensis* upon treatment with ZnO NPs.

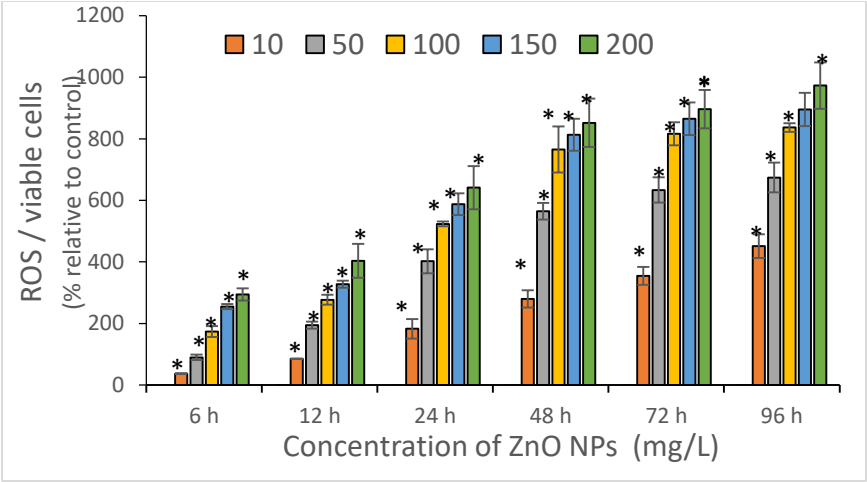

Figure 4.26: Percentage increase in reactive oxygen species from *H. pluvialis* upon treatment with ZnO NPs.

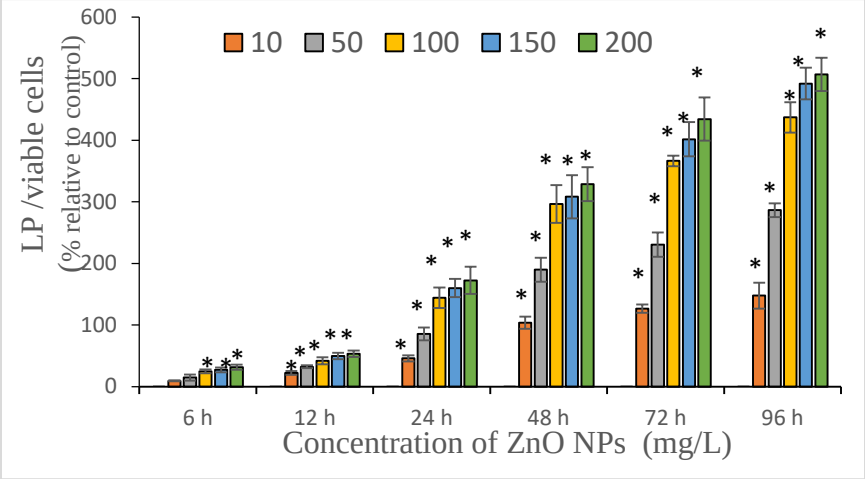

Figure 4.27: Percentage increase in lipid peroxidation level on *S. platensis* upon treatment with ZnO NPs.

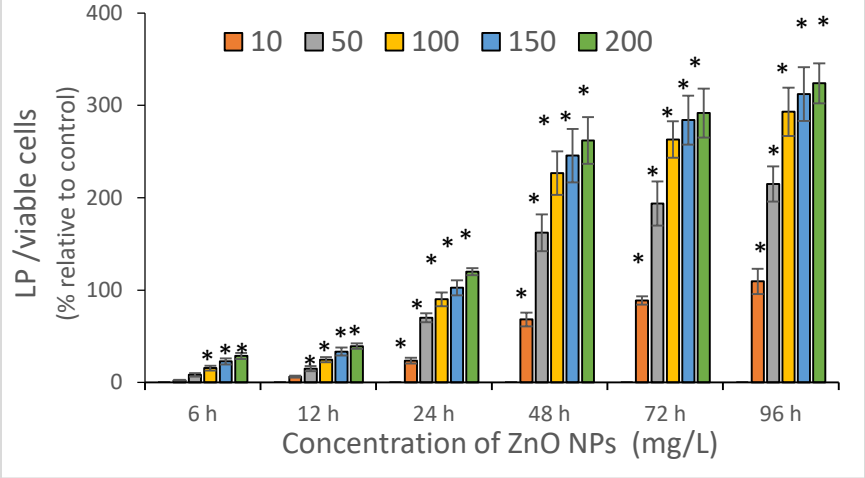

Figure 4.28: Percentage increase in lipid peroxidation level on *H. pluvialis* upon treatment with ZnO NPs.

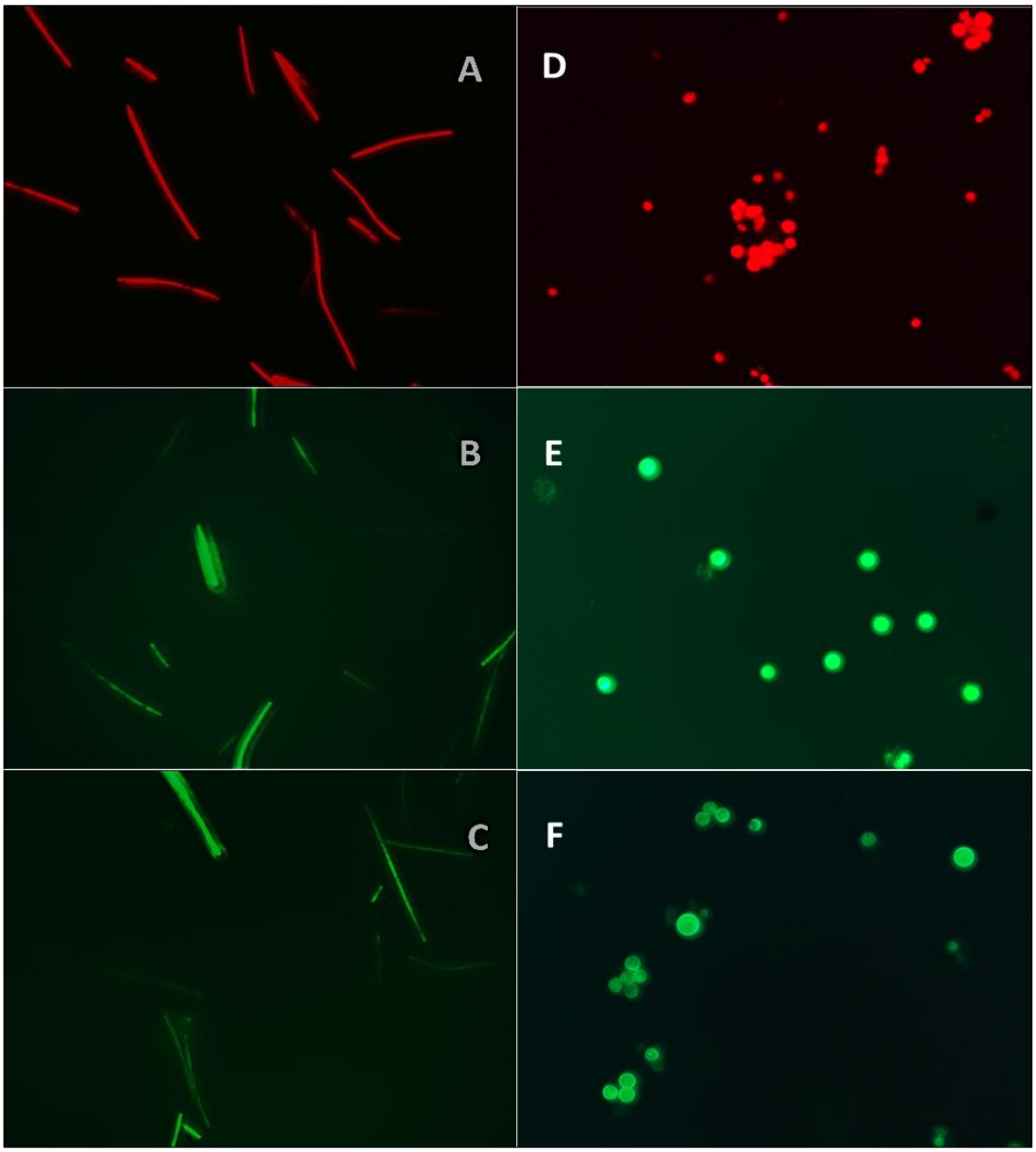

Figure 4.29: Fluorescence images showing the DCF green fluorescence of algal cells due to oxidative stress. (A) Negative control- *S. platensis* without NPs treatment (red fluorescence); (B) Positive control - *S. platensis* treated with hydrogen peroxide (5% v/v) for 20 min (green fluorescence); C) *S. platensis* treated with ZnO NPs (200 mg/L) at 96 h (green fluorescence); (D) Negative control - *H. pluvialis* without NPs treatment (red florescence); (E) Positive control - *H. pluvialis* treated with hydrogen peroxide (5% v/v) for 20 min (green fluorescence) ; (F) *H. pluvialis* treated with ZnO NPs (200 mg/L) at 96 h (green fluorescence).

CHAPTER 5

DISCUSSION

5.1 Cellular Accumulation of ZnO NPS in Algal Cells

Present study confirmed the aggregation of algal cells due to the accumulation of ZnO NPs in the algal biomass through SEM EDX analysis of ZnO NPs treated algal biomass. EDX spectrum of ZnO NPs treated *S. platensis* (Fig. 4.3) and *H. pluvialis* (Fig. 4.4) evidenced the cellular uptake of ZnO NPs in the biomass of *S. platensis* and *H. pluvialis.* Similar results on *S. platensis* were reported by Zinicovscaia et al., (2016) and Dmytryk et al., (2014) who demonstrated the cellular uptake of selenium and zinc in algal biomass of *S. platensis* through EDX analysis upon treating with selenium and zinc solution respectively.

The quantitative analysis of zinc accumulation in the algal cells was carried out using ICP OES. The present study exhibited a dose and time dependent increase in cellular accumulation of zinc in both microalgae *S. platensis* and *H. pluvialis*. In addition, the present study demonstrated nearly three times higher accumulative capacity of *S. platensis* towards ZnO NPs compared to *H. pluvialis*, for instance, *S. platensis* cells accumulated 16.7 to 52.7 pg/cell of zinc (Fig. 4.5) and *H. pluvialis*

cells accumulated 6.27 to 18.36 pg/cell of zinc (Fig. 4.6) for 10 to 200 mg/L of ZnO NPs at 96 h. In accordance with the present study, Gunawan et al., (2013) demonstrated a dose dependent cellular uptake of zinc in green alga *C. reinhardtii* with the cellular uptake of zinc ranged from 5 fg/cell at 1 mg/L to 18 fg/cell at 100 mg/L on day 8 when exposed to the increasing concentrations of nanosized zinc oxide particles from 1 -100 mg/L. Similarly, the exposure of duckweed *Spirodela polyrhiza* to 1, 10 and 50 mg/L of ZnO NPs resulted in dose dependent cellular accumulation of 2.8, 3.6 and 4.5 mg of zinc /g dry weight on day 4 (Hu et al., 2013). Apart from ZnO NPs, *S. platensis* can accumulate various metal ions effectively as reported in the earlier studies. Vannela and Verma, (2006) demonstrated the rapid absorption of zinc by *S. platensis* biomass with a maximum biosorption of Zn 250 mg/g of algal biomass when exposed to 600 mg/L of zinc at 2h. Celekli et al., (2010) showed the active adsorption of copper ions on by *S. platensis* with a reported adsorbed metal value from 13.74 to 52.63 mg/g at 5 h by the exposure of 25 to 200 mg/L of copper respectively. Deniz et al., (2011) reported a dose dependent cellular accumulation of copper ions in *Spirulina* cells by increase in the cellular accumulation of Cu (II) in *Spirulina* sp. as the concentration of Cu (II) exposure increased. Parallel to our study findings that demonstrated the higher accumulation of zinc in *S. platensis* compared to *H. pluvialis*, Shen et al., (2013) reported the higher accumulation of zinc in macrophages with 13, 24.6, 99.4 pg/cell compared to monocytes with accumulated zinc of 0.3, 5.1, 22.9 pg/cell at 24 h when treated with 10, 50 and 100 mg/L of ZnO NPs. The macrophages accumulated nearly four

times of zinc compared to monocytes and the reason for the difference in cell associated zinc between the immunocytes was expected to be the difference in the zinc uptake mechanism. The current study exhibited a strong relationship between the cellular accumulation of zinc and the cell mortality which is analogous with the findings of Shen et al., (2013).

5.2 Cytotoxicity and Oxidative Stress Effects of ZnO NPs on Microalgae

The results of the present study evidently demonstrated a characteristic dose- and time-dependent growth inhibition on both microalgae *S. platensis* and *H. pluvialis.* However, both microalgae were affected differently. *S. platensis* cells exposed to ZnO NPs formed larger aggregates of algal cells in the test system compared to *H. pluvialis* cells (Fig. 4.7 & 4.8) that indicated the higher toxic effects of ZnO NPs on *S. platensis*. Similarly, Sadiq et al. (2011) demonstrated the aggregation of algal cells with growth inhibition of *Scenedesmus* sp. and *Chlorella* sp. upon treating with alumina nanoparticles in the test flask. The possible reason behind the aggregation or flocculation of algal cells by the treatment of nanosized particles was may be due to the release of exopolysaccharides (algal exudates) from the stressed algal cells as a self-defense process. The algal exudates enable the algal cells to become closer and eventually cause cell aggregation or flocculation (Sadiq et al., 2011), this in turn favors the cells to reduce their exposed surface to

nanoparticles in order to reduce the additional binding of nanoparticles to the algal cells (Bhuvaneshwari et al., 2015).

The determination of loss in cell viability revealed the significant cytotoxic effects of ZnO NPs on both microalgae with profound cytotoxic effects in *S. platensis* compared to *H. pluvialis* through higher loss in the viable cells of *S. platensis* in a short time of exposure. ZnO NPs caused a significant ($p < 0.05$) loss in cell viability starting from 24 h on *S. platensis* and from 72 h on *H. pluvialis* for all the tested concentration of ZnO NPs from 10 – 200 mg/L. The maximum loss in cell viability was occurred at 96 h for both microalgae with nearly two times higher percentage of cell death in *S. platensis* with the reported value from 44.3 to 87.3% (Fig. 4.09), compared to *H. pluvialis* which showed cell death from 20.0 to 52.8% (Fig. 4.10) for 10 to 200 mg/L of ZnO NPs at 96 h. In accordance with our study finding, a concentration and time dependent loss in cell viability on the fresh water microalga *C. vulgaris* was reported by Suman et al., when treated with graded concentrations of ZnO NPs (50 - 300 mg/L) from 24 to 72 h. Remarkably, This study reported a higher sensitivity of *S. platensis* and lower sensitivity of *H. pluvialis* towards ZnO NPs' toxicity on comparing with *C. vulgaris* as the percentage of loss in cell viability was reported to be 77.8%, 46.2 % and 64.3 % respectively at 200 mg/L of ZnO NPs on day 3 (Suman et al., 2015).

Exactly similar to the pattern of loss in cell viability, the current results revealed a dose and time dependent reduction in algal biomass with nearly two times higher percentage loss in biomass of *S. platensis* compared to *H. pluvialis*. The highest loss in the biomass of both microalgae was reported at 96 h with greater reduction in the biomass of *S. platensis* i.e. 31.2 to 76% (Fig. 4.11) on comparing with the loss of biomass in *H. pluvialis* from 17.1 to 49.4% (Fig. 4.12) for 10 to 200 mg/L of ZnO NPs at 96 h.

In addition to the results of cell death and loss in biomass, the ecotoxicological parameters such as EC_{10} and EC_{50} have further confirmed the greater toxicity of ZnO NPs on *S. platensis* over *H. pluvialis* through 96 h EC_{10} of 0.83 mg/L and 96 h EC_{50} of 13.97 mg/L for *S. platensis*, and 96 h EC_{10} of 2.37 mg/L and 96 h EC_{50} of 186.67 mg/L for *H. pluvialis* (Table 4.1). The study results established the profound sensitivity of *S. platensis* through the fact that *S. platensis* requires around 13 times lesser dose of ZnO NPs (mg/L) to cause 50% reduction in cell viability compared to *H. pluvialis* based on 96 h EC_{50} values.

Nevertheless, These findings showed more sensitivity of both microalgae *S. platensis and H. pluvialis* when compared with marine alga *Spirodela polyrhiza* towards the toxic effects of ZnO NPs as the percentage of growth inhibition was reported to be 56.5%, 34.56% and 21.1% respectively at 50 mg/L on day 4 (Hu et al., 2013). Further, This study reported a higher percentage loss in biomass of *S.*

platensis with the reported value of 71.7% at 100 mg/L on day 4, when interrelated to the results of Gunawan et al., (2013) which showed 57.1% reduction in biomass of *C. reinhardtii* upon treating with 100 mg/L of ZnO NPs on day 10. However, the study results from Franklin et al., (2007) with 72 h EC_{50} 0.068 mg/L and Aruoja et al., (2009) with 72 h EC_{50} 0.042 mg/L show the greater growth inhibitory effect of ZnO NPs on freshwater microalga *P. subcapitata,* compared to the present study results on *S. platensis* and *H. pluvialis* that showed very high values of 72 h EC_{50} of 6.8 and 241.21 mg/L respectively. Likewise, the salt water microalga *D. tertiolecta* exhibited higher sensitivity to ZnO NPs with 96 h EC_{50} 2.42 mg/L (Hou et al., 2018) compared to our study results of 96 h EC_{50} 13.97 and 118.67 mg/L on *S. platensis* and *H. pluvialis,* respectively. Further, Li et al., (2017) demonstrated more inhibitory effect of ZnO NPs towards marine algae *T. suecica and P. tricornutum* with 72 h EC_{10} 0.047and 0.23 mg/L, respectively, whereas our study reported less inhibitory effect with 72 h EC_{10} 1.29 and 6.8 mg/L for *S. platensis* and *H. pluvialis* respectively. In addition, Wong et al., (2010) reported the higher sensitivity of marine microalgae *Skeletonema costatum* and *Thalassiosia pseudonana* to the treatment of zinc oxide nanomaterial with 96 h EC_{50} of 2.36 and 4.56 mg/L of ZnO NPs, respectively.

Numerous previous research studies have reported the cytotoxic effects of heavy metals and metallic nanoparticles on *S. platensis.* Lone et al., (2013) reported the growth inhibitory effects of ZnO NPs on *S. platensis* with 41.0% reduction in

biomass on the day 10 by the treatment of 10 mg/L of ZnO NPs. Whereas, the exposure of nano TiO_2 particles on *S. platensis* caused 74.1% decrease in biomass on day 15 with 100 mg/L of TiO_2 NPs (Comotto et al., 2014). A treatment of selenium (100 mg/L) reported a significant damage in the nutritional quality of *Spirulina* through 75.6, 69.0 and 98.8% loss in polysaccharides, protein and lipid contents of *Spirulina* cells respectively at 72 h. The results further indicated the degradation and lysis of algal biomass by heavy metal treatment (Zinicovscaia et al., 2016). Another treatment of *Spirulina* cells with 1 mg/L of copper caused 50% decrease in algal biomass on the day 7 (Deniz et al., 2011). A study by Shilpi et al., (2014) reported a dose and time dependent increase in the toxicity of chromium (Cr) (0.01 – 10 mg/L) on *S. platensis* with a maximum growth reduction of 62% at 10 mg/L of Cr during 9 days of exposure. Additionally, the same study reported a concentration and time dependent decrease in carbohydrate and protein content of *S. platensis* upon treating with Cr (0.01 - 10 mg/L). A significantly maximum reduction in carbohydrate and protein content were reported with the resultant values of 61.2% and 65.3% respectively upon treating with 10 mg/L of Cr on day 9 compared to control.

Though numerous studies have reported the major cytotoxic effects of heavy metals and nanosized metal particles on *S. platensis*, only a few studeis have reported the toxicity of metal, metallic nanoparticles and environmental stress on *H. pluvialis*. Exposure of vegetative cells of *H. pluvialis* to different concentrations

of NaCl salt (0.25 to 2 % w/v) for 4 days caused 56.56% loss in cell viability and also resulted in subsequent steep fall of biomass along with the microscopic evidence of cell lysis at the highest concentration of NaCl (Sarada et al., 2002). Li et al., (2008) reported 8.43, 13.08 and 19.72% loss in cell viability of *H. pluvialis* at 48 h under various stress conditions when treated with ferrous sulphate (FS), ferrous sulphate + high intensity light (HL) (200 µmol m^{-2} s^{-1}) and FS+ HL+ sodium acetate respectively. While, the exposure of cyst cells of *H. pluvialis* from the stationary phase to 0.8% NaCl for 11 days resulted in 52.5% decrease in viable cells by salt stress (Cifuentes et al., 2003). A recent investigation by Comotto et al., (2014) reported 18.1% decrease in biomass of *H. pluvialis* on day 9 with the treatment of 100 mg/L of TiO_2 NPs.

Carotenoids and chlorophylls are the essential constituents of the photosynthetic apparatus present in the thylakoid membranes of chloroplast and taking apart in the light harvesting process of photosynthesis (Miazek et al., 2015). Phycocyanin is a blue coloured pigment present in the phycobilisomes attached to the external surface of the thylakoid membranes found in cyanobacteria. It is a photosynthetic an accessory pigment that participates in the energy transfer chain of photosynthesis (Moraes et al., 2011; Horváth et al., 2013). Phycocyanin contributes 30 % of the biomass, however its actual level varies according to the culture conditions (Leema et al., 2010). Similarly, astaxanthin is a secondary carotenoid present in *H. pluvialis* synthesized from the primary carotenoid β carotene by β carotene carboxylase.

Under unfavorable severe stressful conditions, astaxanthin synthesis will be reduced due to the inhibition of β carotene carboxylase enzyme resulting in reduction of astaxanthin content (Huang et al., 2006; Vidhyavathi et al., 2008; Vidhyavathi et al., 2009).

The current investigation demonstrated a dose and time dependent reduction in photosynthetic pigments of microalgae *H. pluvialis* and *S. platensis* corresponding to loss in algal cell viability and reduction in algal biomass as the results of treatment with nanoparticles. Toxicity of ZnO NPs caused a higher fall in photosynthetic pigments of *S. platensis* compared to *H. pluvialis* with the highest percentage reduction of 92.5, 76.2 and 74.1% for chl-a, carotenoids and phycocyanin respectively at 96 h with 200 mg/L of ZnO NPs in *S. platensis* (Fig. 4.13, 4.15 & 4.17). Whereas, *H. pluvialis* showed comparatively lower fall in chl-a, total carotenoids and astaxanthin with the reported value of 63.3, 43.4 and 47.9% respectively at 96 h for the same concentration of NPs (Fig. 4.14, 4.16 & 4.18). The loss in photosynthetic pigments of the present study exhibited a same trend with cell death and reduction in algal biomass in both microalgae with greater loss in photosynthetic pigments in *S. platensis* over *H. pluvialis* upon the treatment with ZnO NPs. Though this study demonstrated a typical dose and time-dependent loss in photosynthetic pigments of both microalgae. A statistically significant ($p < 0.05\%$) decrease in phycocyanin pigment was established from 24 h even with the treatment of 10 mg/L of ZnO NPs. The current results on the loss in photosynthetic

pigments of *S. platensis* are similar to the findings of Lone et al., (2013) which showed that the treatment of ZnO NPs resulted in 93.5% loss of chlorophyll a and 50% decrease in carotenoids of *S. platensis* on day 10 and Leema et al., (2010) which reported that the salt stress on *S. platensis* from the treatment with sea water resulted in 53.1% fall of chlorophyll a, 37.5% decrease in carotenoids and 32.1% loss in phycocyanin during day 25. Shilpi et al., (2014) observed a dose and time dependent fall in chl-a of *S. platensis* with a significantly maximum reduction of 63.9% at 10 mg/L of Cr on day 9. Recently, a treatment with selenium (100 mg/L) resulted in 90% reduction in phycocyanin pigment of *S. platensis* on day 3 (Zinicovscaia et al., 2016). However, the study reported a minimal rise in the phycocyanin pigment of *S. platensis* at the initial duration (6 - 12 h) of exposure to nanoscale zinc oxide particles, possibly because of self-defense activity of *S. platensis* against the oxidative stress induced by the treated nanoscale particles Miazek et al. (2015). Similar to the present study finding, Miazek et al. (2015) evidenced an increase in pigment content of cyanobacteria at low concentrations of lead (Pb). The exposure of *S. platensis* culture heavy metal tellurium resulted in the enhanced production of phycocyanin due to the oxidative stress induced by the metal uptake (Arunakumara et al., 2008)

Parallel to the loss in photosynthetic pigments of *S. platensis*, *H. pluvialis* also showed a same trend on the loss of photosynthetic pigments in the present study. However, the treatment of ZnO NPs caused comparatively less reduction in the

photosynthetic pigments of *H. pluvialis* compared to *S. platensis*. Loss in pigments of *H. pluvialis* under various stress conditions have been reported by previous studies. Li et al., (2008) demonstrated loss in photosynthetic activity of *H. pluvialis* when exposed to ferrous sulphate and high intensity light for 48 h. Exposure of the cyst cells of *H. pluvialis* from the stationary phase to 0.8% NaCl for 11 days resulted in 25.9, 5.27 and 19.0% loss in chlorophyll, carotenoids and astaxanthin respectively (Cifuentes et al., 2003). While the exposure of vegetative cells of *H. pluvialis* to the increasing concentrations of NaCl (0.25 to 2 % w/v) for 4 days, showed increase in astaxanthin and carotenoids at the concentration of NaCl < 1.0 mg/L, but both the pigments reported to be decreased at 1.0% and 2.0 % of NaCl on day 6 and day 9 respectively. The same study reported 90% and 56.68% drop in chlorophyll and astaxanthin with 54.68 % decrease in total carotenoids of *H. pluvialis* when exposed to 17.1 mM of NaCl on day 9 (Vidhyavathi et al., 2008). Similarly, the exposure of *H. pluvialis* to high intensity of light (97 µmol $m^{-2}s^{-1}$) for 48 h resulted in 14. 9% and 8.45% decrease in chlorophyll and total carotenoids. The action of electron transport chain inhibitor diphenylamine (DPA), the herbicide norflurazon (NF) and the antifungal agent cerulenin on the photosynthetic pigments *H. pluvialis* for 48 h resulted in 23.16, 41.34 and 20.8% decrease in total carotenoids, and 17.34, 22.36 and 27.04% decrease in total chlorophylls respectively (Vidhyavathi et al., 2009).

Various studies have described the sensitivity of microalgal pigments towards the toxicity of metal and metallic nanoparticles. Similar to these findings, Sadiq et al., (2011) showed a dose and time dependent loss in chlorophyll of microalgae *Chlorella* sp. and *Scenedesmus* sp. upon treating with alumina nanoparticles for 72 h. In addition, Oukarroum et al., (2012a) demonstrated concentration dependent reduction in chlorophyll of *C. vulgaris* and *D. tertiolecta* when treated with Ag NPs at 24 h. Both the studies showed the lower sensitivity of *Chlorella sp.* compared to *Scenedesmus* sp. and *D. tertiolecta* towards metallic NPs. Nevertheless, Sadiq et al., (2011), Xiaoxiao et al., (2012) and Barhoumi and Dewez (2013) have reported the strongest toxic effects of metallic oxide NPs on the electron transport chain of photosynthesis in *C. vulgaris* that resulted in dose dependent reduction in the photosynthetic pigment. Arunakumara et al., (2009) demonstrated the destruction of thylakoids that are responsible for photosynthetic activities in cyanobacterium *Synechocystis* sp. due to heavy metal treatment and also reported a dose dependent decrease in photosynthetic pigments for cadmium treatment (6 mg/L Cd^{+} at 96 h) with 57.83% fall in chl-a, 48.94% decrease in carotenoids and 56.90% decrease in phycocyanin contents.

The treatment of ZnO NPs results in the aggregation of NPs agglomerates on the surface of algal cells that prevents the algal cells from obtaining the adequate light energy needed for the photosynthetic process which in turn leads to decrease in photosynthetic pigments and growth inhibition (Iswarya et al., 2015). The decline

in the photosynthetic pigments can also result from the onset of oxidative stress which includes the excessive production of reactive oxygen species and the subsequent lipid peroxidation, both destroy the activity of photosynthetic system and causing growth inhibition (Tang et al., 2013). Moreover, unfavorable growth conditions to microalgae such as the stress caused by salt, high light intensity, chemicals, metals and metal nanoparticles result in decrease in chlorophyll content of microalgae which is necessary for photosynthesis could be the reason for growth reduction (Shilpi et al., 2014). Treatment of metals to microalgae results in the destruction of thylakoids that causes reduction in photosynthetic pigments which in turn severely affects the photosynthetic activity and causes growth inhibition or cell death (Arunakumara et al., 2008).

In the present study, the light microscopic and SEM pictures of *S. platensis* cells exposed to ZnO NPs (Fig. 4.19 & 4.20) showed aggregation of NP agglomerates on the algal cells, aggregation of algal cells, cell membrane damage, rupture of *Spirulina* trichrome and cell distortion. Likewise, the exposure of ZnO NPs to freshwater microalga *C. vulgaris* caused morphological alterations and cell membrane and structure damage, which lead to increased levels of lactate dehydrogenase enzyme (Suman et al., 2015) and showed entrapment of NP agglomerates on algal cells, cell aggregation , cell wall breakage and cell membrane damage on *S. obliquus* (Bhuvaneshwari et al., 2015). Previous studies by Ji et al., (2011) and Chen et al., (2012) observed the same effect of ZnO NPs on

Chlorella sp. Xia et al., (2015) and Schiavo et al., (2016) demonstrated the complete wrapping of algal cells with NPs, cell membrane damage and intracellular ROS production in the marine microalga *P. tricornutum* when exposed with ZnO NPs.

On the other hand, light microscopic pictures of *H. pluvialis* cells exposed to ZnO NPs revealed loss in cell motility and presence of ghost cells (Fig. 4.21), in addition to, adsorption of NP agglomerates on algal cells, cell aggregation, alteration in cell structure with wrinkled surface, cell membrane rupture, cell distortion and cell rupture shown by scanning electron microscopic images (Fig. 4.22). In accordance with our findings, Shah et al., (2016) demonstrated the existence of motile flagellated vegetative cells of *H. pluvialis* under favorable growth conditions and non- motile resting vegetative cells with loosing flagellates and expanded cell size under unfavorable environmental or culture conditions such as lack of nutrients, salt stress and high light illumination. Comotto et al., (2014) observed cell aggregation and cell wall degradation in *C. vulgaris, H. pluvialis and S. platensis* upon treating with TiO_2 NPs, and Dong et al., (2014) showed strongly wrinkled and damaged cell wall in hydrochloric acid- acetone (HCl-ACE) treated *H. pluvialis* cells. Harker et al., (1996) reported the fully bleached ghost cells of *H. pluvialis* containing no chlorophyll or carotenoid when exposed to high salt and high light intensity. The number of ghost cells was reported to be increased considerably when the algal cells exposed to increasingly adverse environmental conditions.

Such bleaching of cells is thought to happen due to metabolic imbalance caused by the environmental stress and also may be due to the cytoplasmic leakage of cells through cell membrane rupture. Apart from algal cells, earlier studies have reported the morphological alterations in animal and human cells when exposed to ZnO NPs. Babu et al., (2017) observed the membrane damage and structural deformation in chicken red blood cells and Guan et al., (2012) demonstrated abnormal size, cellular shrinkage and irregular shape of human hepatocytes and human embryonic kidney cells.

The aggregation and adsorption of NPs on the surface of algal cells happens due to large surface area of the NPs and also because of the electrostatic attraction of positively charged ZnO NPs with negatively charged functional groups present in the cell wall of algal cells. The accumulation of nanosized particles on the algal cell surface alters cell membrane permeability and causes cell wall damage, this enables excessive entry of NPs into the cells and brings disturbance in the vital functions of internal organelles which subsequently leads to cell death (Kumar et al., 2011; Bhuvaneshwari et al., 2015). Similarly, the algal growth inhibition results from the penetration of NPs into the cell envelope and the following disruption in cell membrane causing cell rupture and leakage of intracellular contents (Chen et al., 2012). The surface binding and the consequent accumulation of nanosized particles on the algal cell surface compromises the cell membrane integrity and cell morphology and subsequently leads to cell death due to the mechanical damage

(Djearamane et al., 2018) or by the intracellular dissociation of metal ions from the internalized NPs that disturbs the cellular metabolism (Lin and Xing, 2008)). Further, entrapment of large aggregates of nanoparticles on algal cells reduces the light availability to algal cells (Hazeem et al., 2016) and the adsorption of nutrients by nanoparticles impairs the availability of nutrients to algal cells which eventually resulting in growth inhibition (Tang et al., 2013).

In addition, the transmission electron microscopic images of *S. platensis* cells treated with ZnO NPs in the present study evidenced the critical actions of NPs on photosynthetic system of algal cells through the destruction of thylakoid lamellae and also by the reduction and degradation of the photosynthetic pigment phycobilisomes (Fig. 4.23B & D). Further, the TEM examination revealed damage to the cell membrane and degradation of intracellular organelles of S*pirulina* cells exposed to ZnO NPs (Fig. 4.23B&D). Similar to these findings, the results of Vladimirescu, (2010) and Noyma et al., (2015) showed damage to thylakoids and cell memebrane of *S. platensis* exposed to lysozyme enzymes and the cyanobacterium *Cylindrospermopsis raciborskii* subjected to ultraviolet radiation respectively. Similar to the ultrastructural damages of *S. pluvialis* by ZnO NPs, TEM micrographs of ZnO NPs treated *H. pluvialis* showed complete loss of thick cell wall, plasmolysis, destruction of photosynthetic apparatus, degradation and leakage of cytoplasmic contents (Fig. 4.24 B&D). In accordance with the

observation done for this study, the exposure of microalga *S. obliquus* to ZnO NPs resulted in irregular structure, deformed cell membrane, degraded cellular organelles and plasmolysis of cells (Bhuvaneshwari et al., 2015), while the exposure to TiO_2 resulted in uneven cell membrane, shrunken and detached cytoplasm and distorted cellular organelles including chloroplast (Dalai et al., 2013). Kasemets et al., (2009) demonstrated irregularly shaped cells such as shrunken and deformed cells with crushed cell wall and cytoplasmic leakage in yeast *S. cerevisiae* upon treatment with ZnO NPs.

Earlier studies have reported the ultrastructural damage of algal cells caused by heavy metals. Accumulation of cadmium in green alga C*hlamydamonas* resulted in enlargement of cell and decomposition of phosphate bodies (Nishikawa et al., 2003) and presence of lead in *Chlorella sorokimana* induced cytoplasmic lipid droplets and disorganized chloroplasts (Carfagna et al., 2013). Exposure of thallium to *Synchocystis* sp. caused fragmentation of thylakoid membranes (Aoki et al., 2013) and exposure to zinc resulted in enlarged and bloated mitochondria in green alga *Desmidium swartzu* (Andosch et al., 2015). The combined effects of zinc and aluminum on *D. tertiolecta* resulted in cell membrane rupture (Saçan et al., 2007) and the exposure of cyanobactrium *Synechocystis* sp. cells to the heavy metals lead and cadmium was reported to worstly affected the photosynthetic apparatus with deteriorated or completely damaged thylakoid memebrane leaving large empty spaces in the cell interior (Arunakumara et al., 2008). Besides the cell membrane

damage and cytoplasmic leakage, the critical damage to the thylakoids and the photosynthetic pigments are thought to be the key adverse effects of metal ions on algal cells, that results in the failure of photosynthetic process which ultimately leads to cell death (Mohanty, 1989: Deniz et al., 2011).

The widely accepted fact is that the treatment of metals and metallic nanoscale particles induces the oxidative stress in living cells by forming electron reactive oxygen molecules and hydrogen peroxide which are collectively known as reactive oxygen species (ROS) that are responsible for cell death (Lin and Xing, 2008). Exposure to metal ions can also enhance the production of ROS through inhibiting the oxidative stress protective compound metallothione and thus blocking the binding of free metal ions to metallothione, thus free zinc ions catalyze Fenton - type reactions to increase the production of both hydrogen peroxides and hydroxyl ions (Schiavo et al., 2016; Hou et al., 2018).

The exposure of algal cells to ZnO NPs in the present study exhibited a typical time and concentration dependent increase in ROS production on *S. platensis* and *H. pluvialis*. Both microalgae showed significant production of ROS from 6 h even at the lowest concentration of 10 mg/L of ZnO NPs. In accordance with the growth inhibition results, the treatment of ZnO NPs induced greater production of ROS in *S. platensis* compared to *H. pluvialis.* For an example, the level of ROS production

from *S. platensis* was reported to be from 944.7 to 2597.4 % (Fig. 4.25), compared to the ROS production from 250.8 to 540.5% (Fig. 4.26) in *H. pluvialis* at 96 h for ZnO NPs concentration from 10 to 200 mg/L, respectively. This finding demonstrated the higher level of oxidative stress induction in *S. platensis* through the toxic effects of ZnO NPs over *H. pluvialis.* Further, the intracellular production of reactive oxygen species was demonstrated by the green fluorescence of dichlorofluorescein diacetate in *S. platensis* and *H. pluvialis* cells treated with ZnO NPs (Fig. 4.29). In vivo production of ROS was demonstrated in both the microalgae exposed to ZnO NPs through the emission green fluorescence. Similar findings were documented by Bhuvaneshwari et al., (2015) who reported a concentration dependent increase in ROS production in freshwater alga *S. obliquus* with the reported values of 99.5, 113.8 and 125.5% for 1, 0.5, 0.25 mg/L of ZnO NPs at 72 h. In addition, authors demonstrated the intracellular production of ROS by DCF fluorescence from the algal cells treated with nanoscale zinc oxide particles. Also, Dalai et al., (2013) reported a concentration and time dependent rise in ROS production from *S. obliquus* with the resultant values of 5.21 and 15.24 % at 24 h, and 7.46 and 20.39 % at 72 h upon treating with 0.5 and 1 mg/L of nanosized TiO_2 particles respectively. Another study by the same authors reported the induction of dose dependent ROS production by 147.43, 161.24 and 190.2% respectively during the treatment with 0.05, 0.5 and 1 mg/L of mixed TiO_2 and Al_2O_3 nanoparticles on the same microalga at 72 h (Dalai et al., 2014). A study by Oukarroum et al., (2012a) demonstrated a does dependent enhanced ROS formation

in *D. tertiolecta* over *C. vulgaris* upon 24 h treatment with 1 - 10 mg/L of Ag NPs. Besides microalgae, Zhang et al., (2016b) and Dalai et al., (2012) showed the intracellular production of ROS in yeast *S. cerevisiae* with ZnO NPs (2 mg/L) at 48 h and in freshwater bacteria with 2 h treatment of TiO_2 NPs (1 mg/L) respectively.

Various studies demonstrated the induction of ROS through the adverse effects of ZnO NPs in animal and human cells. Babu et al., (2017) demonstrated a dose dependent increase in ROS production in chicken red blood cells with the treatment of ZnO NPs (50 mg/L) at 24 h. Similarly, exposure to cerium oxide NPs resulted in a dose and time dependent rise in ROS production in human lung alveolar epithelium cell line with the reported ROS production from 171 – 286 % for 25 – 100 mg/L of cerium oxide NPs at 6 h (Mittal and Pandey, 2014). The excessive production of reactive oxygen species through the initiation of oxidative stress targets and damages the biomolecules of algal cells resulting in irreversible cell damage. The failure in restoration of ROS production will eventually cause oxidative stress and cell death (Wong et al., 2010). Alternatively, the production of huge amount of ROS causes cell membrane damage which spontaneously results in the intracellular accumulation of NPs and the corresponding cell death (Boussiba, 2000; Brayner et al., 2006).

Metals ions are proven to induce oxidative stress through the excessive production and accumulation of hydrogen peroxide and the resulting lipid peroxidation (Choudhary et al., 2007). ROS reacts on cell membrane and oxidizes the membrane proteins, cholesterol and more predominantly the poly unsaturated fatty acids through a process called lipid peroxidation (Dingjan et al., 2016). Lipid peroxidation spontaneously results in damaging the cell membrane that enables the entry of excessive NPs into the cells and leading to disturbance in the vital functions of cellular organelles (De Jesus and Kapila, 2014; Gallo et al., 2016). As a consequence, disintegration of cell membrane and the leakage of intracellular contents results in death of algal cells (Valko et al., 2006; Dingjan et al., 2016).

Malondialdehyde (MDA) is a well-known biomarker of lipid peroxidation (LPO) and thus the quantification of MDA can be used to evaluate the status of lipid peroxidation (Melegari et al., 2012). The previous investigations by Choudhary et al., (2007) and Deniz et al., (2011) measured MDA (Malondialdehyde) level as a measure of the degree of lipid peroxidation and showed that the level of MDA increased with the increasing concentration of trace metals including copper and zinc on *Spirulina* cells. In addition, the authors also conveyed that the lipid peroxidation is linked to the concentration dependent free radical formation in *Spirulina* sp. A study Li et al., (2008) demonstrated a linear relationship between the loss in photosynthetic activity and increase in MDA level in *Spirulina* cells when exposed to intense light (250 µmol m^{-2} s^{-1}) indicating the photooxidative

stress of photosynthetic apparatus (thylakoid membranes), that in turn resulted in reduction and or impairment of photosynthesis.

The present study results demonstrated a dose and time dependent increase in lipid peroxidation level with a significant ($p < 0.05\%$) increase in lipid peroxidation from 12 h in *S. platensis* and from 24 h in *H. pluvialis* for all the tested concentrations of ZnO NPs from 10 - 200 mg/L. In agreement with increase in ROS production, *S. platensis* exhibited a greater level of lipid peroxidation compared to *H. pluvialis*, for an instance, the level of lipid peroxidation in *S. platensis* ranged from 147.7% to 506.8% (Fig. 4.27), whereas from 109.5%, to 323.9% in *H. pluvialis* (Fig. 4.28) at 96 h for 10 - 200 mg/L of ZnO NPs. Similarly, Oukarruom et al. (2012a) demonstrated a dose and time dependent increase in ROS and lipid peroxidation, with higher level of ROS and lipid peroxidation in *C. vulgaris* compared to the cell wall lacking microalga *D. tertiolecta* when treated with Ag NPs. The authors also reported a positive relationship between ROS production and lipid peroxidation. This finding is in accordance with the findings that the thick cell wall containing *H. pluvialis* exhibited lower ROS production and lipid peroxidation compared to smooth cell wall containing *S. platensis*. Besides microalgae, toxicity of ZnO NPs has been reported to cause oxidative stress through a dose dependent increase in MDA formation as a result of lipid peroxidation on human hepatocytes and human embryonic kidney cells treated with 50, 75 and 100 mg/L of nanoscale ZnO particles at 24 h (Guan et al., 2012). Apart from the measurement of ROS and LPO

to assess the oxidative stress, Comotto et al., (2014) used quantification of phenolic compounds to assess the oxidative stress caused by the treatment of TiO_2 on *S. platensis* and *H. pluvialis*. The phenolic compounds are the group of antioxidants that are produced to scavenger the oxidative stress. Exposure of pure anatase TiO_2 to both microalgae resulted in the increased release of extracellular phenolic compounds to scavenger the free radicals produced by oxidative stress. Additionally, Choudhary et al., (2007) reported a dose dependent increase in anti-oxidant enzyme superoxide dismutase level as the indicator of oxidative stress induced by heavy metals such as lead, zinc and copper in *S. platensis*.

5.3 Comparison of Toxic Effects of ZnO NPs Between *S. platensis* and *H. pluvialis*

The treatment ZnO NPs caused larger aggregates of *S. platensis* (Fig. 4.7) while these aggregates were less in *H. pluvialis* (Fig. 4.8). This difference in the formation of aggregates may due to the higher zinc absorptive and accumulative capacity of *Spirulina* cells (Zinicovscaia et al., 2013). The formation of aggregates of algal cells upon treating with SiO_2 and alumina NPs was reported by the previous studies by Van Hoecke et al., (2008) and Sadiq et al., (2011). Formation of cell aggregates might inhibit algal growth (Navarro et al., 2008, Wei et al., 2010, Perreault et al., 2012). It is believed that algal cell aggregation involved in the toxicological effects of metallic NPs through the strong reduction in chlorophyll

content and viable cells (Oukarroum et al., 2012a). Formation of larger cell aggregates of *S. platensis* upon the treatment with ZnO NPs may have resulted in higher growth inhibition and the corresponding higher reduction in photosynthetic pigments in *S. platensis* compared to *H. pluvialis*. Further, motile nature of *H. pluvialis* is belied to be the fact behind the lower toxicity of ZnO NPs on *H. pluvialis*. It is likely due to the slight interaction of ZnO agglomerates with motile algal cell, while this interaction could easily happen with non-motile *S. platensis*. Similar findings were reported by Li et al., (2017) who reported higher sensitivity of ZnO NPs to non-motile microalga *P. tricornutum* (96 h EC_{10} 0.23 mg/L) compared to motile microalga *T. suecica* (96h EC_{10} 0.47). Motility renders reduced contact time with ZnO NPs agglomerates and consequently less entrapment of algal cells with NP agglomerates. At the same time, complete entrapment of non- motile algal cells with ZnO NPs renders less light availability and results in higher growth inhibition (Aruoja et al., 2009; Gong et al., 2011). In addition, since the interaction of NPs happens on the cell surface, the greater surface area to volume ratio of *S. platensis* against *H. pluvialis* are also a very important factor to be considered for higher toxicity of ZnO NPs on *S. platensis* (Moraes et al., 2013). Involvement of higher surface area to volume ratio of microalgae in exerting higher toxic effects of ZnO NPs has been previously reported by Li et al., (2017).

Oxidative stress due to high levels of ROS production can lead to lipid peroxidation and cell damage. Lipid peroxidation can result in impaired cellular function and

alterations in cell membrane permeabilities that in turn disrupt vital functions of cell (Reiter et al., 1999; Halliwell and Gutteridge, 2015). This study demonstrated higher accumulation of ZnO NPs and the subsequent stronger production of ROS and LPO in *S. platensis* that high levels of oxidative stress might have led to enhanced growth inhibition on *S. platensis* compared to *H. pluvialis*. Similar findings were reported by Oukarroum et al., (2012a) who reported higher reduction in cell viability and chlorophyll in *D. tertiolecta* which showed greater production of ROS and LPO compared to *C. vulgaris*. The same study opined that the higher toxicity of ZnO NPs was may be due to lack of cell wall in *D. tertiolecta*. Accordingly, this study reported lower toxicity on *H. pluvialis* which has thick cell wall covered with more two layers of smooth wall compared to a smooth cell wall of *S. pluvialis*. The comparative analysis of toxicity parameters of the present study revealed the substantially greater sensitive of ZnO NPs on prokaryotic marine microalga *S. platensis* (96 h EC_{50} 13.97 mg/L) compared to eukaryotic freshwater microalga *H. pluvialis* (96h EC_{50} 186.67 mg/L) (Table 4.1). Study results demonstrated that *S. platensis* requires nearly 13 times lesser concentration of ZnO NPs to kill 50% viable cells when compared to *H. pluvialis*. Profound metal accumulative capacity, large surface area and the non-motile nature of *S. platensis* are believed to be the reasons behind the higher accumulation of ZnO NPs and the corresponding greater oxidative stress induction and the cell death of *S. platensis* compared to *H. Pluvialis*.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In a nutshell, the treatment of zinc oxide nanoparticles on *S. platensis* and *H. pluvialis* resulted in a characteristic dose and time dependent accumulation of nano-scale zinc oxide particles in the algal cells and the corresponding cytotoxicity through loss in cell viability, algal biomass and photosynthetic pigments with increase in ROS and lipid peroxidation levels. The induction of oxidative stress due to the treatment of ZnO NPs on algal cells resulted in the loss of viable cells and subsequently caused growth inhibition of algal cells through reduction in photosynthetic pigments. Further, the study results revealed nearly three times greater accumulative capacity of *S. platensis* and about two folds' higher cytotoxicity and oxidative stress effects to ZnO NPs compared to *H. pluvialis*. 96 h EC_{50} values reported in our study show almost 13 times greater sensitivity of *S. platensis* over *H. pluvialis* towards ZnO NPs toxicity effects. Because of the profound higher sensitivity of *S. platensis,* this study recommends the microalga or cyanobacterium *S. platensis* to function as a bioindicator for the ecotoxicological assessment of zinc oxide nanoparticles in the aquatic environment including

marine ecosystem since *S. platensis* grows both in fresh and salt water. Furthermore, results from this study may bring insights into suggesting the methods for screening ZnO NPs contamination in the nutrient microalgae.

6.2 Limitation and Recommendation

This study was unable to confirm the internalization of ZnO NPs in the ultra-thin sections of microalgae through TEM analysis. This is because of unavailability of EDX facility in TEM technique used in this study. Since our study reported the cellular accumulation of ZnO NPs on algal cells, future study is required to distinguish the adsorption and the cellular uptake of ZnO NPs in algal cells. Additionally, the toxic mechanisms behind the different sensitivity of our test microalgae to ZnO NPs needs to be studied further.

Since both of the study on microalgae showed substantial cellular adsorption and accumulation of ZnO NPs in the present study. The adsorption and accumulation of metallic NPs will ultimately increase the cellular weight of algae and lead to sedimentation of algal cells, this will help to remove pollutants from the medium (Hazeem et al., 2016). This technique of bioremediation of NPs by using our test microalgae needs to be studied in detail with different concentrations of ZnO NPs and with different densities of algal biomass to optimize the parameters that could

effectively remove NPs from the polluted water bodies. Further studies can be conducted with our test microalgae to investigate the bio-remedial capacity on other most commonly used metallic nanoparticles such as silver and titanium di oxide nanoparticles.

REFERENCES

ABDULQADER, G., BARSANTI, L. & TREDICI, M. R., 2000. Harvest of *Arthrospira platensis* from Lake Kossorom (Chad) and its household usage among the Kanembu. *Journal of applied phycology,* 12**,** pp. 493-498.

AITKEN, R., et al., 2009. EMERGNANO: A review of completed and near completed environment, health and safety research on nanomaterials and nanotechnology. *DEFRA Report CB0409* (downloaded on 15.01.2018 fromhttp://www.iom-support.co.uk/Portals/3/SNContent/Documents/ EMERGNANO CB0409 Full.pdf).

AL-DHABI, N. A. 2013. Heavy metal analysis in commercial *Spirulina* products for human consumption. *Saudi journal of biological sciences,* 20**,** pp. 383-388.

ANDERSSON-WILLMAN, B., et al., 2012. Effects of subtoxic concentrations of TiO 2 and ZnO nanoparticles on human lymphocytes, dendritic cells and exosome production. *Toxicology and applied pharmacology,* 264**,** pp.94-103.

ANDOSCH, A., HÖFTBERGER, M., LÜTZ, C. & LÜTZ-MEINDL, U., 2015. Subcellular sequestration and impact of heavy metals on the ultrastructure and physiology of the multicellular freshwater alga *Desmidium swartzii*. *International journal of molecular sciences,* 16**,** pp. 10389-10410.

AOKI M., et al., 2013. Thallium Induces Morphological Changes in the Photosynthetic Apparatus of *Synechocystis* sp. PCC6803. In: Photosynthesis Research for Food, Fuel and the Future. *Advanced Topics in Science and Technology in China*. Berlin Heidelberg: Springer, pp. 586-589.

APPLEROT, G., et al., 2009. Enhanced antibacterial activity of nanocrystalline ZnO due to increased ROS-mediated cell injury. *Advanced Functional Materials,* 19**,** pp 842-852.

ARUNAKUMARA, K. & ZHANG, X., 2009. Effects of heavy metals (Pb 2+ and Cd 2+) on the ultrastructure, growth and pigment contents of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. *Chinese Journal of Oceanology and Limnology,* 27**,** pp. 383-388.

ARUNAKUMARA, K., ZHANG, X. & SONG, X., 2008. Bioaccumulation of Pb 2+ and its effects on growth, morphology and pigment contents of *Spirulina (Arthrospira) platensis*. *Journal of Ocean University of China,* 7**,** pp. 397-403.

ARUOJA, V., DUBOURGUIER, H.-C., KASEMETS, K. & KAHRU, A., 2009. Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. *Science of The Total Environment,* 407**,** pp. 1461-1468.

ASHARANI, P., WU, Y. L., GONG, Z. & VALIYAVEETTIL, S., 2008. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology,* 19**,** pp. 1-8.

ATES, M., DANIELS, J., ARSLAN, Z., FARAH, I. O. & RIVERA, H. F., 2013. Comparative evaluation of impact of Zn and ZnO nanoparticles on brine shrimp (*Artemia salina*) larvae: effects of particle size and solubility on toxicity. *Environmental Science: Processes & Impacts,* 15, pp. 225-233.

AUFFAN, M. at al., 2011. Ecotoxicology: Nanoparticle reactivity and living organisms. *Nanoethics and nanotoxicology,* Berlin Heidelberg: Springer, pp. 325-357.

BABU, E. P. et al., 2017. Size Dependent Uptake and Hemolytic Effect of Zinc Oxide Nanoparticles on Erythrocytes and Biomedical Potential of ZnO-Ferulic acid Conjugates. *Scientific reports,* 7, p. 4203.

BACCHETTA, R., MARAN, B., MARELLI, M., SANTO, N. & TREMOLADA, P., 2016. Role of soluble zinc in ZnO nanoparticle cytotoxicity in *Daphnia magna*: A morphological approach. *Environmental research,* 148**,** pp. 376-385.

BAEK, Y.-W. & AN, Y.-J., 2011. Microbial toxicity of metal oxide nanoparticles (CuO, NiO, ZnO, and Sb_2O_3) to *Escherichia coli, Bacillus subtilis,* and *Streptococcus aureu*s. *Science of the total environment,* 409**,** pp. 1603-1608.

BAI, W. et al., 2010. Toxicity of zinc oxide nanoparticles to zebrafish embryo: a physicochemical study of toxicity mechanism. *Journal of Nanoparticle Research,* 12**,** pp. 1645-1654.

BAKER, T. J., TYLER, C. R. & GALLOWAY, T. S., 2014. Impacts of metal and metal oxide nanoparticles on marine organisms. *Environmental Pollution,* 186**,** pp. 257-271.

BARHOUMI, L. & DEWEZ, D. 2013., Toxicity of superparamagnetic iron oxide nanoparticles on green alga *Chlorella vulgaris*. *BioMed Research International,* p. 647974.

BATTIN, T. J., KAMMER, F. V., WEILHARTNER, A., OTTOFUELLING, S. & HOFMANN, T., 2009. Nanostructured TiO_2: transport behavior and effects on aquatic microbial communities under environmental conditions. *Environmental science & technology,* 43**,** pp. 8098-8104.

BAUN, A., HARTMANN, N. B., GRIEGER, K. & KUSK, K. O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotoxicology,* 17**,** pp. 387-395.

BELAY, A., OTA, Y., MIYAKAWA, K. & SHIMAMATSU, H. 1993. Current knowledge on potential health benefits of *Spirulina*. *Journal of applied phycology,* 5**,** pp**.** 235-241.

BHUVANESHWARI, M. et al., 2015. Cytotoxicity of ZnO NPs towards fresh water algae *Scenedesmus obliquus* at low exposure concentrations in UV-C, visible and dark conditions. *Aquatic toxicology,* 162**,** pp. 29-38.

BIANCHINI, A., GROSELL, M., GREGORY, S. M. & WOOD, C. M., 2002. Acute silver toxicity in aquatic animals is a function of sodium uptake rate. *Environmental science & technology,* 36**,** pp**.** 1763-1766.

BLABY-HAAS, C. E. & MERCHANT, S. S., 2012. The ins and outs of algal metal transport. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1823**,** pp. 1531-1552.

BLASER,S.A., 2006. *Environmental Risk Analysis for Silver-Containing Nanofunctionalized Products.* Master Thesis, Swiss Federal Institute of Technology, Zürich.

BLINOVA, I., IVASK, A., HEINLAAN, M., MORTIMER, M. & KAHRU, A., 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environmental Pollution,* 158**,** pp. 41-47.

BONDARENKO, O. et al., 2013. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. *Archives of toxicology,* 87**,** pp. 1181-1200.

BORM, P. J. et al., 2006. The potential risks of nanomaterials: a review carried out for ECETOC. *Particle and fibre toxicology,* 3 (11), pp. 1-35.

BOTELHO, M. C. et al., 2014. Effects of titanium dioxide nanoparticles in human gastric epithelial cells in vitro. *Biomedicine & Pharmacotherapy,* 68**,** pp. 59-64.

BOUSSIBA, S., 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. *Physiologia Plantarum,* 108(2)**,** pp. 111-117.

BOXALL, A. B. et al., 2007. *Current and future predicted environmental exposure to engineered nanoparticles*. Report by the Central Science Laboratory (CSL) York for the Department of the Environment and Rural Affairs (DEFRA), UK.

BRAYNER, R. et al., 2006. Toxicological Impact Studies Based on *Escherichia coli* Bacteria in Ultrafine ZnO Nanoparticles Colloidal Medium. *Nano Letters,* 6(4), pp. 866-870.

BRAYNER, R., 2008. The toxicological impact of nanoparticles. *Nano Today,* 3**,** pp. 48-55.

BRAYNER, R. et al., 2010. ZnO nanoparticles: synthesis, characterization, and ecotoxicological studies. *Langmuir,* 26**,** pp. 6522-6528.

BRUNNER, T. J. et al., 2006. In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility. *Environmental science & technology,* 40**,** pp. 4374-4381.

BURCHARDT, L., BALCERKIEWICZ, S., KOKOCINSKI, M., SAMARDAKIEWICZ, S. & ADAMSKI, Z., 2006. Occurrence of *Haematococcus pluvialis* Flotow emend. Wille in a small artificial pool on the university campus of the collegium biologicum in poznan (Poland). *Biodiversity Research and Conservation,* 1**,** pp. 163-166.

CARDOSO et al., 2012. Comparison of *Spirulina platensis* microalgae and commercial activated carbon as adsorbents for the removal of Reactive Red 120 dye from aqueous effluents. *Journal of Hazardous Materials,* 241–242, pp. 146-153.

CARFAGNA, S. et al., 2013. Physiological and morphological responses of Lead or Cadmium exposed *Chlorella sorokiniana* 211-8K (Chlorophyceae). *SpringerPlus,* 2**,** p. 147.

CARLSON, C. et al., 2008. Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. *The Journal of Physical Chemistry B,* 112**,** pp. 13608-13619.

CASALS, E., GONZALEZ, E. & PUNTES, V., 2012. Reactivity of inorganic nanoparticles in biological environments: insights into nanotoxicity mechanisms. *Journal of Physics D: Applied Physics,* 45**,** p. 443001.

CASSIER-CHAUVAT, C. & CHAUVAT, F., 2014. Responses to oxidative and heavy metal stresses in cyanobacteria: recent advances. *International journal of molecular sciences,* 16**,** pp. 871-886.

ÇELEKLI, A. & BOZKURT, H., 2011. Bio-sorption of cadmium and nickel ions using *Spirulina platensis*: Kinetic and equilibrium studies. *Desalination,* 275**,** pp. 141-147.

CELEKLI, A., YAVUZATMACA, M. & BOZKURT, H., 2010. An eco-friendly process: Predictive modelling of copper adsorption from aqueous solution on *Spirulina platensis*. *Journal of Hazardous Materials,* 173**,** pp. 123-129.

CHAN, C. Y. S. & CHIU, J. M. Y., 2015. Chronic Effects of Coated Silver Nanoparticles on Marine Invertebrate Larvae: A Proof of Concept Study. *PloS one,* 10**,** p. e0132457.

CHEKANOV, K. et al., 2014. Accumulation of astaxanthin by a new *Haematococcus pluvialis* strain BM1 from the White Sea coastal rocks (Russia). *Marine drugs,* 12**,** pp. 4504-4520.

CHEN, P., POWELL, B. A., MORTIMER, M. & KE, P. C., 2012. Adaptive interactions between zinc oxide nanoparticles and *Chlorella sp*. *Environmental Science & Technology,* 46**,** pp. 12178-12185.

CHEN, R. et al., 2014. Endoplasmic reticulum stress induced by zinc oxide nanoparticles is an earlier biomarker for nanotoxicological evaluation. *ACS nano,* 8**,** pp. 2562-2574.

CHEN, T.-H., LIN, C.-Y. & TSENG, M.-C., 2011. Behavioral effects of titanium dioxide nanoparticles on larval zebrafish (*Danio rerio*). *Marine pollution bulletin,* 63**,** pp. 303-308.

CHOUDHARY, M., JETLEY, U. K., KHAN, M. A., ZUTSHI, S. & FATMA, T., 2007. Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. *Ecotoxicology and environmental safety,* 66**,** pp. 204-209.

CIFERRI, O. & TIBONI, O., 1985. The biochemistry and industrial potential of *Spirulina*. *Annual Reviews in Microbiology,* 39**,** pp. 503-526.

CIFUENTES, A. S., GONZALEZ, M. A., VARGAS, S., HOENEISEN, M. & GONZALEZ, N., 2003. Optimization of biomass, total carotenoids and astaxanthin production in *Haematococcus pluvialis Flotow* strain Steptoe (Nevada, USA) under laboratory conditions. *Biological Research,* 36**,** pp. 343-357.

COLVIN, V. L., 2003. The potential environmental impact of engineered nanomaterials. *Nature biotechnology,* 21**,** pp. 1166-1170.

COMOTTO, M. et al., 2014. Influence of TiO2 nanoparticles on growth and phenolic compounds production in photosynthetic microorganisms. *The Scientific World Journal,* p. 961437.

CRUZ-MARTÍNEZ, L, C. et al., 2015. Growth and composition of *Arthrospira (Spirulina) platensis* in a tubular photobioreactor using ammonium nitrate as the nitrogen source in a fed-batch process. *Brazilian Journal of Chemical Engineering,* 32 (2) , pp. 347 - 356.

CZYGAN, F., 1970. Blood-rain and blood-snow: nitrogen-deficient cells of *Haematococcus pluvialis* and *Chlamydomonas nivalis*. *Archiv fur Mikrobiologie,* 74**,** pp. 69-76.

DABRUNZ, A. et al., 2011. Biological surface coating and molting inhibition as mechanisms of TiO 2 nanoparticle toxicity in *Daphnia magna*. *PloS one,* 6**,** p. e20112.

DALAI, S., PAKRASHI, S., KUMAR, R. S. S., CHANDRASEKARAN, N. & MUKHERJEE, A. A, 2012. Comparative cytotoxicity study of TiO_2 nanoparticles under light and dark conditions at low exposure concentrations. *Toxicology Research,* 1, pp. 116– 130.

DALAI, S. et al., 2013. Cytotoxicity of TiO_2 nanoparticles and their detoxification in a freshwater system. *Aquatic toxicology,* 138**,** pp. 1-11.

DALAI, S. et al., 2014. Toxic effect of Cr(VI) in presence of n-TiO_2 and n-Al_2O_3 particles towards freshwater microalgae. *Aquatic toxicology,* 146 (2014), pp. 28-37.

DASTJERDI, R. & MONTAZER, M., 2010. A review on the application of inorganic nano-structured materials in the modification of textiles: focus on anti-microbial properties. *Colloids and Surfaces B: Biointerfaces,* 79**,** pp. 5-18.

DAUGHTON, C. G., 2004. Non-regulated water contaminants: emerging research. *Environmental Impact Assessment Review,* 24**,** pp. 711-732.

DAVIES, B. 1976., Carotenoids. *Chemistry and biochemistry of plant pigments***,** London: Academic Press, pp. 149-153.

DAZHI, W., ZHAODI, C., SHAOJING, L. & YAHUI, G., 2003. Toxicity and accumulation of selenite in four microalgae. *Chinese journal of oceanology and limnology*, 21**,** pp. 280-285.

DE JESUS M.B. & KAPILA Y.L., 2014. Cellular Mechanisms in Nanomaterial Internalization, Intracellular Trafficking, and Toxicity. In: Durán N., Guterres S., Alves O. (eds) *Nanotoxicology. Nanomedicine and Nanotoxicology*. New York: Springer, pp. 201-227.

DENG, X. et al., 2009. Nanosized zinc oxide particles induce neural stem cell apoptosis. *Nanotechnology*, 20**,** p. 115101.

DENIZ, F., SAYGIDEGER, S. & KARAMAN, S., 2011. Response to copper and sodium chloride excess in *spirulina* sp.(cyanobacteria). *Bulletin of environmental contamination and toxicology*, 87**,** pp. 11-15.

DEWEZ, D. & OUKARROUM, A., 2012. Silver nanoparticles toxicity effect on photosystem II photochemistry of the green alga *Chlamydomonas reinhardtii* treated in light and dark conditions. *Toxicological & Environmental Chemistry*, 94**,** pp. 1536-1546.

DINGJAN, I. ET AL., 2016. Lipid peroxidation causes endosomal antigen release for cross-presentation. *Scientific Reports*, 6, p. 22064.

DJEARAMANE, S., WONG, L. S., MOOI, L. Y. & LEE, P. F., 2016. A review on bio-distribution and toxicity of sliver, titanium dioxide and zinc nanoparticles in aquatic environment. *Pollution Research*, 35(4), pp. 701-712.

DJEARAMANE, S., WONG, L. S., MOOI, L. Y. & LEE, P. F., 2018. Cytotoxic effects of zinc oxide nanoparticles on cyanobacterium *Spirulina (Arthrospira) platensis*. PeerJ, 6, p. e4682.

DMYTRYK, A., SAEID, A. & CHOJNACKA, K., 2014. Biosorption of microelements by *spirulina*: Towards technology of mineral feed supplements. *The Scientific World Journal*, p. 356328.

DONG, S., HUANG, Y., ZHANG, R., WANG, S. & LIU, Y., 2014. Four different methods comparison for extraction of astaxanthin from green alga *Haematococcus pluvialis*. *The Scientific World Journal*, p. 694305.

DORE, J. E. & CYSEWSKI, G. R., 2003. *Haematococcus algae meal as a source of natural astaxanthin for aquaculture feeds*. Hawaii, USA :Cyanotech Corporation.

DU, W. et al., 2011. TiO 2 and ZnO nanoparticles negatively affect wheat growth and soil enzyme activities in agricultural soil. *Journal of Environmental Monitoring,* 13**,** pp. 822-828.

FANG, L. et al., 2011. Binding characteristics of copper and cadmium by cyanobacterium Spirulina platensis. *Journal of hazardous materials,* 190**,** pp. 810-815.

FEDERICI, G., SHAW, B. J. & HANDY, R. D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquatic toxicology,* 84**,** pp. 415-430.

FERIS, K. et al., 2009. Electrostatic interactions affect nanoparticle-mediated toxicity to gram-negative bacterium *Pseudomonas aeruginosa* PAO1. *Langmuir,* 26**,** pp. 4429-4436.

FERRARI, S. G., SILVA, P. G., GONZÁLEZ, D. M., NAVONI, J. A. & SILVA, H. J., 2013. Arsenic tolerance of cyanobacterial strains with potential use in biotechnology. *Revista Argentina de microbiologia,* 45**,** pp. 174-179.

FISHER, N. S. & WANG, W. X., 1998. Trophic transfer of silver to marine herbivores: A review of recent studies. *Environmental Toxicology and Chemistry,* 17**,** pp. 562-571.

FRANKLIN, N. M. et al., 2007. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl2 to a freshwater microalga (*Pseudokirchneriella subcapitata*): the importance of particle solubility. *Environmental Science & Technology,* 41**,** pp. 8484-8490.

FRENCH, R. A. et al., 2008. Influence of ionic strength, pH, and cation valence on aggregation kinetics of TiO2 nanoparticles. *Geochimica et Cosmochimica Acta Supplement,* 72**,** p.283.

FRÖHLICH, E. & ROBLEGG, E., 2012. Models for oral uptake of nanoparticles in consumer products. *Toxicology,* 291**,** pp. 10-17.

GAISER, B. K. et al., 2011. Effects of silver and cerium dioxide micro-and nano-sized particles on *Daphnia magna. Journal of Environmental Monitoring,* 13**,** pp. 1227-1235.

GALLO, A., BONI, R., BUTTINO, I. & TOSTI, E., 2016. Spermiotoxicity of nickel nanoparticles in the marine invertebrate *Ciona intestinalis* (ascidians). *Nanotoxicology,* 10, pp. 1096-1104.

GERSHWIN, M. E. & BELAY, A., 2007. *Spirulina* in human nutrition and health, 1st ed. Raton: CRC Press.

GÖKSAN, T. & AK, I., 2006. Vegetative growth of the green alga *Haematococcus pluvialis* cultivated on different light-path lengths. *Asian Journal of Plant Sciences,* 5**,** pp. 455-460.

GOLBAMAKI, N. et al., 2015. Genotoxicity of metal oxide nanomaterials: review of recent data and discussion of possible mechanisms. *Nanoscale,* 7**,** pp. 2154-2198.

GONDAL, M. A., ALZAHRANI, A. J., RANDHAWA, M. A. & SIDDIQUI, M. N., 2012. Morphology and antifungal effect of nano-ZnO and nano-Pd-doped nano-ZnO against *Aspergillus* and *Candida*. *Journal of Environmental Science and Health, Part A,* 47**,** pp. 1413-1418.

GONG, N., SHAO, K., FENG, W., LIN, Z., LIANG, C. & SUN, Y., 2011. Biotoxicity of nickel oxide nanoparticles and bio-remediation by microalgae *Chlorella vulgaris*. *Chemosphere,* 83, pp**.** 510-516.

GOTTSCHALK, F., SONDERER, T., SCHOLZ, R. W. & NOWACK, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental science & technology,* 43**,** pp. 9216-9222.

GRIFFITT, R. J., LUO, J., GAO, J., BONZONGO, J. C. & BARBER, D. S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry,* 27**,** pp. 1972-1978.

GUAN, R. et al., 2012. Cytotoxicity, oxidative stress, and genotoxicity in human hepatocyte and embryonic kidney cells exposed to ZnO nanoparticles. *Nanoscale research letters,* 7**,** p. 602.

GUNAWAN, C., SIRIMANOONPHAN, A., TEOH, W. Y., MARQUIS, C. P. & AMAL, R., 2013. Submicron and nano formulations of titanium dioxide and zinc oxide stimulate unique cellular toxicological responses in the green microalga *Chlamydomonas reinhardtii*. *Journal of hazardous materials,* 260**,** pp. 984-992.

HALLIWELL, B. & GUTTERIDGE, J. M., 2015. *Free radicals in biology and medicine,* 5th ed. USA : Oxford University.

HANNA, S. K., MILLER, R. J., MULLER, E. B., NISBET, R. M. & LENIHAN, H. S., 2013. Impact of engineered zinc oxide nanoparticles on the individual performance of *Mytilus galloprovincialis*. *PLoS One,* 8**,** p. e61800.

HAO, L. & CHEN, L., 2012. Oxidative stress responses in different organs of carp (*Cyprinus carpio*) with exposure to ZnO nanoparticles. *Ecotoxicology and environmental safety,* 80**,** pp. 103-110.

HARDMAN, R., 2006. A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. *Environmental health perspectives***,** 114 (2), pp. 165-172.

HARKER, M., TSAVALOS, A. J. & YOUNG, A. J., 1996. Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. *Bioresource Technology,* 55**,** pp. 207-214.

HATA, N., OGBONNA, J. C., HASEGAWA, Y., TARODA, H. & TANAKA, H., 2001. Production of astaxanthin by *Haematococcus pluvialis* in a sequential heterotrophic-photoautotrophic culture. *Journal of applied phycology,* 13**,** pp. 395-402.

HAZEEM, L. J. et al., 2016. Cumulative effect of zinc oxide and titanium oxide nanoparticles on growth and chlorophyll a content of *Picochlorum* sp. *Environmental Science and Pollution Research,* 23**,** pp. 2821-2830.

HAYASHI, T. & HAYASHI, K.,1996. Calcium spirulan, an inhibitor of enveloped virus replication, from a blue-green alga *Spirulina platensis*. *Journal of Natural Products, 59,* pp. 83–87.

HEINLAAN, M., IVASK, A., BLINOVA, I., DUBOURGUIER, H.-C. & KAHRU, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO 2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere,* 71**,** pp. 1308-1316.

HONGA, M, E., CHOIB,Y, Y. & SIMB, S, J., 2016. Effect of red cyst cell inoculation and iron(II) supplementation onautotrophic astaxanthin production by *Haematococcus pluvialis* underoutdoor summer conditions. *Journal of Biotechnology,* 218, pp. 25–33.

HORVÁTH, H., KOVÁCS, A. W., RIDDICK, C. & PRÉSING, M., 2013. Extraction methods for phycocyanin determination in freshwater filamentous cyanobacteria and their application in a shallow lake. *European journal of phycology,* 48**,** pp. 278-286.

HOSEINI, S., KHOSRAVI-DARANI, K. & MOZAFARI, M., 2013. Nutritional and medical applications of *spirulina* microalgae. *Mini reviews in medicinal chemistry,* 13**,** pp. 1231-1237.

HOU, J. et al., 2018. Toxic effects of different types of zinc oxide nanoparticles on algae, plants, invertebrates, vertebrates and microorganisms. *Chemosphere,* 193**,** pp. 852-860.

HU, C., LIU, Y., LI, X. & LI, M., 2013. Biochemical responses of duckweed (*Spirodela polyrhiza*) to zinc oxide nanoparticles. *Archives of environmental contamination and toxicology,* 64**,** pp. 643-651.

HUANG, J.-C., CHEN, F. & SANDMANN, G., 2006. Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*. *Journal of biotechnology,* 122**,** pp. 176-185.

HUSSAIN, S., HESS, K., GEARHART, J., GEISS, K. & SCHLAGER, J., 2005. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicology in Vitro,* 19**,** pp. 975-983.

IMAMOGLU, E., SUKAN, F. V. & DALAY, M. C., 2007. Effect of different culture media and light intensities on growth of *Haematococcus pluvialis. International journal of natural and engineering sciences,* 1**,** pp. 5-9.

ISWARYA, V. et al., 2015. Combined toxicity of two crystalline phases (anatase and rutile) of Titania nanoparticles towards freshwater microalgae: *Chlorella sp. Aquatic Toxicology, 161***,** *pp. 154-169.*

JANER, G. et al., 2014. Cell uptake and oral absorption of titanium dioxide nanoparticles. *Toxicology Letters,* 228**,** pp. 103-110.

JI, J., LONG, Z. & LIN, D., 2011. Toxicity of oxide nanoparticles to the green algae *Chlorella sp*. *Chemical Engineering Journal,* 170**,** pp. 525-530.

JIN, T., SUN, D., SU, J., ZHANG, H. & SUE, H. J., 2009. Antimicrobial efficacy of zinc oxide quantum dots against *Listeria monocytogenes, Salmonella enteritidis,* and *Escherichia coli O157: H7. Journal of food science,* 74(1), pp. 46-52.

JOHNSTON, B. D. et al., 2010. Bioavailability of nanoscale metal oxides TiO_2, CeO2, and ZnO to fish. *Environmental science & technology,* 44**,** pp. 1144-1151.

JONES, N., RAY, B., RANJIT, K. T. & MANNA, A. C., 2008. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. *FEMS microbiology letters,* 279, pp. 71-76.

KAHRU, A. & DUBOURGUIER, H. C., 2010. From ecotoxicology to nanoecotoxicology. *Toxicology,* 269**,** pp. 105-119.

KANG, C., LEE, J., PARK, T. & SIM, S. J., 2005. Comparison of heterotrophic and photoautotrophic induction on astaxanthin production by *Haematococcus pluvialis*. *Applied microbiology and biotechnology*, 68**,** pp. 237-241.

KASEMETS, K., IVASK, A., DUBOURGUIER, H.-C. & KAHRU, A., 2009. Toxicity of nanoparticles of ZnO, CuO and TiO2 to yeast *Saccharomyces cerevisiae*. *Toxicology in vitro,* 23**,** pp. 1116-1122.

KASSIM, H. et al., 2013. *The European Commission of the twenty-first century*, 1st ed. Oxford : OUP.

KAVITHA, G., KURINJIMALAR, C., THEVANATHAN, R. & RENGASAMY, R., 2015. Impact of UV-B Radiation on *Haematococcus pluvialis* Flotow Isolated from Himachal Pradesh under Laboratory Conditions. *Journal of Academia and Industrial Research (JAIR),* 3 (11), pp. 581-585.

KAZAMIA, E., RISELEY, A,S., HOWE, C, J. & SMITH1, A,G., 2014. An Engineered Community Approach for Industrial Cultivation of Microalgae. *Industrial Biotechnology*, 10 (3), pp. 184-190.

KELLER, A. A., MCFERRAN, S., LAZAREVA, A. & SUH, S., 2013. Global life cycle releases of engineered nanomaterials. *Journal of Nanoparticle Research*, 15**,** p. 1692.

KHAN, A.K., RASHID, R., MURTAZA,G.& ZAHRA, A., 2014. Gold Nanoparticles: Synthesis and Applications in Drug Delivery. *Tropical Journal of Pharmaceutical Research,* 13 (7), pp. 1169-1177.

KLAINE, S. J. et al., 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environmental toxicology and chemistry*, 27**,** pp. 1825-1851.

KLINGSHIRN, C., 2007. ZnO: From basics towards applications. *physica status solidi (b),* 244**,** pp. 3027-3073.

KLOCHKOVA, T. A. et al., 2013. Cold-tolerant strain of *Haematococcus pluvialis* (Haematococcaceae, Chlorophyta) from Blomstrandhalvøya (Svalbard). *Algae,* 28 (2)**,** pp. 185-192.

KOUHIA, S, M, M., LAHOUTIA, M., GANJEALIA, A. & ENTEZARIB, M, ., 2014. Comparative phytotoxicity of ZnO nanoparticles, ZnO microparticles, and Zn2+ on rapeseed (*Brassica napus L.*): investigating a wide range of Concentrations. *Toxicological & Environmental Chemistry*, 2014.

KUMAR, A., PANDEY, A. K., SINGH, S. S., SHANKER, R. & DHAWAN, A., 2011. Engineered ZnO and TiO_2 nanoparticles induce oxidative stress and DNA damage leading to reduced viability of *Escherichia coli. Free Radical Biology and Medicine,* 51**,** pp. 1872-1881.

KUMAR, H. & RANI, R., 2013. Structural and optical characterization of ZnO nanoparticles synthesized by microemulsion route. *International Letters of Chemistry, Physics and Astronomy,* 14**,** pp. 26--36.

KUMARI, M., KHAN,S,S., PAKRASHI,S., MUKHERJEE,A. & CHANDRASEKARAN, N., 2011. Cytogenetic and Genotoxic Effects of Zinc Oxide Nanoparticles on Root Cells of *Allium cepa*. *Journal of Hazardous Materials* , 190, p. 613621.

KUNHIKRISHNAN, A., SHON, H, K., BOLAN,N,S., SALIBY, I, E. & VIGNESWARAN, S., 2015. Sources, Distribution, Environmental Fate, and Ecological Effects of Nanomaterials in Wastewater Streams. C*ritical Reviews in Environmental Science and Technology*, 45, pp. 277–318.

LANLAN, Z. et al., 2015. Attached cultivation for improving the biomass productivity of *Spirulina Platensis*. *Bioresource Technology*, 181, pp. 136–142.

LEEMA, J. M., KIRUBAGARAN, R., VINITHKUMAR, N., DHEENAN, P. & KARTHIKAYULU, S., 2010. High value pigment production from *Arthrospira (Spirulina) platensis* cultured in seawater. *Bioresource Technology,* 101**,** pp. 9221-9227.

LI, J. et al., 2017. Comparative toxicity of nano ZnO and bulk ZnO towards marine algae *Tetraselmis suecica* and *Phaeodactylum tricornutum*. *Environmental Science and Pollution Research,* 24**,** pp. 6543-6553.

LI, M., LIN, D. & ZHU, L., 2013. Effects of water chemistry on the dissolution of ZnO nanoparticles and their toxicity to *Escherichia coli*. *Environmental Pollution,* 173**,** pp. 97-102.

LI, X., ZHOU, S. & FAN, W., 2016. Effect of nano-Al_2O_3 on the toxicity and oxidative stress of copper towards *Scenedesmus obliquus*. *International journal of environmental research and public health,* 13**,** p. 575.

LI, Y., SOMMERFELD, M., CHEN, F. & HU, Q., 2008. Consumption of oxygen by astaxanthin biosynthesis: a protective mechanism against oxidative stress in *Haematococcus pluvialis* (Chlorophyceae). *Journal of plant physiology,* 165**,** pp. 1783-1797.

LIN, D. & XING, B., 2007. Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. *Environmental Pollution,* 150**,** pp. 243-250.

LIN, D. & XING, B., 2008. Root uptake and phytotoxicity of ZnO nanoparticles. *Environmental science & technology,* 42**,** pp. 5580-5585.

LINHUA, H., ZHENYU, W. & BAOSHAN, X., 2009. Effect of sub-acute exposure to TiO 2 nanoparticles on oxidative stress and histopathological changes in juvenile carp (*Cyprinus carpio*). *Journal of Environmental Sciences,* 21**,** pp. 1459-1466.

LIPOVSKY, A., NITZAN, Y., GEDANKEN, A. & LUBART, R., 2011. Antifungal activity of ZnO nanoparticles—the role of ROS mediated cell injury. *Nanotechnology,* 22**,** p. 105101.

LIU, J. et al., 2014. Effects of ZnO, CuO, Au, and TiO_2 nanoparticles on *Daphnia magna* and early life stages of zebrafish *danio rerio*. *Environment Protection Engineering,* 40, pp. 139-149.

LONE, J., KUMAR, A., KUNDU, S., LONE, F. & SUSEELA, M., 2013. Characterization of Tolerance Limit in *Spirulina platensis* in Relation to Nanoparticles. *Water, Air, & Soil Pollution,* 224**,** pp. 1-6.

LONG, T. C., SALEH, N., TILTON, R. D., LOWRY, G. V. & VERONESI, B., 2006. Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. *Environmental science & technology,* 40**,** pp. 4346-4352.

LORENZ, R.T., 1999. A review of *Spirulina* and *Haematococcus* algae meal as a carotenoid and vitamin supplement for poultry. *Spirulina Pacifica Technical Bulletin,* 53, pp. 1-14.

MA, H., WILLIAMS, P. L. & DIAMOND, S. A., 2013. Ecotoxicity of manufactured ZnO nanoparticles–a review. *Environmental Pollution,* 172**,** pp. 76-85.

MAGDOLENOVA, Z. et al., 2014. Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. *Nanotoxicology,* 8**,** pp. 233-278.

MANZO, S., MIGLIETTA, M. L., RAMETTA, G., BUONO, S. & DI FRANCIA, G., 2013. Toxic effects of ZnO nanoparticles towards marine algae *Dunaliella tertiolecta*. *Science of The Total Environment,* 445**,** pp. 371-376.

MANZO, S. et al., 2011. Investigation of ZnO nanoparticles' ecotoxicological effects towards different soil organisms. *Environmental Science and Pollution Research,* 18**,** pp. 756-763.

MARIN, S. et al., 2015. Applications and Toxicity of Silver Nanoparticles: A Recent Review. *Current Topics in Medicinal Chemistry*, 15, pp. 1596-1604.

MATOS, J., CARDOSO,C., BANDARRA, N, M. & AFONSO, C., 2017. Microalgae as a healthy ingredient for functional food: A review, *Food & Function,* 2017.

MAYNARD, A. D. et al., 2006. Safe handling of nanotechnology. *Nature,* 444**,** pp. 267-269.

MELEGARI, S. P. et al., 2012. Induction to Oxidative Stress by Saxitoxin Investigated through Lipid Peroxidation in Neuro 2A Cells and *Chlamydomonas Reinhardtii* Alga. *Chemosphere,* 89 (1) , pp. 38– 43.

MENEZES, R, S., 2016. Culture medium influence on growth, fatty acid, and pigment composition of *Choricystis minor* var. minor: a suitable microalga for biodiesel production. *Journal of Applied Phycology*, 2016.

MIAO, A.-J. et al., 2009. The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. *Environmental Pollution,* 157**,** pp. 3034-3041.

MIAZEK, K., IWANEK, W., REMACLE, C., RICHEL, A. & GOFFIN, D., 2015. Effect of metals, metalloids and metallic nanoparticles on microalgae growth and industrial product biosynthesis: a review. *International journal of molecular sciences,* 16**,** pp. 23929-23969.

MILLER, R. J. et al., 2010. Impacts of metal oxide nanoparticles on marine phytoplankton. *Environmental science & technology,* 44**,** pp. 7329-7334.

MITTAL, S. & PANDEY, A.K., 2014. Cerium oxide nanoparticles induced toxicity in human lung cells: role of ROS mediated DNA damage and apoptosis. *BioMed Research International,* p. 891934.

MOEZZI, A., MCDONAGH, A. M. & CORTIE, M. B., 2012. Zinc oxide particles: Synthesis, properties and applications. *Chemical engineering journal*, 185**,** pp. 1-22.

MOHANTY, P., 1989. Effect of elevated levels of zinc on growth of *Synechococcus 6301*. *Zentralblatt fuer Mikrobiologie,* 144**,** pp. 531-536.

MONTEIRO, C. M., CASTRO, P. M. & MALCATA, F. X., 2012. Metal uptake by microalgae: underlying mechanisms and practical applications. *Biotechnology progress,* 28**,** pp. 299-311.

MONTES, M. O., HANNA, S. K., LENIHAN, H. S. & KELLER, A. A., 2012. Uptake, accumulation, and biotransformation of metal oxide nanoparticles by a marine suspension-feeder. *Journal of hazardous materials,* 225**,** pp. 139-145.

MOORE, M., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environment international,* 32**,** pp. 967-976.

MORAES, C. C., SALA, L., CERVEIRA, G. P. & KALIL, S. J., 2011. C-phycocyanin extraction from *Spirulina platensis* wet biomass. *Brazilian Journal of Chemical Engineering,* 28**,** pp. 45-49.

MORAES, I. D. O., ARRUDA, R. D. O. M., MARESCA, N. R., ANTUNES, A. D. O. & MORAES, R. D. O., 2013. *Spirulina platensis*: process optimization to obtain biomass. *Food Science and Technology (Campinas),* 33**,** pp. 179-183.

MORRIS, J., WILLIS, J. & GALLAGHER, K., 2007. Nanotechnology white paper. *US Environmental Protection Agency, Washington, DC, www. epa. gov/osa/pdfs/nanotech/epa-nanotechnology-whitepaper-0207. pdf (Feb. 2007).*

MORTIMER, M., KASEMETS, K. & KAHRU, A., 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. *Toxicology,* 269**,** pp. 182-189.

NAGARAJ, S., ARULMURUGAN, P., RAJARAM, M., SUNDARARAJ, R. & RENGASAMY, R., 2012. Enhanced production of astaxanthin at different physico-chemical parameters in the green alga *Haematococcus pluvialis* Flotow. *Phycological Society India,* 42, pp. 59-71.

NAKANISHI, K. & DEUCHI, K., 2014. Culture of a high-chlorophyll-producing and halotolerant *Chlorella vulgaris. Journal of bioscience and bioengineering,* 117**,** pp. 617-619.

NALIMOVA, A., POPOVA, V., TSOGLIN, L. & PRONINA, N., 2005. The effects of copper and zinc on *Spirulina platensis* growth and heavy metal accumulation in its cells. *Russian Journal of Plant Physiology,* 52**,** pp. 229-234.

NATIONS, S. et al., 2011. Acute effects of Fe2O3, TiO_2, ZnO and CuO nanomaterials on *Xenopus laevis*. *Chemosphere,* 83**,** pp. 1053-1061.

NAVARRO, E. et al., 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. *Environmental Science & Technology,* pp. 42**,** 8959-8964.

NISHIKAWA, K., YAMAKOSHI, Y., UEMURA, I. & TOMINAGA, N., 2003. Ultrastructural changes in *Chlamydomonas acidophila* (Chlorophyta) induced by heavy metals and polyphosphate metabolism. *FEMS Microbiology Ecology,* 44**,** pp. 253-259.

NOYMA, N. P. et al., 2015. Potential effects of UV radiation on photosynthetic structures of the bloom-forming cyanobacterium *Cylindrospermopsis raciborskii* CYRF-01. *Frontiers in microbiology,* p. 6.

OSMOND, M. J. & MCCALL, M. J., 2010. Zinc oxide nanoparticles in modern sunscreens: an analysis of potential exposure and hazard. *Nanotoxicology,* 4**,** pp. 15-41.

OUKARROUM, A., BRAS, S., PERREAULT, F. & POPOVIC, R., 2012a. Inhibitory effects of silver nanoparticles in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*. *Ecotoxicology and environmental safety,* 78**,** pp. 80-85.

OUKARROUM, A., POLCHTCHIKOV, S., PERREAULT, F. & POPOVIC, R., 2012b. Temperature influence on silver nanoparticles inhibitory effect on photosystem II photochemistry in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*. *Environmental Science and Pollution Research,* 19**,** pp. 1755-1762.

PANDURANGAN , M. & KIM, D, H., 2015. In vitro toxicity of zinc oxide nanoparticles: a review. *Journal of Nanoparticles Research*, 17, pp. 158.

PARK, E.-K. & LEE, C.-G., 2001. Astaxanthin production by *Haematococcus pluvialis u*nder various light intensities and wavelengths. *Journal of microbiology and biotechnology,* 11**,** pp. 1024-1030.

PATIL, S, S. et al., 2016. Nanoparticles for environmental clean-up: A review of potential risks and emerging solutions. *Environmental Technology & Innovation* , 5, pp. 10–21.

PERALES-VELA, H. V., PENA-CASTRO, J. M. & CANIZARES-VILLANUEVA, R. O., 2006. Heavy metal detoxification in eukaryotic microalgae. *Chemosphere,* 64, pp. 1-10.

PERREAULT, F., OUKARROUM, A., MELEGARI, S. P., MATIAS, W. G. & POPOVIC, R. 2012., Polymer coating of copper oxide nanoparticles increases nanoparticles uptake and toxicity in the green alga *Chlamydomonas reinhardtii. Chemosphere,* 87**,** pp. 1388-1394.

PICCINNO, F., GOTTSCHALK, F., SEEGER, S. & NOWACK, B., 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *Journal of Nanoparticle Research,* 14**,** pp. 1-11.

POPOV, A., PRIEZZHEV, A., LADEMANN, J. & MYLLYLÄ, R., 2005. TiO_2 nanoparticles as an effective UV-B radiation skin-protective compound in sunscreens. *Journal of Physics D: Applied Physics,* 38**,** pp. 2564-2570.

PREMANATHAN, M., KARTHIKEYAN, K., JEYASUBRAMANIAN, K. & MANIVANNAN, G., 2011. Selective toxicity of ZnO nanoparticles toward Gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. *Nanomedicine: Nanotechnology, Biology and Medicine,* 7**,** pp. 184-192.

QUIK, J. T., VONK, J. A., HANSEN, S. F., BAUN, A. & VAN DE MEENT, D., 2011. How to assess exposure of aquatic organisms to manufactured nanoparticles? *Environment international,* 37**,** pp. 1068-1077.

RAMESH, A, M., ANBUVANNAN, M, B., VIRUTHAGIRI, G, B., 2015. Green synthesis of ZnO nanoparticles using *Solanum nigrum* leaf extract and their antibacterial activity. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 136, pp. 864–870.

RATTE, H. T., 1999. Bioaccumulation and toxicity of silver compounds: a review. *Environmental Toxicology and Chemistry,* 18**,** pp. 89-108.

REDDY, K. M. et al., 2007. Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems. *Applied physics letters,* 90**,** p. 213902.

REIDY, B., HAASE, A., LUCH, A., DAWSON, K. A. & LYNCH, I., 2013. Mechanisms of silver nanoparticle release, transformation and toxicity: a critical review of current knowledge and recommendations for future studies and applications. *Materials,* 6**,** pp. 2295-2350.

REITER, R. J. et al., 1999. Augmentation of indices of oxidative damage in life-long melatonin-deficient rats. *Mechanisms of Ageing and Development,* 110**,** pp. 157-173.

ROCO, M. C., MIRKIN, C. A. & HERSAM, M. C., 2011. Nanotechnology research directions for societal needs in 2020: summary of international study. *Journal of Nanoparticle Research,*13 (3), pp. 897-919.

ROH, J.-Y. et al., 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. *Environmental science & technology,* 43**,** pp. 3933-3940.

RUAN, J., LONG, C. & GUO, B., 1988. *Spirulina* prevented damage induced by radiation. *Journal of Genetics,* 10**,** pp. 27-30.

SABIR, S., ARSHAD, M. & CHAUDHARI, S. K., 2014. Zinc oxide nanoparticles for revolutionizing agriculture: synthesis and applications. The Scientific World Journal, p. 925494.

SAÇAN, M. T., OZTAY, F. & BOLKENT, S., 2007. Exposure of *Dunaliella tertiolecta* to lead and aluminum: toxicity and effects on ultrastructure. *Biological trace element research,* 120**,** pp. 264-272.

SADIQ, I. M., PAKRASHI, S., CHANDRASEKARAN, N. & MUKHERJEE, A., 2011. Studies on toxicity of aluminum oxide (Al2O3) nanoparticles to microalgae species: *Scenedesmus sp.* and *Chlorella sp*. *Journal of Nanoparticle Research,* 13**,** pp. 3287-3299.

SARADA, R., TRIPATHI, U. & RAVISHANKAR, G., 2002. Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. *Process Biochemistry,* 37**,** pp. 623-627.

SCHIAVO, S., OLIVIERO, M., MIGLIETTA, M., RAMETTA, G. & MANZO, S., 2016. Genotoxic and cytotoxic effects of ZnO nanoparticles for *Dunaliella tertiolecta* and comparison with SiO2 and TiO2 effects at population growth inhibition levels. *Science of the Total Environment,* 550, pp. 619-627.

SHAH, M., MAHFUZUR, R., LIANG, Y., CHENG, J. J. & DAROCH, M., 2016. Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high value commercial products. *Frontiers in plant science,* 7**,** p. 531.

SHEN, C. et al., 2013. Relating cytotoxicity, zinc ions, and reactive oxygen in ZnO nanoparticle–exposed human immune cells. T*oxicological sciences,* 136**,** pp. 120-130.

SHILPI, G., SUNITA, S., & SWETA, S., 2014. Hexavalent chromium toxicity to cyanobacterium *Spirulina platensis*. *International research journal of pharmacy,* 5 (12), pp. 910-914.

SHUKLA, M. et al., 2009. Responses of cyanobacterium *Anabaena doliolum* during nickel stress. *Journal of Environmental Biology,* 30(5), pp. 871-876.

SONG, W. et al., 2010. Role of the dissolved zinc ion and reactive oxygen species in cytotoxicity of ZnO nanoparticles. *Toxicology Letters,* 199**,** pp. 389-397.

SRIVASTAVA, V., GUSAIN, D. & SHARMA, Y. C., 2015. Critical review on the toxicity of some widely used engineered nanoparticles. *Industrial & Engineering Chemistry Research,* 54, pp. 6209-6233.

STEVENSON, L. M. et al., 2013. Environmental feedbacks and engineered nanoparticles: mitigation of silver nanoparticle toxicity to *Chlamydomonas reinhardtii* by algal-produced organic compounds. *PloS one,* 8**,** p. e74456.

SUMAN, T., RAJASREE, S. R. & KIRUBAGARAN, R., 2015. Evaluation of zinc oxide nanoparticles toxicity on marine algae *Chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis. *Ecotoxicology and Environmental Safety,* 113, pp. 23-30.

SUN, J. et al., 2015. Microstructures and functional groups of *Nannochloropsis* sp. cells with arsenic adsorption and lipid accumulation. *Bioresource technology,* 194**,** pp. 305-311.

TAKAICHI, S., 2011. Carotenoids in algae: distributions, biosyntheses and functions. *Marine drugs,* 9**,** pp. 1101-1118.

TANG, Y., LI, S., QIAO, J., WANG, H. & LI, L., 2013. Synergistic effects of nano-sized titanium dioxide and zinc on the photosynthetic capacity and survival of *Anabaena sp*. *International Journal of Molecular Sciences,* 14**,** pp. 14395-14407.

TANGAHU, B. V. et al., 2011. A review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. *International Journal of Chemical Engineering,* p. 939161.

TOCQUIN, P., FRATAMICO, A. & FRANCK, F., 2012. Screening for a low-cost *Haematococcus pluvialis* medium reveals an unexpected impact of a low N/P ratio on vegetative growth. *Journal of applied phycology,* 24**,** pp. 365-373.

TREVISAN, R. et al., 2014. Gills are an initial target of zinc oxide nanoparticles in oysters *Crassostrea gigas*, leading to mitochondrial disruption and oxidative stress. *Aquatic toxicology,* 153**,** pp. 27-38.

TRIPATHI, U., SARADA, R., RAO, S. R. & RAVISHANKAR, G., 1999. Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. *Bioresource Technology,* 68, pp. 197-199.

VALKO, M., RHODES, C.J., MONCOL, J., IZAKOVIC, M. & MAZUR, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions,*160 (1), pp. 1-40.

VAN HOECKE, K., DE SCHAMPHELAERE, K. A., VAN DER MEEREN, P., LCUCAS, S. & JANSSEN, C. R., 2008. Ecotoxicity of silica nanoparticles to the green alga *Pseudokirchneriella subcapitata*: importance of surface area. *Environmental Toxicology and Chemistry,* 27**,** pp. 1948-1957.

VANCE, M. E. & MARR, L. C., 2015. Exposure to airborne engineered nanoparticles in the indoor environment. *Atmospheric Environment,* 106**,** pp. 503-509.

VANNELA, R. & VERMA, S. K., 2006. Co^{2+}, Cu^{2+}, and Zn^{2+} accumulation by cyanobacterium *Spirulina platensis*. *Biotechnology progress,* 22**,** pp. 1282-1293.
VELZEBOER, I., KUPRYIANCHYK, D., PEETERS, E. & KOELMANS, A., 2011. Community effects of carbon nanotubes in aquatic sediments. *Environment international,* 37**,** pp. 1126-1130.

VIDHYAVATHI, R., SARADA, R. & RAVISHANKAR, G. A., 2009. Expression of carotenogenic genes and carotenoid production in *Haematococcus pluvialis* under the influence of carotenoid and fatty acid synthesis inhibitors. *Enzyme and Microbial Technology,* 45**,** pp. 88-93.

VIDHYAVATHI, R., VENKATACHALAM, L., SARADA, R. & RAVISHANKAR, G. A., 2008. Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. *Journal of Experimental Botany,* 59**,** pp. 1409-1418.

VLADIMIRESCU, A. F., 2010. Isolation of permeaplasts and spheroplasts from *Spirulina platensis*. *Romanian Biotechnological Letters,* 15 (3), pp. 5361-5368.

VON MOOS, N. & SLAVEYKOVA, V. I., 2014. Oxidative stress induced by inorganic nanoparticles in bacteria and aquatic microalgae–state of the art and knowledge gaps. *Nanotoxicology,* 8**,** pp. 605-630.

VONSHAK, A., 1997. *Spirulina platensis arthrospira: physiology, cell-biology and biotechnolog.* London: Taylor& Francis Ltd.

WAHAB, R., MISHRA, A., YUN, S.-I., KIM, Y.-S. & SHIN, H.-S., 2010. Antibacterial activity of ZnO nanoparticles prepared via non-hydrolytic solution route. *Applied microbiology and biotechnology,* 87**,** pp. 1917-1925.

WANG, B. et al., 2008. Acute toxicological impact of nano-and submicro-scaled zinc oxide powder on healthy adult mice. *Journal of Nanoparticle Research,* 10**,** pp. 263-276.

WANG, H., WICK, R. L. & XING, B., 2009. Toxicity of nanoparticulate and bulk ZnO, Al2O3 and TiO2 to the nematode *Caenorhabditis elegans*. *Environmental Pollution,* 157**,** pp. 1171-1177.

WANG, N., TONG, T., XIE, M. & GAILLARD, J.-F., 2016. Lifetime and dissolution kinetics of zinc oxide nanoparticles in aqueous media. *Nanotechnology,* 27, p. 324001.

WANG, Z & ZHAO,Y., 2005. Morphological reversion of *Spirulina (Arthrospira) platensis* (Cyanophyta): from linear to helical. *Journal of Phycology,* 41, pp. 622-628.

WANG, Z. L., 2004. Zinc oxide nanostructures: growth, properties and applications. *Journal of physics: condensed matter,* 16**,** pp. 829-858.

WEI, C. et al., 2010. Effects of silica nanoparticles on growth and photosynthetic pigment contents of *Scenedesmus obliquus*. *Journal of Environmental Sciences,* 22**,** pp. 155-160.

WEIR, A., WESTERHOFF, P., FABRICIUS, L., HRISTOVSKI, K. & VON GOETZ, N., 2012. Titanium dioxide nanoparticles in food and personal care products. *Environmental science & technology,* 46**,** pp. 2242-2250.

WIESNER, M. R., LOWRY, G. V., ALVAREZ, P., DIONYSIOU, D. & BISWAS, P., 2006. Assessing the risks of manufactured nanomaterials. *Environmental science & technology,* 40**,** pp. 4336-4345.

WONG, S. W., LEUNG, P. T., DJURIŠIĆ, A. & LEUNG, K. M., 2010. Toxicities of nano zinc oxide to five marine organisms: influences of aggregate size and ion solubility. *Analytical and bioanalytical chemistry,* 396, pp. 609-618.

XIA, B. et al., 2015. Interaction of TiO_2 nanoparticles with the marine microalga *Nitzschia closterium*: growth inhibition, oxidative stress and internalization. *Science of the Total Environment,* 508**,** pp. 525-533.

XIAOXIAO, C. et al., 2012. Photosynthetic toxicity and oxidative damage induced by nano-Fe3O4 on *Chlorella vulgaris* in aquatic environment. *Open Journal of Ecology,* 2, pp. 21-28.

XIE, Y., HE, Y., IRWIN, P. L., JIN, T. & SHI, X., 2011. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against *Campylobacter jejuni*. *Applied and environmental microbiology,* 77**,** pp. 2325-2331.

XIONG, D., FANG, T., YU, L., SIMA, X. & ZHU, W., 2011. Effects of nano-scale TiO2, ZnO and their bulk counterparts on zebrafish: acute toxicity, oxidative stress and oxidative damage. *Science of the Total environment,* 409**,** pp. 1444-1452.

XU, M. et al., 2013. Challenge to assess the toxic contribution of metal cation released from nanomaterials for nanotoxicology–the case of ZnO nanoparticles. *Nanoscale,* 5**,** pp. 4763-4769.

YEO, M.-K. & PAK, S.-W., 2008. Exposing zebrafish to silver nanoparticles during caudal fin regeneration disrupts caudal fin growth and p53 signaling. *Molecular and Cellular Toxicology,* 4**,** pp. 311-317.

YEO, M.-K. & YOON, J.-W., 2009. Comparison of the effects of nano-silver antibacterial coatings and silver ions on zebrafish embryogenesis. *Molecular and Cellular Toxicology,* 5, pp. 23-31.

YU, L.-P. et al., 2011. Comparative toxicity of nano-ZnO and bulk ZnO suspensions to zebrafish and the effects of sedimentation, OH production and particle dissolution in distilled water. *Journal of Environmental Monitoring,* 13**,** pp. 1975-1982.

YUNG, M. M. N., MOUNEYRAC, C. & LEUNG, K. M. Y., 2014. Ecotoxicity of zinc oxide nanoparticles in the marine environment. In: Bhushan B. (eds), *Encyclopedia of Nanotechnology*. Dordrecht : Springer, pp. 1-17.

ZHANG, C., WANG, J., TAN, L. & CHEN, X., 2016. Toxic effects of nano-ZnO on marine microalgae *Skeletonema costatum*: Attention to the accumulation of intracellular Zn. *Aquatic Toxicology,* 178**,** pp. 158-164.

ZHANG, W., BAO, S. & FANG, T., 2016. The neglected nano-specific toxicity of ZnO nanoparticles in the yeast Saccharomyces cerevisiae. *Scientific reports,* 6**,** p.24839.

ZHOU, H., WANG, X., ZHOU, Y., YAO, H. & AHMAD, F., 2014. Evaluation of the toxicity of ZnO nanoparticles to *Chlorella vulgaris* by use of the chiral

perturbation approach. *Analytical and Bioanalytical Chemistry,* 406**,** pp. 3689-3695.

ZHU, X., CHANG, Y. & CHEN, Y., 2010. Toxicity and bioaccumulation of TiO_2 nanoparticle aggregates in *Daphnia magna*. *Chemosphere,* 78**,** pp. 209-215.

ZHU, X. et al., 2008. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health, Part A,* 43**,** pp. 278-284.

ZINICOVSCAIA, I. et al., 2016. Selenium uptake and assessment of the biochemical changes in *Arthrospira (Spirulina) platensis* biomass during the synthesis of selenium nanoparticles. *Canadian journal of microbiology,* 63**,** pp. 27-34.

ZINICOVSCAIA, I. et al., 2013. *Spirulina platensis* as biosorbent of zinc in water. *Environmental Engineering & Management Journal,* 12 (5), pp. 1079-1084.

LIST OF PUBLICATIONS, CONFERENCE PRESENTATIONS AND RESEARCH AWARD

PUBLICATIONS:

1. **Sinouvassane Djearamane**, Ling Shing Wong, Yang Mooi Lim, Poh Foong Lee. (2016). "A Review of Bio-distribution & Toxicity of Silver, Titanium Dioxide and Zinc Oxide Nanoparticles in Aquatic Environment*". Pollution Research*, 6 (4), 139-150.

2. **Sinouvassane Djearamane**, Ling Shing Wong, Yang Mooi Lim, Poh Foong Lee. (2018). "Cytotoxic Effects of Zinc Oxide Nanoparticles on Cyanobacterium *Spirulina platensis*". *PeerJ*, e4682; DOI 10.7717/peerj.4682.

3. **Sinouvassane Djearamane**, Ling Shing Wong, Yang Mooi Lim, Poh Foong Lee. (2019). "Short Term Toxicity of Zinc Oxide Nanoparticles on the Fresh Water Microalga *Chlorella vulgaris*". *Sains Malaysiana*, 48(1): 69–73.

4. **Sinouvassane Djearamane**, Ling Shing Wong, Yang Mooi Lim, Poh Foong Lee. "Cytotoxic Effects of Zinc Oxide Nanoparticles on *Chlorella Vulga*ris". Pollution Research. Accepted in February 2019.

TRAVEL GRANT AWARD

"**KSMCB Travel Grant Award**" in the International Conference: Korean Society for Molecular and Cellular Biology 2018 (KSMCB) held at COEX Centre, Seoul in Korea from 17 to 19 September 2018".

CONFERENCE PRESENTATIONS:

1. **Sinouvassane Djearamane**, Yang Mooi Lim, Ling Shing Wong, Poh Foong Lee." Induction of Morphological Alterations and Intracellular Damages in Cyanobacterium *Spirulina platensis* by Zinc Oxide Nanoparticles". *International conference on Recent Trends in Humanities and Science* held at Universiti Tunku Abdul Rahman, 31900 Kampar, Malaysia on 26 October 2018. Abstract is published in American J of Bio-pharmacy Biochemistry and Life Sciences 2018 December, Vol. 6: OP14. (Oral presentation).

2. **Sinouvassane Djearamane**, Yang Mooi Lim, Ling Shing Wong, Poh Foong Lee. "The Oxidative Stress Effects of Zinc Oxide Nanoparticles on Cyanobacterium *Spirulina platensis*". *International conference on Recent Trends in Science and Technology* on 21-23 September 2018, AIMST University, 08100 Bedong, Kedah, Malaysia. (Oral presentation).

3. **Sinouvassane Djearamane**, Yang Mooi Lim, Ling Shing Wong, Poh Foong Lee. "Biochemical Responses of Nutrient Microalgae *Spirulina platensis* to Zinc Oxide Nanoparticles' Toxicity". *International Conference: Korean Society for Molecular and Cellular Biology 2018 (KSMCB)* held at COEX Centre, Seoul in Korea from 17 to 19 September 2018. (Poster Presentation & Received Travel Grant Award).

4. **Sinouvassane Djearamane**, Yang Mooi Lim, Ling Shing Wong, Poh Foong Lee. "Cytotoxicity of Zinc Oxide Nanoparticles on Nutrient Microalga". *Utar Intervarsity Science Symposium 2018* held at Universiti Tunku Abdul Rahman, 31900 Kampar, Malaysia from 04-05 August 2018 (Invited Speaker).

5. **Sinouvassane Djearamane**, Ling Shing Wong, Yang Mooi Lim, Poh Foong Lee (2016). "Oxidative Stress of Nutritional Supplement *Chlorella vulgaris* by Zinc Oxide Nanoparticles Treatment". *Malaysia-Thailand-Japan International Conference on Science and Technology* on 28 - 29 September 2016 at Graduate Centre, Universiti Kebangsaan Malaysia, 43600 Bangi, Malaysia. (Oral Presentation).