Toxicity Evaluation of TiO_2 Nanoparticles in Earthworm (*Eisenia fetida*)

THESIS

Submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI

2017

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis entitled **"Toxicity Evaluation of TiO_2 Nanoparticles in Earthworm (*Eisenia fetida*)"** and submitted by **Mr. Bitragunta Siva Prasad,** ID No **2011PHXF0006H** for the award of Ph.D. degree of the Institute embodies original work done by him under my supervision.

Signature of the Supervisor

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Date:

Acknowledgement

Working towards my doctoral degree has been a period of various challenges that have led to a great deal of personal and professional growth.

Making it through would not have been possible without the help and support of family and friends.

Great appreciation goes to my supervisor, ***Prof. P. Sankar Ganesh****, who has been great inspiration during this tenure by providing critical scientific feedback. In this regard, it gives me immense pleasure to express my profound sense of gratitude and heartfelt indebtedness to my supervisor for his scrupulous guidance, pertinent encouragement, inspiring discussions and valuable suggestions throughout my research work. His observations and critical comments helped me to establish the overall direction of the research and to explore in depth investigations constantly. I sincerely thank him for giving the opportunity to evolve as an independent researcher.*

In addition, I would like to thank my doctoral advisory committee members ***Prof. Kumar Pranav Narayan*** *and* ***Dr. Jayati Ray Dutta*** *for their valuable suggestions and guidance in the execution of the work.*

I am delighted to thank the Director, ***Prof. G. Sundar*** *and the former Director,* ***Prof. V.S. Rao*** *BITS Pilani Hyderabad Campus for providing research ambience to carry out my research work.*

I also want to deeply thank Department Research Committee (DRC), Department of Biological Science and Academic Research Division (ARD), BITS Pilani, Hyderabad Campus for their great patience in helping my study experience throughout the course of my research regardless of limitation of time.

I express my profound regards to ***Prof. K. Naga Mohan****, Head, Department of Biological Sciences, for his constant encouragement throughout my research.*

I am grateful to all faculty members and staff of Department of Biological Sciences for their inspiration and assistance respectively during my course work and research.

I am also grateful to ***Prof. Venugopal Reddy, the former Director, Central Facilities for Research and Development Osmania University*** *and* ***Dr. Vamsi Krishna Venuganti****,* ***Department of Pharmacy, BITS Pilani, Hyderabad Campus*** *for their valuable suggestions during my research.*

I sincerely thank Mr. Ramaiah for his profound help in providing the test organisms, E. fetida from the Vermiculture Project, Hyath Nagar, RR District.

I would like to thank my close friends Sri Chamarthi Suresh and Smt Chamarthi Sridevi and family for their valuable support and inspiring discussions at any point of time.

I invariably fall short of words to express my heart-felt gratitude and profound thanks to the management, Ideal Degree College for Women, Hyderabad for their valuable support for my research.

I have been blessed with incessant moral support and unlimited encouragement of my beloved parents, Father Sri B.V. Raju, Mother Smt. B.V. Raghavamma; Grand Parents Sri B.V. Narayana Raju and Smt. Aadilaxmi; my Wife Mrs. Usha Rani and other family members. I am thankful for their unflinching support, love and affection, which really meant for what I am today.

I thank Department of Biotechnology, Ministry of Science and Technology, Govt. of India for financial assistance in the form of JRF and SRF, vide project titled "Novel biomarker responses to validate acute toxicity of engineered nanoparticles and their physicochemical interactions in earthworm as a model bio indicator of nano (eco) toxicology" (No. BT/PR6592/GBD/27/446/2012).

Finally, I thank one and all who supported me directly and indirectly.

Bitragunta Siva Prasad

ABSTRACT

Steady growth of nanotechnology and extensive use of engineered nanoparticles (ENP) in personal care products, pharmaceutics, and biomedical applications have raised concerns about their implications on environment and human health. However, recent toxicological studies in this regard raised more questions than answers. There exists paucity of data pertaining to toxicity evaluation of metal oxide nanoparticles including rutile crystal phase of Titanium dioxide engineered nanoparticles (r-TiO_2-ENP) in the invertebrates. Hence, in the light of developing an invertebrate based toxicity assessment and biomarkers specifically for r-TiO_2-ENP, earthworm, *Eisenia fetida* is chosen as a sentinel organism in the present study. Besides toxicity evaluation, this study also sheds light on advancements in the analytical techniques for the detection and characterization of Ti and TiO_2 nanoparticles from environmental matrices using single-particle ICP-MS.

The current study quantified the concentrations of Ti (1085 and 298 mg/kg) and Titanium dioxide nanoparticles (TNP) (13.6 and 3.3 mg/kg) in the municipal sewage. The size of TNP ranged between 71 and 145 nm in the sewage sludge fraction. As per OECD-207 guidelines, median lethal concentration of r-TiO_2-ENP was found to be at 0.13 mg/cm^2 trough dermal exposure. Soil exposure of the organism (0.05-0.4 mg/kg) for 2, 7 and 14 days did not cause mortality of the worms. Interdependency of mass concentration (0.05-0.25 mg/ml), z-average of size (341-480 nm) and charge (-6 to -23 mV) upon agglomeration of the nanoparticles is demonstrated using dynamic light scattering. ICP-OES studies revealed that presence of electrolyte and natural organic matter causes agglomeration of r-TiO_2-ENP in aqueous media. Thus r-TiO_2-ENP could act as strong pro-oxidant to alter the activities of antioxidant enzymes and lipid peroxidation. From this study, it shall be concluded that increased activity of SOD, decreased CAT and GR activities can serve as sensitive biomarkers of oxidative stress caused by lethal concentrations of r-TiO_2-ENP exposed for 48h through dermal route in earthworm, Eisenia fetida. But r-TiO_2-ENP caused lipid peroxidation in earthworm. Moreover, these nanoparticles activated acetylcholinesterase

(AChE) activity through soil exposure. Label free quantitation revealed the role of 26 proteins that aid to understand stress-responsive system and behavior of earthworm upon exposure to r-TiO_2-ENP. Moreover, to the best of our knowledge, this study is the first of its kind in presenting the results on proteomic profiles of the earthworm when exposed to r-TiO_2-ENP through skin.

Present study also aids in the advancement of analytical methods such as SP-ICP-MS to detect and characterize nanoparticles in environmental samples. Results of the study can serve as cues to design comprehensive label free quantitation approach for considering earthworms as early warning indicators or sentinels for assessing health hazards of engineered nanoparticles such as TiO_2-ENP. Further the responses of antioxidant enzymes, acetylcholinesterase and lipid peroxidation along with proteomics approach to r-TiO_2-ENP help in the transition from conventional biomarkers such as mortality to a mechanism-based risk assessment biomarkers which also aid in hazard ranking of nanoparticles. Results of the study will be useful in documenting ontology of r-TiO_2-ENP in nanoparticle databases that facilitate computational modeling and development of predictive nanotoxicolgy. Consequently, the study will be useful in advancing the science of nanoecotoxicology for developing safety assessment paradigm in the era of nanotechnology.

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Abbreviations

°C	Degree Celsius
µg	Micro gram
µL	Micro liter
µM	Micro mole
AChE	Acetylcholinesterase
ACN	Acetonitrile
APHA	American Public Health Association
ATC	Acetylthiocholine iodide
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CAT	Catalase
CDNB	1-chloro-2,4-dinotrobenzene
cm	Centi meters
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenolhydrazine
DOM	Dissolved organic matter
DTNB	5,5'-Dithio-bis-(2- nitrobenzoic acid)
EC	European Communities
EDTA	Ethylene diamine tetra acetic acid
EEC	European Economic Community
ENP	Engineered nanoparticles
g	Gram
GR	Glutathione reductase
GSH	Glutathione
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione *S*-transferase
h	hours
H_2O	Water
H_2O_2	Hydrogen peroxide
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
ICPMS	Inductively Coupled Plasma Mass Spectrometer
ICP-OES	Inductively Coupled Optical Emission Spectrometer
ISO	International Organization for Standardization

kDa	Kilo Dalton
kg	Kilogram
kHz	Kilohertz
K_m	Michaelis-Menten constant (half the maximum velocity)
L	Litre
LC_{50}	Lethal Concentration 50 or Median Lethal Concentration
LC-MS	Liquid chromatography–mass spectrometry
LPO	Lipid peroxidation
LTQ	Linear Trap Quadropole
m	Milli
M	Molar
MASCOT	Database for peptides/ protein profiles
MDA	Malondialdehyde
mg	Milli gram
min.	Minutes
mL	Milliliter
mm	Milli meter
mM	Milli molar
MQ	Milli-Q
MT	Metallothionein
MW	Molecular weight
Na_2EDTA	Ethylene diamine tetra acetic acid disodium salt
NaCl	Sodium chloride
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NaOH	Sodium hydroxide
nm	Nano meters
NOM	Natural organic matter
O_2	Molecular oxygen
$O2^{\bullet-}$	Superoxide anion radical
OECD	Organization for Economic Cooperation and Development
$OH^{\bullet}$	Hydroxyl radical
OP	Organophosphorus
PBS	phosphate-buffered saline
ppb	Parts per billion
ppm	Parts per million
ROS	Reactive oxygen species
rps	Revolution per second
r-TiO_2-ENP	Rutile Titanium dioxide Engineered nanoparticles
SD	Standard deviation
SOD	Superoxide dismutase

SP-ICPMS	Single Particle-ICPMS
TBA	Thiobarbituric acid
TiO_2	Titanium dioxide
ToxCast	EPA Toxicity Fore Caster
TRA	Time-resolved analysis
Tris-HCl	Tris (hydroxymethyl) amino methane hydrochloride
UniProt	UniProt Knowledgebase
USEPA	United States Environmental Protection Agency
W	Watt
WPMN	Working Party on Manufactured Nanomaterials

Chapter 1

Introduction

1. Introduction

Engineered nanoparticles (ENPs) are widely used in various commercial products and in the industries owing to their unique properties such as size, surface area and crystal phase (Cumberland and Lead, 2009; Klaine et al., 2008). For instance, application of silver nanoparticles as antibacterial agents led to their projected market value from $0.79 billion in 2014 to $2.54 billion by 2022 (Grand View Research, 2015; Peyrot et al., 2014). Nanoparticles are used as components in burn treatment creams, dental compositions and cosmetic lotions (Bindhu and Umadevi, 2015). Currently, the market potential of TiO_2 production is approximately $2000 per ton (ICI, 2015). Production of ENP is has doubled every three years. In parallel to the growth of nanotechnology, assessment of nanomaterial safety and its significant contributions to understand impact of nanomaterials on environment and human health is important (Krug, 2014). In continuation, attempts to characterize them in environmental matrices thereby evaluating toxicological effects of ENP in various sentinel organisms is gaining prominence across the globe.

Release of ENP into the environment is inevitable during production, transportation, utilization and final disposal (Soni et al. 2015; Bakshi et al. 2015; Sun et al. 2016). Thereby their entry into various environmental compartments is imperative. However, standard analytical methods to detect and characterize Ti and associated nanomaterials in the environment are still emerging (Tourinho et al. 2012; Gottschalk et al. 2013). Application of dynamic light scattering (Topuz et al. 2015) and atomic emission spectroscopy (Westerhoff et al. 2011) cannot facilitate simultaneous

measurement of ENP properties such as size, concentration, and number (Pachapur et al. 2015). In this pursuit, single particle-ICP-MS which enables rapid yet simultaneous analyses of elemental composition, number and size distribution of nanoparticles including TiO_2 (Kim et al. 2012; Laborda et al. 2014; Proulx et al. 2016), Ag and Au (Lee et al. 2014; Yang et al. 2016), ZnO (Hadioui et al. 2015) in environment is gaining prominence. In light of advancing the analytical methods for quantifying ad characterizing ENP in environment, there is a great need to optimize techniques such as single particle ICP-MS that could aid in risk assessment.

In the backdrop of information and data on risk assessment of nanomaterials, research efforts in this pursuit are being focused to divulge the toxicity thereby safety management of ENP across the globe. Working Party on Manufactured Nanomaterials (WPMN) of the Organization for Economic Co-operation and Development (OECD) is involved in reviewing the efforts that are relevant to the use of alternatives such as *in vitro* analysis to support or if required to replace traditional animal toxicology studies to obtain significant confidence in results (A. Clunan, 2014). Such efforts can provide important information regarding the potential toxicity mechanisms of ENP under a given dose/ concentration. Hence a comprehensive testing strategy for hazard and risk analysis of ENP is possible in the future (OECD WPMN, 2017).

Although standard methods exist for hazard and risk analysis of chemicals, the suitability of using these tools for nanoparticles needs to be evaluated and adapted, where appropriate. In addition, development of alternative testing systems that are less reliant on vertebrate testing is required owing

to the vast array of nanoparticles under development and use. There is an urgent need to develop reliable and predictive approaches for risk assessment of engineered nanoparticles to be integrated into a coherent strategy both for the regulators and industry. However, it is not possible to test the safety of all engineered nano materials (NM). Consequently, within hazard investigations few "representative" engineered nanoparticles or nanomaterials are often selected. Based on the OECD-Working party on manufactured nanomaterials (WPMN) (OECD WPMN, 2010), four representative nanoparticles *viz.* Titanium dioxide (TiO2); Gold (Au); Polystyrene and Dendrimers were selected for the current PhD thesis work. Criteria for selecting these representative nanoparticles were production volume and availability of such materials for testing. Toxicological assessment and associated end points in soil sentinels are not documented for the above said representative nanoparticles. Toxicity potential of these nanoparticles was screened by OECD-207 method. Out of the 4 representative nanoparticles chosen, only TiO_2 showed toxic response during the study. Additional data on screening of Gold (Au); Polystyrene and Dendrimers is presented in Appendix-1. Hence further investigation was carried out on TiO_2 to: (i) study its environmental occurrence (ii) physico-chemical characterization (iii) toxicological evaluation and (iv) biochemical responses in earthworm, *Eisenia fetida.*

In recent times, metal oxide nanoparticles including titanium dioxide nanoparticles (also called as nano-TiO_2 or TiO_2-ENPs) are extensively used in various industrial and agriculture applications due to their enhanced surface properties and quantum effect. TiO_2-ENPs are most commonly used

in cosmetics, pharmaceutics and paint industries due to their high stability, high transparency to visible light, high absorption of UV spectrum, anti-corrosiveness and photocatalytic effect (Bindhu and Umadevi, 2015; Pachapur et al., 2015).

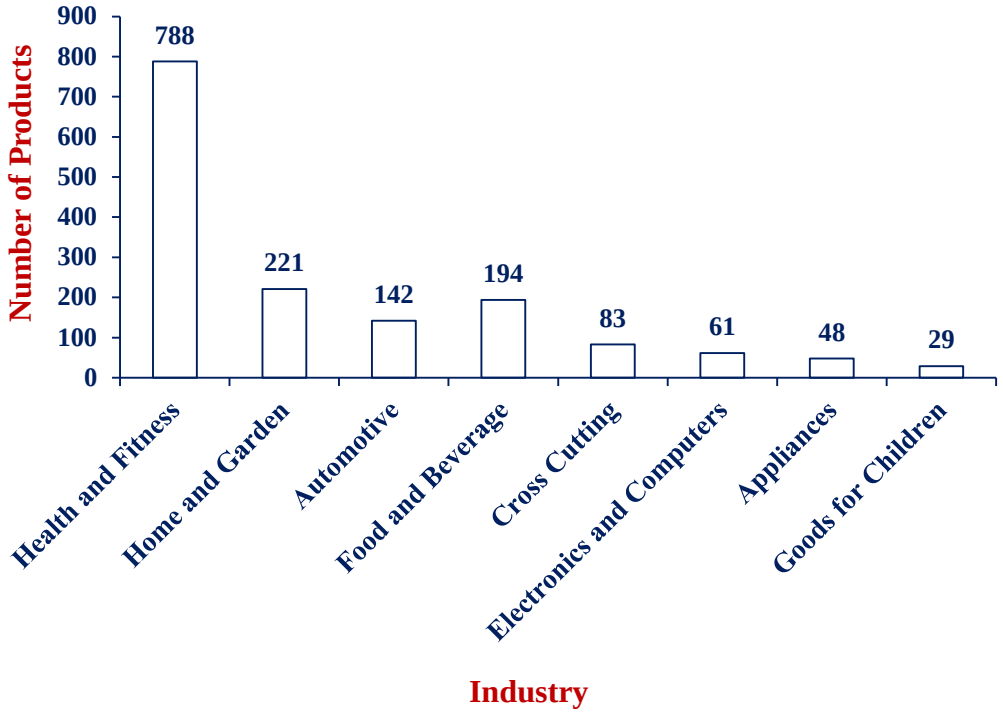

Figure 1.1 Categories of products that are incorporating nanomaterials during their manufacture. *http://www.nanotechproject.org/cpi/site/assets/files/3551/chart_2.png*

Out of the total TiO_2-ENPs produced, 68% utilized in manufacturing of personal and pharmaceutical products, whereas 6% in manufacturing of plastics, 14% in paints, and 12% other applications (e.g., cement) (Piccinno et al., 2012). For instance, the number of nanoparticles that are being integrated to various products in the year 2013 is presented in **Figure 1.1**. Most of the currently produced bulk form of TiO_2 will be substituted by TiO_2-ENPs by end of the year 2026, reaching an overall production rate of 2.5 million tons per year.

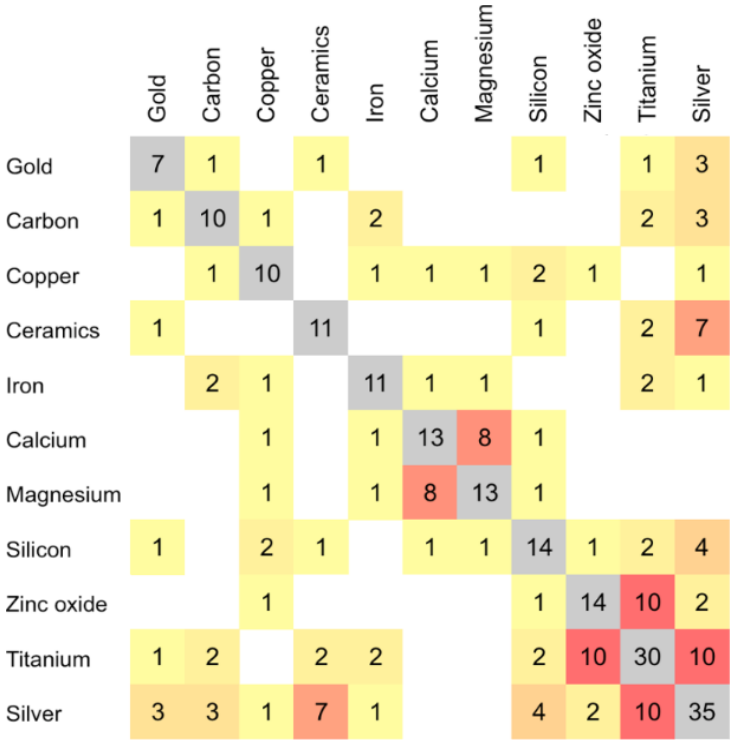

Figure 1.2 Major nanomaterial composition pairs in consumer products. Carbonaceous nanomaterials (carbon black, carbon nanotubes, fullerene, and graphene) were combined into the same category (carbon). Grey boxes in the diagonal represent the total times each nanomaterial composition has been listed with other compositions in the same product. *Culled from* (Vance et al., 2015).

Significant number of studies to address challenges pertaining to the properties of nanomaterials, their implications and specific needs for regulation are under progress (Ahlbom et al., 2009; Rasmussen et al., 2016; Singh et al., 2014). Analysis of the data that were derived for evaluating environmental fate and toxicity testing of nanomaterials concluded that environmental targets tested for conventional chemicals were applicable to nanomaterials. However, there is an urgent need for (i) characterization of the physico-chemical properties of nanomaterials, (ii) development of testing strategy on agglomeration behavior, (iii) preparation of dispersions of nanomaterials and (iv) spiking and dose metrics of nanomaterials during

environmental toxicity assessment of nanomaterials (Kühnel and Nickel, 2014; OECD, 2014). Though (biological) scientists argue that behavior of nanomaterials resembles "classic" pollutants such as organic molecules and metals, physico-chemical properties of nanoparticles and their association with adverse effects gained considerable importance in physics, chemistry and geology (Bottero et al., 2015). However, major challenge in nano-ecotoxicity relies on interdisciplinary approach, encompassed with meagre data pertaining to the effects of pristine forms of nanomaterials, quantities of nanomaterials that are released during their life cycle in environment. Despite evolution of ecotoxicity testing of a wide range of nanomaterials including Ag, Carbon nanotubes, CuO, ZnO and $TiO_{2,}$ ethical challenges in employing bioassays that were conducted in vertebrates still needs to be addressed. Though the existing guidelines for conventional toxicity testing shall be considered to be adopted for ENP, there is a need for concurrent development of emerging *in vitro* tests to address specific toxicity endpoints for ENP (e.g. reactive oxygen species generation). Studying small variations across ENP help in understanding the correlations between physicochemical properties and ENP toxicity. Outcomes of such studies definitely shed light on quantitative, mechanistic, pathway-based data generation (Nel et al., 2013). Moreover they also provide data for early stages of hazard assessment, strategies for advanced testing, and determination of starting dose or concentrations of ENP corresponding to further experiments (Kavlock et al., 2012; Lin et al., 2011) Therefore, combination of *in vitro* and *in vivo* for developing integrated approaches to testing and assessment (IATA) for ENP will further allow the development of predictive toxicology approaches. For screening toxicity of ENP, there is a need for adequate

qualitative and/or quantitative data so as to develop weight of evidence based approaches. Such strategies are needed in support of decision and risk assessment purposes. In this regard, comprehensive analysis of the data published on evaluation of TiO_2 nanoparticle toxicity by WPMN (OECD WPMN, 2017) revealed that: (i) Despite prioritizing important data relevant to routes of exposure, relatively few of such studies are documented with regard to TiO_2 nanoparticles, (ii) Till date, the TiO_2 nanoparticles were studied under inhalation exposure than from other exposure routes (such as dermal or ingestion). Furthermore, *in vivo* studies were performed mostly in animal models such as rats, whereas *in vitro* experiments were conducted largely on human cells. Furthermore, reports on physicochemical properties including crystalline structure (anatase and rutile) which can play a vital role in toxicity evaluation are meagre (Braydich-Stolle et al., 2009). Therefore, toxicological evaluation of TiO_2 nanoparticles with respect to their physico-chemical properties such as size, charge and crystal phase will be a right approach in this way forward. However, most of the available data in the literature, even for the same test species are highly variable (Juganson et al., 2015).

Titanium dioxide can exhibit amorphous or polymorphous crystalline states that include anatase, rutile, and brookite forms. All these three forms of TiO_2 are present in natural minerals. Phase composition of TiO_2 nanomaterials and their impact on environment is important to conclude the toxicological effects of these materials especially at nanoscale. However, risk assessment methods that are based on environmental modeling (Gottschalk et al., 2009; Mueller and Nowack, 2008), life-cycle assessment (Keller et al., 2013) were

excluding various forms of TiO_2. As a result, there exists a paucity of data regarding the effects of TiO_2 phase composition on various environmental compartments. Direct evidence of the coexistence of anatase and rutile forms of TiO_2 in wastewater samples is reported in the literature elsewhere (Tong et al., 2015). The study also stated that Ti released from wastewater treatment plant's sludge and final effluent, is composed of approximately 30% anatase and 60% rutile (the remaining 10% is ilmenite) and contains a significant fraction of TiO_2 that is <100 nm. Furthermore, different TiO_2 phase composition with a reversed relative abundance of anatase to rutile is possible in sediments located upstream and downstream of wastewater treatment plants. TiO_2-ENPs that are used in commercial sunscreens and allied products exhibit rutile crystalline structure rather than the anatase crystalline structure (which is dominant in the industrially produced TiO_2) (Lewicka et al., 2011).

Data pertaining to the toxicological effects related to various crystal phases of nano TiO_2 on sentinels is meagre (Handy et al., 2008; Kahru and Dubourguier, 2010; Klaine et al., 2012). Despite the progress in nanotoxicology, advancement in assessing the potential of ENPs to induce toxicity in sentinels raised more hypotheses than true answers. Therefore, knowledge of the fundamental aspects of environmental risks associated with ENPs is to be substantiated in several key areas of toxicity assessment. However, due to the relative newness of the nanotechnology per se, there exist knowledge gaps pertaining to potential hazards posed by TiO_2-ENPs on environmental sentinels such as earthworm. Elucidation of toxicity in environmentally relevant sentinels is lagging far behind the *in vitro* studies

that are conducted on human and other vertebrate cell lines. Hence evaluation of ENPs in environmental sentinel such as earthworm is imperative.

In lieu of the scenario presented above, present study is designed to (i) elucidate the presence of TiO_2 nanoparticles in environmental compartments using advanced methods such as Single Particle ICP-MS (ii) characterize r-TiO_2-ENP in terms of size, charge and agglomeration and (iii) develop an invertebrate based toxicity assessment for r-TiO_2-ENP by employing OECD-207 and identifying corresponding biomarkers in earthworm, *Eisenia fetida.*

The study comprehends (i) detection of TiO_2-ENPs in untreated and treated sewage and sludge (ii) physicochemical characterization of rutile TiO_2-ENPs (iii) Toxicity assessment of TiO_2-ENPs by OECD-207 guidelines and (iv) Measuring the biochemical responses *viz.* antioxidant enzymes and proteomic profiles of earthworms that were exposed to TiO_2-ENPs.

Outcomes of the study facilitate unveiling the role of size and charge in eliciting toxicity. Besides evaluating toxicity through dermal exposure, bioaccumulation of ENPs in earthworm and assessing their subsequent effects on oxidative stress were evaluated by studying the antioxidant enzyme activities [catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR)] in the earthworm. Moreover, release of thiobarbituric acid reactive substances (TBARS) measure will help in understanding the probable membrane perturbations caused by the nanoparticles in earthworm. The study will definitely provide more insights into the toxicity evaluation of other metal oxide nanoparticles which are

having similar characteristic features to TiO_2-ENPs. Antioxidant enzyme responses and proteomic profiles of earthworms that are exposed to TiO_2-ENPs will serve as the basis for establishing survival mechanisms and hints/ starting points for paraphrasing mechanisms of TiO_2-ENPs nanoparticles in soil environment.

Chapter 2

Review of Literature

2. Review of Literature

2.1 Detection and characterization of TiO_2 nanoparticles in the environment

Application of nanomaterials including titanium and silver in various sectors increased their market potential in recent times (Grand View Research, 2015). However, application of nanomaterials in various industrial, pharmaceutical and environmental processes inevitably release these particles into environment via sewage and biosolids (Benn and Westerhoff, 2008; Cornelis, 2015a; Landsiedel et al., 2010). Wastewater including treated effluent and bio-solids are the vital sources of nanomaterial discharge into the environment. Excessive production and use of TiO_2-ENPs may lead to their discharge into the environment during their production, transport, use and final disposal (Gottschalk et al., 2013; Lin et al., 2010). Currently, the market potential of TiO_2 production is approximately $2000 per ton (ICI, 2015). TiO_2 nanoparticles are used as one of the components in burn treatment creams, dental compositions and cosmetic lotions (Bindhu and Umadevi, 2015). Starting from their manufacture to disposal, various unit processes release TiO_2 nanomaterials into environment (Bakshi et al., 2015; Soni et al., 2015; Sun et al., 2016).

As a result, implications of TiO_2-ENPs have raised the concerns about their impact on environment and humans. Recent studies on exposure modeling of nanomaterials indicated that predicted concentrations of nano-TiO_2 in wastewater effluents (0.7–16 µg/L) were higher than the predicted no-effect concentration level (1 µg/L) (Gottschalk et al., 2009).

TiO_2-ENP is one of the most widely used metal oxide nanoparticles till date (Robichaud et al., 2009). In the year 2010, the production of TiO_2-ENP had increased to 5000 metric tons and is expected to increase by leaps and bounds by the year 2025 (Landsiedel et al., 2010). The study conducted by Weir et al., 2012 highlights the presence of nano TiO_2 in a variety of food and personal care products. These nanoparticles have been incorporated into a number of branded consumer products such as Neutrogena sensitive sunblock cream, M & M's chocolate candy, Colgate toothpaste, Mentos mint, Nestle coffee creamer etc. Thus widespread usage of TiO_2 nanoparticles leads to their occurrence in the environment and interaction with various components of ecosystems. It is therefore highly necessary to detect, quantify and assess the toxicological impacts of these nanoparticles in different environmental matrices such as organic material and conditions including pH and ionic strength. It is imperative that a framework for appropriate monitoring and risk assessment of these metals and associated nanoparticles in environmental matrices especially in biosolids and wastewater is gaining prominence across the globe.

Despite their wide application, information or reports regarding the occurrence of metals including Ti, and associated nanomaterials in wastewater and bio-solids is meagre (Tourinho et al., 2012). Hence, in recent times, studies that predict the fate and behavior of TiO_2 nanomaterials in wastewater and sludge, bio-solids amended soils are gaining attention. The major routes of entry of TiO_2 nanoparticles into the

terrestrial ecosystem would be application of sewage sludge on land, effluent from wastewater treatment plants, leachates and surface runoffs from exterior façade paints etc. (Menard et al., 2011). Hence, a terrestrial ecosystem that majorly encompasses soil is considered as one of the major sinks for ENPs at the end of their lifecycle. However little data is available with regard to environmentally relevant concentrations of TiO_2 nanoparticles in the terrestrial ecosystem. The concentration of metallic Ti in surface runoffs was estimated to be 600µg/L (Kaegi et al., 2008). In raw sewage, the concentration of Ti was found to be as high as 100-3000µg/L and 5-15µg/L (Kiser et al., 2009) in effluents majorly generated from wastewater treatment plants. Data pertaining to the ability of water treatment systems to deal with nanoparticle contaminants is unclear because of non-availability of standard methods to characterize nanomaterials in wastewater and sludge. Studies to address the knowledge gaps in this area remains as a top priority in environmental monitoring. Though few studies could elucidate the occurrence of TiO_2 nanoparticles in wastewater and sludge, (Pachapur et al., 2016) there is a need for developing methods to address knowledge gaps in this regard. Hence design of studies on detection and characterization of nanomaterials from environmental matrices have gained the prominence. The occurrence of TiO_2 nanomaterials in wastewater can also serve as a tracer or indicator for monitoring other widely used nanomaterials in environment (Kiser et al., 2009). Therefore, information on methods to characterize and quantify nanoparticles in environmental compartments is crucial to understand their transport, behavior, and fate in the environment. Such data also assists in arriving at toxicological evaluation of nanoparticles at environmentally realistic concentrations.

2.2 Toxicity assessment of engineered nanoparticles

Previous investigations on the health risk and environmental impacts of TiO_2 nanoparticles reported their potential to cause toxicities in a variety of living beings aquatic fish, daphnia, algae and microbes. Toxicity of TiO_2 nanoparticles in benthic species was also evaluated (Li et al., 2014). Recent reports on TiO_2 nanoparticle toxicity (Coll et al., 2016; Cornelis, 2015b) demonstrated their potential to cause harmful effects on humans and the environment. Moreover, occurrence of Ti and other heavy metals such as Zn and Cu and their potential to cause environmental toxicity is emphasized in recent reports (Cupi et al., 2015; Holden et al., 2014). Experimental data about acute and chronic toxicity and environmental fate of engineered nanomaterials score lower by far: tested materials are less than 20% in this respect. Toxicity assessment of ENP relies heavily on accurate physical and chemical characterization of these materials. This concern has been addressed by many authors (Domingos et al., 2009; Klaine et al., 2008; Oberdorster et al., 2005; Powers et al., 2007). Hence a list of essential characterization parameters has been developed. The characterization challenges include accurate determination of size, surface properties, aggregation characteristics & mechanisms, solubility characteristics, variability in ENP preparation, and behavior & interaction of ENMs in various matrices (Pettitt and Lead, 2013). Apart from characterization, toxicity studies in terms of exposure, dose, hazard and risk are to be studied for engineered nanoparticles. To date, however, nanoparticle toxicity studies have mainly focused on *in vitro* examinations due to the ease of execution, control and interpretation of the experiments compared to *in vivo* tests. Therefore, consideration must be placed on

improving *in vivo* experimental procedures of nanoparticle toxicity assays in order to gain reliable data (Kong and Zepp, 2012). Methods of toxicity assessment of nanomaterials should essentially comprise a list of short and long term effects on species that inhabit aquatic and terrestrial habitats (Gourmelon et al., 2007). Firstly, acute toxicity studies in the sentinel organisms should be performed, which should be followed by studies that focus on morphological, ethological and biochemical changes. This would provide a comprehensive outlook on the ecotoxicity of nanoparticles in a given ecosystem.

All these intricate requirements that are specific to ENP make it difficult to adopt existing methodologies those were put forth by Organization for Economic Cooperation and Development (OECD) guidelines for the risk assessment of ENP. Hence necessitates a comprehensive and strategic framework for the assessment of ecotoxicity of ENPs. There is an unprecedented need for establishing such a comprehensive and robust risk assessment framework, especially for metal oxide based nanomaterials that are rapidly proliferating in the consumer markets.

2.3 Toxicity evaluation of TiO_2 nanoparticle in invertebrates as sentinels/ indicators

Among the crystal phases of TiO_2-ENPs, rutile phase of TiO_2 has the highest refractive index and relative scattering power when compared to other forms *viz.* anatase and brookite. Hence it is used as an agent in bringing opacity to paints, inks, toothpaste etc. It is also used as a pigment and thickener in cosmetic products. Another useful property is its capacity to

absorb potentially damaging ultraviolet light (UV) light. Hence it is employed in manufacturing of plastics, sunscreens and other applications. The anatase phase, on the other hand, has comparatively less refractive index and light scattering capacity but is well known for its photocatalytic property, hence used in catalytic and photo-catalytic applications (Mueller and Nowack, 2008). On a global scale nano TiO_2 (rutile form) has much wider applications especially in consumer products in comparison to the anatase form. Due to all the above mentioned reasons, titanium dioxide nanoparticle in rutile crystal phase has higher potential to get released into the environment and cause potential hazards during exposure. This is one of the major reasons for that rutile TiO_2 nanoparticles are the most extensively studied metal oxide nanoparticles in terms of toxicity assessment (Cattaneo et al., 2010; Kahru and Dubourguier, 2010). Data pertaining to the toxicity of TiO_2-ENP evaluation using invertebrates and plants in terrestrial environment is lagging.

Nanoparticles undergo aggregation and sedimentation after being introduced into water (Boxall et al., 2007). This could facilitate the interaction between nanoparticles and soil dwelling organisms. The exposure of these organisms and hence bioavailability of nanoparticles would depend on several factors such as concentration, size, surface characteristics, state of aggregation and agglomeration as well as adsorption to various environmental media such as sediments, biofilms etc. (Fent, 2010). Till date, there is meagre data available on toxicity evaluation of TiO_2 nanoparticles in the terrestrial ecosystem that addresses the influence of some of the factors mentioned on the interaction

of these particles with terrestrial biota. Scarce ecotoxicological data exists and future emission rates of such engineered nanoparticles are still unknown. These facts compromise the ability to predict the impact of ENP on terrestrial communities (Roh et al., 2010). Data on the mobility and transport of ENPs into the soil matrix is meagre (Doshi et al., 2008). In this context, ecotoxicity assessment studies on nanomaterials should essentially comprise a list of short and long term effects on species that inhabit aquatic and terrestrial habitats (Gourmelon et al., 2007). Hence preliminary studies on exposure assessment of nanomaterials should ideally begin with invertebrate models of any ecosystem followed by sentinels of higher trophic levels in the food chain. This would help in understanding the effect of nanoparticles on simple organisms and facilitate the estimation of parameters like bioaccumulation at different trophic levels in the food chain. However, studies that are focusing on toxicity evaluation of nano TiO_2 on terrestrial invertebrates are limited compared to aquatic invertebrates. Till date, only a few studies have been documented with regard to ecotoxicity of TiO_2 nanoparticles on terrestrial invertebrates such as oligochaetes (earthworms, *Eisenia fetida*), nematodes (roundworm, *Caenorhabditis elegans*) and isopods (woodlouse, *Porcellio scaber).* Amongst these invertebrate organisms, earthworms have long been considered for ecotoxicological testing of industrial chemicals and pesticides by global organizations such as OECD and Food and Agriculture Organization (FAO). Despite belonging to the lower class of invertebrates, earthworms also possess highly differentiated organs, tissues and an immune system that are almost comparable to humans, hence serve as an appropriate indicator organism for evaluating toxicity of TiO_2 nanoparticles. Whereas some studies related to the toxicity of TiO_2 nanoparticles on terrestrial isopod,

Porcellio scaber and soil nematode, *Caenorhabditis elegans* are discussed below. A detailed discussion on earthworm is given in the section 2.3.1.

In the study done by Jemec et al., (2008), the effect of ingested TiO_2 (anatase) nanoparticles (particle size 15nm) on *Porcellio scaber* was studied for a duration of 3 days. Concentrations of 0.5, 1, 10, 100, 1000, 2000 and 3000µg nano TiO_2 per gram food were used. Characterization of TiO_2 nanoparticles was carried out by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) and aggregation of particles were also observed. Lower end points such as the activity of antioxidant enzymes catalase and glutathione-S-transferase were estimated and correlated with higher level endpoints such as weight change, feeding rate, feeding level efficiency and survival of the organism. It was observed that activities of catalase and glutathione-S-transferase enzymes affected the digestive glands. However, the response was not dose-dependent. Sonication seemed to alter the toxic potential of TiO_2 suspensions. The authors also suggest that the influence of other properties of TiO_2 nanoparticles on the response of enzymes needs to be understood, hence warrant further studies on similar lines.

Uptake of TiO_2 leads to disruption of cell membrane integrity in the digestive gland cells of *Porcellio scaber* following a 14 day exposure period to food dosed with nano TiO_2 (Novais et al., 2012). Exposure concentrations used were 100, 1000, 3000 or 5000 µg nano TiO_2 per gram food and the particles were characterized by TEM, DLS, Brauner Emmett and Teller (BET) method

and X-ray powder diffraction techniques. Endpoints such as feeding parameters, survival, weight change and digestive gland membrane stability were studied. Significant differences could not be observed in feeding parameters, survival and weight change. However, digestive gland epithelial cell membrane destabilization and hence internalization of nano TiO_2 was reported. The authors also reported that cell membrane destabilization was not dose dependent but it influences the internalization of nano TiO_2 to a large extent.

In the study done by Wang et al., (2009), toxicity of nano TiO_2 in comparison to its bulk counterpart, to the soil nematode *Caenorhabditis elegans* was tested. The tested concentrations of TiO_2 nanoparticle suspensions were 24.0, 47.9, 95.9, 167.8, and 239.6 mg/L and the particles were characterized by TEM and DLS. Aggregation of the particles was reported. The endpoints assessed were lethality, variations in worm length, number of eggs and offspring produced per worm. The authors of this study indicated that the nano TiO_2 exerted significant toxicity on *C.elegans* in comparison to its bulk form. They also suggested that toxicity of nanoparticles could be influenced by parameters such as chemical composition, shape, size, surface area and dissolution characteristics. The study is first of its kind to evaluate the toxicity of metal oxide nanoparticles in *C.elegans* and also highlights the need for integrated toxicity assessment strategies for the terrestrial and aquatic ecosystems. Ji-Yeon Roh *et al* (2010) investigated the effect of different sizes (7 & 20nm) of nano TiO_2 on *C.elegans* by studying multiple toxicity endpoints such as growth, fertility, stress-response gene expression and survival. Nanoparticle characterization was carried out by

DLS and TEM. Particle aggregation was reported to be controlled by sonication. The study indicated that smaller sized TiO_2 nanoparticles are more toxic to the test organism than larger sized particles. In this study, the authors had tried to establish a correlation between variation in certain gene expression patterns and organismal level responses in the worm after exposure to nanoparticles. However, a definitive correlation could not be established between these endpoints and hence proposed further investigation. The authors concluded that nano TiO_2 exerts a toxic effect on fertility and survival of *C.elegans.* They also highlighted the need to understand the mechanism of toxicity of nano TiO_2 and various parameters that influence the same. Q. Wu et al., (2013) studied the toxicity of TiO_2 nanoparticles (particle size 30nm) on *C.elegans* with respect to growth, reproduction, locomotion behavior, reactive oxygen species (ROS) production and lethality as toxicological endpoints. Particle characterization was done by TEM and DLS and sonication of the nanoparticle suspensions were carried out to obtain homogeneous suspensions. The study reports that locomotion behavior and ROS production were more sensitive endpoints than the others in *C.elegans* that were exposed to environmentally relevant TiO_2 nanoparticle concentrations. The authors also reported the association between induction of oxidative stress and alterations in other endpoints and suggest the application of antioxidants such as ascorbate to retrieve the toxicity exerted by the nanoparticles in the worm. The authors concluded that nano TiO_2 causes adverse effects in *C.elegans* and underscores the role of oxidative stress in the induction of toxicity.

TiO_2 nanoparticles (particle size 10nm)induce acute toxicity to *C.elegans* with mutations of certain susceptible genes that are associated with

oxidative stress and stress response (Rui et al., 2013). Nano TiO_2 concentration found in environmental matrices (20µg/L) and food products (25 mg/L) have been considered for the study. Characterization of nanoparticles was carried out by TEM and DLS and sonication were done to obtain homogenized suspensions of nanoparticles. The authors report that acute exposure to TiO_2 nanoparticles at the above stated concentrations did not affect the development and survival of the worms. However, acute exposure at 25 mg/L adversely affected reproduction, locomotive behavior and significantly induced ROS production (as observed from gene expression levels) in mutant worms. From this study, the authors suggest that genetic mutations of susceptible genes involved in oxidative stress response together with other sensitive endpoints could be applied for toxicity assessment of nanoparticles. They also warranted complete characterization of nanoparticles and use of predicted environmentally relevant concentrations of nanoparticles during investigation of such susceptible genes.

In an article by Angelstorf et al., (2014), the influence of particle size on toxicity of anatase TiO_2 nanoparticles (21nm) in comparison to its bulk form (~160 nm) was investigated in *C.elegans*. The induction of oxidative stress by TiO_2 nanoparticles under light and dark conditions was also investigated by the measurement of ROS production. DLS and Scanning Electron Microscopy (SEM) were used to characterize both nano and bulk particles. Agglomeration of both particle types was reported. However, a clear correlation between nanoparticle concentration and particle size could not be observed. The study also reports the inhibition of growth and

reproduction of the worm by nano TiO_2 in comparison with its bulk counterpart. In addition, the authors reported an increase in toxicity of nano TiO_2 after irradiation, which was not the case with its bulk form. Prolonged exposure of *C.elegans* to nano TiO_2 resulted in agglomeration and severe accumulation of the particles in the intestine leading to its deformation. In contrast, prolonged exposure of the worm to bulk TiO_2 resulted in even distribution of particles in the intestine with no formation of large agglomerates. In this study, the authors attribute differences in physicochemical characteristics between bulk and nano TiO_2 to the varied toxicological response of the worms. The authors also concluded that primary particle size and crystalline structure could influence the toxicity of titanium dioxide.

The studies mentioned above have investigated toxicity of titanium dioxide nanoparticles in two important bioindicators of the terrestrial ecosystem namely terrestrial isopod, *Porcellio scaber* and soil nematode, *Caenorhabditis elegans*. Different endpoints were assessed in each study for the evaluation of TiO_2 nanoparticle toxicity. Most of the studies are conducted using anatase form which is of 20 nm size. However, the details of physicochemical properties of nano TiO_2 to the response of various toxicological endpoints in order to develop a holistic toxicity assessment approach for nano TiO_2 in soil sentinels is not reported. This necessitates the need for studies that investigate ecotoxicity of nano TiO_2 in soil sentinels with regard to physicochemical parameters and their influence on toxicological endpoints in order to better understand the interactions of nano TiO_2 with such indicator organisms in the environment.

2.3.1 Earthworm as a sentinel for TiO_2 ecotoxicity assessment

The use of earthworms for toxicity testing is highly recommended by many toxicologists and is considered as a much preferred indicator organism for monitoring pollution in the environment (Fourie et al., 2007; Rao and Kavitha, 2004). Earthworms are key species within soils and can get exposed to anthropogenic compounds through both their external and internal surfaces. For these reasons and others, these invertebrates are widely recognized as useful indicators of soil quality and are widely used as model organisms in terrestrial ecotoxicology (Tourinho et al., 2012). Over the last few decades, a large number of useful toxicological biomarkers that are applicable under both laboratory and field conditions have been developed in earthworms (Bundy et al., 2002; Scott-Fordsmand and Weeks, 2000). *Eisenia fetida* and *Eisenia andrei* are the commonly used species for standard toxicity tests (OECD, 1984) and ISO ecotoxicological studies, under acute and chronic exposure. *Eisenia fetida* is recommended in a number of standardized tests (OECD, 1984), USEPA OCSPP 850.3100 Earthworm Subchronic toxicity test (USEPA 2012), ISO 11268-1:2012 acute toxicity test (ISO 2015). This is due to its short life cycle, high fecundity rate, relative ease of cultivation and commercial availability (Brami et al., 2017).

In recent times, a battery of biomarkers suitable for proving pollutant induced physiological changes at different levels of functional complexity has been developed in this earthworm species. Since little information is available on the toxicity produced by engineered nanoparticles, simple *in vivo* toxicity models are need of the hour. Earthworms as model organisms,

to study the ecotoxicological effects as per the OECD 207 guidelines, have been widely used in many studies. But such studies scarcely have focused on engineered nanoparticles as toxicants. Moreover, studies that are focusing on toxicity evaluation of TiO_2 nanoparticles with earthworm as the test organism are scarce. In this regard, few studies that have assessed biochemical responses of earthworm exposed to TiO_2 nanoparticles are provided below:

In the study done by Hu et al., (2010) *Eisenia fetida* was exposed to TiO_2 rutile nanoparticles having an average diameter of 10 - 20nm in artificial soil test (OECD 207 guidelines) for a duration of 7 days. The concentrations of TiO_2 nanoparticles used were 0.1, 0.5, 1 and 5mg/Kg in artificial soil containing distilled water. The endpoints such as bioaccumulation, activity of enzymes such as superoxide dismutase (SOD), catalase (CAT), cellulase, lipid peroxidation and genotoxicity were estimated. It was observed that TiO_2 nanoparticles were indeed toxic to the worms when the concentration exceeded 1g/Kg. Bioaccumulation was observed beyond 5g/Kg. The activity of cellulase decreased with increase in concentration of TiO_2 nanoparticles. The authors had also studied the activity of antioxidant enzymes, lipid peroxidation and DNA damage. Oxidative stress through ROS production was thought to be the main mechanism of toxicity elicitation by nanoparticles (Kohen and Nyska, 2002). The activity of CAT was highest at 1g/Kg but a decrease in activity was observed at 0.5g/Kg. The activity of SOD was highest at 0.5g/Kg after which a gradual decrease in activity with an increase in the concentration of nanoparticles was observed. This indicated that clear dose

response relationship between enzyme activity and concentration of nanoparticles is meagre. However, at higher concentrations (1g/Kg), the activity of SOD and CAT decreased slightly as the antioxidant defense system was overwhelmed. As a result, increase in malondialdehyde indicated lipid peroxidation and DNA damage at 1g/Kg and 5g/Kg. The authors concluded that TiO_2 nanoparticles could accumulate and induce harmful effects in the worm.

In the study done by Canas et al., (2011), acute and reproductive toxicity of anatase TiO_2 nanoparticles to *Eisenia fetida* were studied for a duration of 14 days by filter paper contact test, sand acute test and artificial soil test. The study was conducted at concentrations 0.1, 1, 10, 100, 1000, 5000 and 10,000 mg/L. The nanoparticles were characterized by SEM and DLS. Aggregation of particles with increase in concentration was evident at 1-100mg/L. Earthworms survived at all concentrations of TiO_2 nanoparticles on filter paper. In sand test, a significant acute effect of TiO2 nanoparticles on worms was not observed. In artificial soil test, a decrease in cocoon production with an increase in the concentration of nanoparticles was reported. However, a significant dose response relationship could not be established. Aggregation of particles was considered as the possible mechanisms of TiO_2 nanoparticle toxicity.

Bioaccumulation of TiO_2 nanocomposite coated with $Al(OH)_3$ and PDMS (Poly dimethyl siloxane) affected the frequency of apoptosis in the model organism *Lumbricus terrestris* (Lapied et al., 2011). The study was carried out in three different exposure media with the following concentrations of

TiO_2 nanoparticles: Water (1, 10, 100mg/L); dry food (10 & 100mg/Kg); soil (15mg/Kg). The duration of exposure was 7 days (water) and 2-8 weeks (soil). Characterization studies were not carried out. The apoptotic frequency was highest at 100mg/L in water as exposure media. In soil test, at 15mg/Kg, an increase in apoptosis was observed in cuticle tissue. The authors suggested that there is no significant difference in the bioavailability of the compound in water and soil. They also highlighted that apoptosis is tissue specific response and it could be a better and more sensitive endpoint for ecotoxicity assessment of nanoparticles. No bioaccumulation of TiO_2 nanocomposite was reported. The study concluded that coated TiO_2 nanocomposites have reduced the propensity of ROS generation thereby causing oxidative stress.

In the study done by McShane et al., (2012), two test organisms *Eisenia fetida* and *Eisenia andrei* were exposed to TiO_2 nanoparticles of different sizes and composition (5 nm-100% anatase; 10 nm-100% anatase & 21nm-80% anatase and 20% rutile). Field test and artificial soil test were carried out at concentration 200-10,000mg/Kg and endpoints such as mortality, reproduction, juvenile growth and avoidance behavior were assessed. TEM, DLS and BET methods were used to characterize the particles. The authors reported that survival of earthworm adults as well as their offspring was not affected by TiO_2 nanoparticles even at concentrations as high as 10,000mg/Kg. The authors explained the effects of constituents of a complex matrix such as soil on the characteristics and behavior of the particles. Earthworms avoided the soil amended with nano TiO_2 at concentration >1000mg/Kg. No avoidance behavior was

observed in soil amended with micro-sized TiO_2 (control). The authors thereby emphasized the role of particle size and specific surface area in the avoidance behavior of the worms. The study also suggested that soil chemistry and adsorptive properties of the nanoparticles could have significant influence on behavior of the worms.

Design and development of rapid, sensitive, cost-effective, and socially non-controversial biomarkers in invertebrates is need of the hour. They assist in understanding the survival mechanisms when exposed to nanoparticles. Such invertebrate based biomarkers aid in divulging the toxicity mechanisms in parallel with existing protocols that are designed for non-invertebrate models. The emergence of molecular biology techniques led to the development of rapid and sensitive methods to identify biomarkers in toxicological evaluation of various compounds including nanoparticles.

Assessment of responses such as antioxidant enzymes, genes and proteins in earthworms that are exposed to nanoparticles help in predicting their detrimental effects (Forbes et al., 2006; Naaby-Hansen et al., 2001). In order to arrive at a comprehensive understanding of nanoparticle toxicity and associated perturbations, a global analysis of the biological responses to nanoparticle exposures and corresponding biomarkers should be performed at the molecular level. In recent times, advancements in analytical chemistry have greatly improved the process of identification of biomarkers in toxicity evaluation by employing global biomarker approaches such as genomics, proteomics, transcriptomics and metabolomics. These 'omics'

serve as a promising tool in ascertaining gene, protein or metabolite alterations which are indicative of the mechanistic action of compound or chemical on the organisms.

Divulging the expression of genes at protein level is called as proteomics. It provides glimpses of direct measurement of protein expression and insight into the activity state of all relevant proteins (Naaby-Hansen et al., 2001). Hence application of proteomics approach will aid in identification of proteins to decipher the toxicological effects of stressors (Dowling and Sheehan, 2006; Lemos et al., 2010; López-Barea and Gómez-Ariza, 2006). However, proteomics research in earthworms lags far behind when compared to other model organisms such as *Mus musculus* and *Caenorhabditis elegans*. Furthermore, proteomic approach was used in demonstrating the specific signatures of the proteins in *Eisenia fetida* exposed to chemical warfare agents [14]. In recent times, proteomic studies on earthworm, *Eisenia fetida* that is exposed metal based drugs (Guo et al., 2015), Cadmium (Wang et al., 2010) and an organic compound, phenanthrene (S. Wu et al., 2013) is documented. But considerable information on protein profiling using proteomic approaches in earthworms in response to TiO_2 nanoparticles is scanty. On the basis of the studies discussed above, there exist knowledge gap pertaining with respect to the following:

- Data on detection and characterization of TiO_2 nanoparticles in environmental compartments is still emerging and there is a need for such studies for benchmarking the concentrations of nanoparticles in toxicity evaluation.

- Though physico-chemical characterization of nanoparticles is carried out, supplementing such studies is needed to for comprehending the role of agglomeration of TiO_2 nanoparticles in a given medium.
- Toxicity evaluation of TiO_2 nanoparticles in soil sentinels will contribute the repository of guidelines that are employing earthworms as sentinels.
- Divulging of oxidative stress and other molecular markers when exposed TiO_2 nanoparticles via diverse routes of exposure such as skin contact and ingestion contribute to the identification of possible biomarkers of acute toxicity TiO_2 nanoparticles.
- Information on proteomic profiles of a soil sentinel, earthworm to TiO_2 nanoparticles to aid in mechanism based toxicity assessment.

In summary, vast and diverse applications of TiO_2 nanoparticles in the industrial and consumer domains have undoubtedly raised concerns about their fate and unpredictable interactions and impacts on flora and fauna. As the soil is one of the major sinks for TiO_2 nanoparticles, the exposure of its biota to these nanoparticles becomes inevitable. In addition, these interactions are governed by the characteristics of the nanoparticle as well as physiological and environmental factors. Although there are studies that are documenting the ecotoxicity potential of TiO_2 nanoparticles in terrestrial invertebrates including earthworms, supplementing such studies with recent findings in this regard is important to comprehend the future needs of nanoparticle's toxicity assessment *viz.* designing guidelines for toxicity assessment, identification of biomarkers of toxicity and sentinels to evaluate

toxic potential of nanoparticles etc. Hence it underscores the need for more comprehensive studies that encompass a multitude of factors that govern their toxicity. In this regard, knowledge gaps are described in the following chapter.

Chapter 3

Knowledge Gaps in Existing Research and Objectives

3.1 Knowledge gaps in TiO_2 nanoparticle toxicity evaluation

In the backdrop of information on risk assessment of nanomaterials, efforts in this pursuit are being focused on safety management of ENP across the globe. Working Party on Manufactured Nanomaterials (WPMN) of the Organization for Economic Co-operation and Development (OECD) is involved in reviewing the efforts with regard to the use of *in vitro* and other alternatives to traditional animal toxicology studies (A. Clunan, 2014). In this context, incorporation of *in vitro* tests with alternative testing strategies will assist in obtaining significant confidence in results. It can provide important information with regard to potential toxicity mechanisms of ENP under a given dose/ concentration. Hence a comprehensive testing strategy for hazard and risk analysis of ENP is possible in the future (OECD WPMN, 2017).

In the state of the art of the literature available, reported doses of TiO_2 nanoparticles/nanomaterials and the method used to prepare the nanomaterials for toxicity testing found to be varied among the laboratories utilizing similar organisms. Currently, the investigation of behavior and effects of ENPs in the environment still have explorative character and raise more hypothesis than true answers (Nowack and Bucheli, 2007). Therefore, new testing strategies are needed to delineate the emerging toxicity mechanisms of nanomaterials. Quantitative measures of both exposure and effects are need of the hour to implement environmental and safety management of nanomaterials.

Behra and Krug (Behra and Krug, 2008) substantiated the important problems and associated factors to address the gaps in nanoecotoxicology within the next few years:

i. the choice of nanoparticles in biological experiments, and the tests needed to characterize them before, during and after these experiments, need to be determined
ii. the need to examine the route of uptake of synthetic NPs by organisms in different environments (important for the behavior of synthetic NPs in the food-chain)
iii. the choice of organisms and endpoints measured
iv. The progress of the study about the nanomaterials behavior, fate and effects in the environment depends on the standardization of following issues [7]:
    - Development of standardized methods for formulating test media (both soil/sediment and water) for assessing fate and effects of nanoparticles during toxicity testing.
    - Standardization of characterization requirements for particles and particle suspensions.

Addressing the above issues is very important to interpret results of various nanoecotoxicity studies. Key challenges and knowledge gaps in the toxicity testing are presented **Figure 3.1** to derive a right strategy for environmental risk assessment and monitoring of nanoparticles.

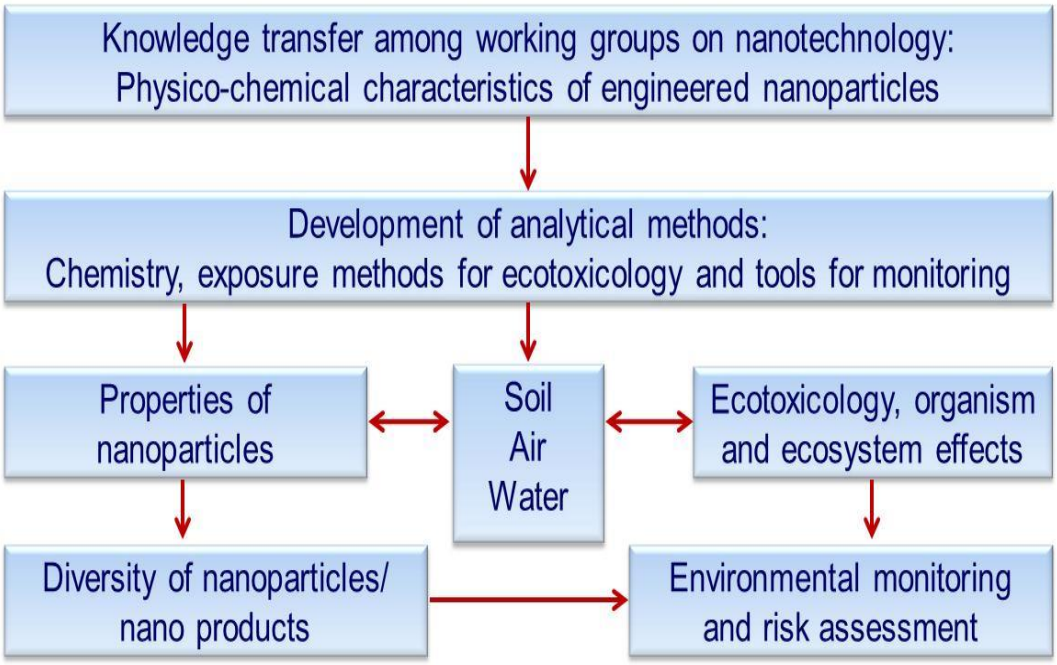

Figure 3.1 Key challenges and knowledge gaps in the toxicity evaluation of nanoparticles.

Occurrence, behavior and fate of engineered nanomaterials in the environmental matrices are largely unknown (A. Clunan, 2014). Evaluation of potential environmental hazards of various nanoparticles should be performed before their prior use in various products and ultimate release into the environment. Although the standardized protocols for evaluation of the toxicity of conventional chemicals are available from various guidelines, validating their applicability for testing nanomaterials has gained prominence(Rasmussen et al., 2016). Hence characterization of nanomaterials in environmentally relevant media is needed to establish their behavior and environmental fate. There is also a need for developing methods to detect and characterize nanoparticles in various media.

Comparing and validating outcomes of such studies aid in framework for nanoecotoxicology studies (Stone et al., 2010).

The behavior and effects of nanomaterials on the environment are largely unknown. Recent investigations demonstrated the possible effects of nanomaterials are of human concern. Most of the investigations are referred to the effects of nanoparticles on large wildlife. However, basis of many food chains depends on the benthic and soil flora and fauna, which could be dramatically affected by the release of nanomaterials in the environment. As the ultimate sink of nanomaterials is the sediment or soil, research is needed in soil environment/ compartment. It necessitates studies on effects of nanomaterial on terrestrial environment and organisms (S J Klaine et al., 2008). Standard guidelines designed for toxicity testing of various nanomaterials are based on the survival and reproduction rate of sentinels (Kühnel and Nickel, 2014). But data pertaining to the effect of nanomaterials at the sub cellular levels are scarce in the terrestrial species including earthworm. Moreover, the studies which link lethal concentrations of ENPs and the toxicity end points are lacking to address challenges in toxicity evaluation of nanoparticles in terrestrial ecosystem.

From the literature, it is evident that research is needed to validate the applicability of existing methodologies to assess environmental toxicity of nanoparticles. Experimental data on correlating physicochemical characteristics, fate, behavior of nanoparticles and their potential to induce

toxicity in different environmental receptors are still needs to be explored (Rasmussen et al., 2016).

3.2 Choice of nanoparticle and characteristics

According to Stone et al., (2010), six parameters have been prioritized for toxicity assessment of nanoparticles. They are size, dissolution, surface area, surface charge, and surface chemistry. Till date, out of the 4 studies that investigated toxicity of TiO_2 nanoparticles on earthworm, only McShane et al., (2012) reported near to complete characterization of the TiO_2 nanoparticles in terms of particle size, agglomeration and specific surface area. In most of the other studies soil has been used as the exposure medium. However the behavior of nanoparticles in such complex matrices was inadequate to understand the effect of parameters like pH, ionic strength, nature of electrolytes (Navarro et al., 2008; Sharma and Sharma, 2012) and the presence of organic materials (Domingos et al., 2009; Pettibone et al., 2008) on agglomeration of nanoparticles. Two other characteristics of TiO_2 nanoparticles include: (i) crystal phase viz. anatase, rutile and brookite and (ii) the presence or absence of external coating on the nanoparticle that dictates the interaction of the nanoparticles with the exposure medium, target organisms such as earthworms. Subsequently, there exist scarce data with regard to physicochemical characterization and assessment of agglomeration behavior of rutile nanoparticles.

3.3 Nature of sentinel organism

Till date, ecotoxicology developed mostly as aquatic toxicology. Terrestrial ecotoxicological studies lag behind aquatic ones. It is also evident from the literature that the variability of experimental conditions was found to be the

basis of inconsistent (eco) toxicological data of specific nanomaterials. Therefore, it is essential to perform the validation of the collected data rather than providing quantitative information on ecotoxicological effects of nanoparticles (Kahru and Dubourguier, 2010). These aspects have been scarcely assessed in soil sentinels. Future emission rates of TiO_2 NPs are still unknown and scarce ecotoxicological data exists, compromising the ability to predict their impact on terrestrial communities (Mueller and Nowack, 2008; Roh et al., 2010). Earthworms are one among the ecosystem engineers that play a key role in soil functioning, and are also used extensively in ecotoxicity studies. But examination of the effects of TiO_2 nanoparticles on earthworms is just emerging (Canas et al., 2011; Jemec et al., 2008; Lapied et al., 2011). Furthermore, three earthworm species viz. *Eisenia fetida, Eisenia andrei* and *Lumbricus terrestris* have been considered for ecotoxicity assessment nanoparticles till date. Though the organisms belong to the same class, species level difference would influence their behavior and biochemical response to rutile TiO_2 nanoparticles. Hence this could also be attributed as one of the reasons for dose response variations. At the molecular level, however, the dissimilar biological response of the worms in terms of different endpoints such as activity of antioxidant enzymes, lipid peroxidation, and proteomic profiles to varying concentrations of rutile TiO_2 nanoparticles needs attention. In order to enrich such data, there is a need for studies with regard to toxicity evaluation of rutile TiO_2 nanoparticles in earthworm.

3.4 Bio-monitoring and risk assessment of ENPs: need of the hour

Though the toxicity assessment strategies for TiO_2 nanoparticles is in soil sentinels is emerging, studies pertaining to the rutile type of

nanoparticles is not well defined and are based on traditional chemical risk assessment strategies without taking into consideration their unique properties compared to their bulk counterparts. Hence understanding the inherent behavior of these particles in various exposure scenarios, which in turn is dictated by the physicochemical characteristics, is important. Though physicochemical characterization of nanoparticles including TiO_2, by various investigators (Domingos et al., 2009; Stephen J Klaine et al., 2008; Oberdorster et al., 2005), there is a need for lack of particle specific information during toxicity assessment (Stone *et al.,*2010). A review by Michala Pettitt and Jamie Lead (2013) underscores the importance of these issues that need to be addressed for accurate characterization of nanoparticles. They defined nanomaterials in terms of size, core properties, surface properties, aggregation and solubility characteristics, accounting for variability in nanomaterial preparation and dynamics of nanomaterial in various environmental matrices. This would aid in accurate and reliable characterization of the particles. The next step in risk assessment framework would be to assess the impact of these pollutants on organisms' representative of various ecosystems. Interaction of these pollutants with the organisms would vary temporally and spatially and hence would aid in accurate quantification of the effects of ENMs in various exposure scenarios. However, this is a farfetched benchmark for risk assessment as it is interdependent on particle characteristics, fate, transport, behavior, bioavailability in the environment. Other crucial factors to consider would be the effect of pH, ionic strength, nature of electrolytes, presence of organic acids etc. on the particles as they could alter particle characteristics. In order to understand the interdependent effects of nanoparticles and their interacting matrices, sensitive endpoints such as variation in the activity of

antioxidant enzymes, lipid peroxidation, genotoxicity, apoptosis, mitochondrial damage etc., in the model organisms should be considered as potential biomarkers. Such studies already exist for various ecosystems but there are gaps that have to be addressed by future research.

In this regard, ecotoxicity data which considered sensitive endpoints for TiO_2 nanoparticles especially with respect to the rutile crystal phase, the most commercially exploited metal oxide nanoparticle are available for the aquatic ecosystem. Similar type of data is very scarce for the terrestrial ecosystem which is one of the major sinks for these nanoparticles. There are few documented studies involving three types of invertebrates (oligochaetes, isopods and nematodes) representative of the terrestrial ecosystem that are available. This situation highlights the urgency and need for exposure assessment data on ecotoxicity of TiO_2 nanoparticles in the terrestrial ecosystem.

The ecotoxicological effects of nanoparticles are still poorly documented while their commercialization for industrial and household applications increases (Simon-Deckers et al., 2009). TiO_2 nanoparticles are the second most widely used metal oxide nanoparticles after SiO_2 (Piccinno et al., 2012). However, it is still not clear exactly how, at which concentrations, and in what form TiO_2 nanomaterials will show their impact on the environment. In this context, there exist data gap with regard to rutile TiO_2 nanoparticle's toxicity. Hence understanding toxic effects of these nanoparticles including sub lethal effects is essential to comprehend the ecosystem responses. Outcomes of such experiments will provide insights for nanotoxicology

studies that are aimed at divulging the toxicity of metal oxide nanoparticles that are possessing similar characteristics as TiO_2 nanoparticle in the future. In this context, the following **Figure 3.2** represents the key question and gaps in toxicological evaluation of rutile TiO_2 nanoparticles.

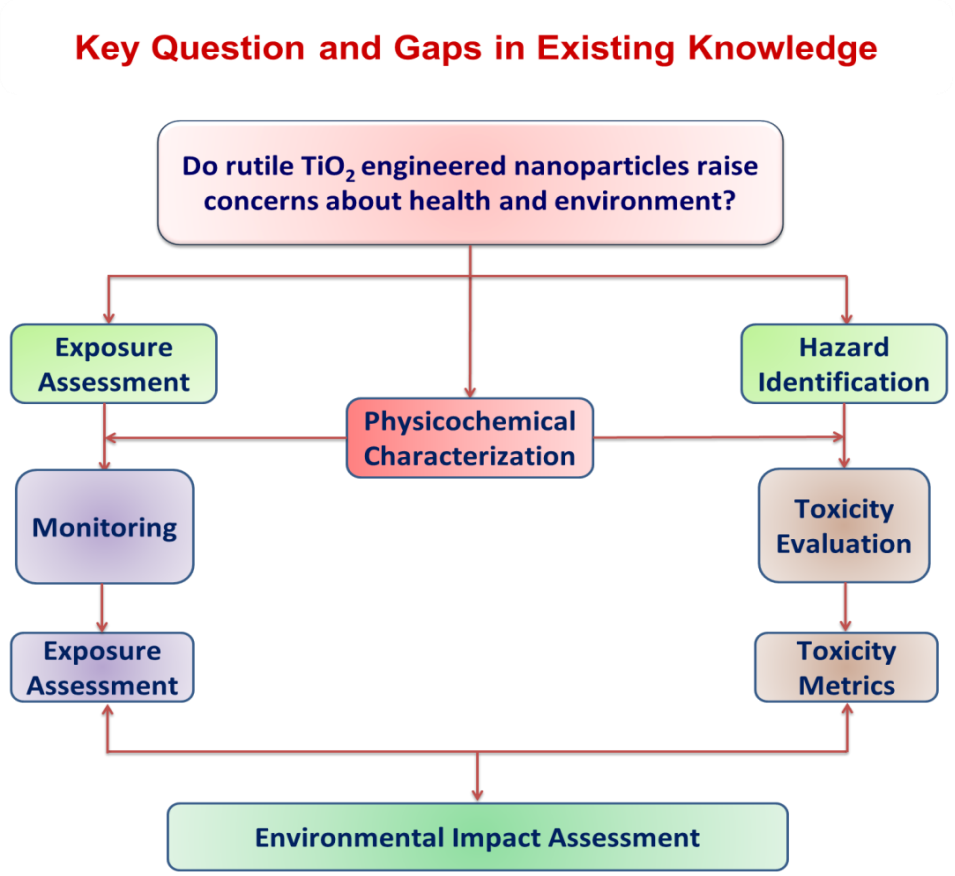

Figure 3.2 Key question and research gaps in the toxicity testing of TiO_2 nanoparticles

3.5 Objectives and Key Questions

1. Detection and characterization TiO_2 nanoparticles in environmental samples including wastewater and sludge.
2. Physicochemical characterization of rutile TiO_2 Engineered Nanoparticles (r-TiO2-ENP) in terms of size, charge and agglomeration.

3. Toxicity evaluation of r-TiO2-ENP in earthworm, *Eisenia fetida* by:

a. Quantifying Ti in earthworm thereby its influence on trace elements and electrolytes (metallomics)
b. Assessing antioxidant enzyme responses including CAT, SOD,GR and lipid peroxidation in earthworm
c. Divulging the expression of protein levels using LTQ-Orbitrap-label free quantitation (proteomic profiles)

Outcomes of the study will help in establishing and the implementation of effective, protective regulatory methods for environmental impact assessment of nanoparticles that resemble TiO_2 nanoparticles. It will also provide data pertaining to proteomic profiles and antioxidant enzymes that assist in divulging the survival mechanism during the exposure of TiO_2 engineered nanoparticles.

Chapter 4

Materials and Methods

4. MATERIALS AND METHODS

4.1 Test chemicals

All the chemicals, and reagents used in the present study are of analytical grade and used without any further purification.

Nanoparticles: Rutile Titanium dioxide engineered nanoparticles (r-TiO_2-ENP) [titanium (IV) oxide, rutile, <100nm, 99.5% trace metals, Sigma Aldrich] was purchased and suspension of the same was prepared in ultrapure water.

4.2 Sentinel Organism

In the light of development of an invertebrate based toxicity assessment and biomarkers that are rapid, sensitive and cost-effective, an obvious model organism is earthworm, *Eisenia fetida*. Reasons for using the earthworm in toxicity studies and biomarker identification (GOVEN et al., 1994) are given below::

- Extensive literature on basic biology and ecology of earthworms is available.
- Appropriate sentinel species for laboratory and *in situ* acute toxicity and/or bioaccumulation studies.
- Standard guidelines such as OECD and ISO employ earthworms as model /sentinel organisms.
- Considered as engineers of natural ecosystems and comprise key links in food chains.
- They are cost-effective and socially non-controversial sentinel organisms.
- Their morphology and behavior enable exposure studies in the laboratory or *in situ* with a wide variety of matrices.

- Their high surface area/volume ratio, feeding, and behavior facilitate understanding the uptake of contaminants.
- Their tissue can be readily compartmentalized and easily isolated for chemical characterization using conventional analytical techniques, enabling determination of actual tissue-level dose of contaminants and concomitant biological responses.

Hence, *Eisenia fetida* (Savigny, 1826, *E. fetida*) which plays an important role in the soil macrofauna biomass, the species is most commonly used in ecotoxicology. It is recognized as a useful bio-monitor in assessing chemical toxicity in the soil caused by various chemicals especially metals. Therefore, it has been chosen as an appropriate bio-monitor organism for assessing the toxicity of r-TiO_2-ENP in the present study.

Taxonomy of Sentinel

Kingdom:	Animalia
Phylum:	Annelida
Class:	Clitellata
Sub Class:	Oligochaeta
Order:	Haplotaxida
Family:	Lumbricidae
Genus:	*Eisenia*
Species:	*fetida*

http://www.uniprot.org/taxonomy/6396

Earthworms (*Eisenia fetida)*, were purchased from the Vermiculture Project at Hayathnagar and Mallapur, Hyderabad, India. Worms were carefully brought to the laboratory within an hour along with the moist soil in a

perforated jute bag. Before proceeding to toxicity evaluation, worms were acclimated for a minimum of 72 h and maximum for a period of 7 days under laboratory conditions in feed boxes (36"x18"x24"). Earthworms were acclimated in soil composed of sterilized red soil at the bottom (base soil). A thin layer of dried leaves, cow dung collected from uncontaminated sites along with a thin layer of moss peat were used in preparing the diet for the worm. Wet gunny bags were used to cover the feed boxes to maintain the optimum moisture (30-35%).

4.3 METHODS

4.3.1 Quantification of Titanium from environmental Samples

4.3.1.1 Collection and acid digestion of wastewater samples

Wastewater samples were collected from a common effluent treatment facility, activated sludge treatment system (Hyderabad India). *American Public Health Association* guideline No. 1060 (APHA, 2005) was followed to collect wastewater samples. Wastewater and bio-solid samples were obtained from influent, aeration tank and effluent samples from a common effluent treatment plant. The samples were subjected to digestion in a closed Teflon vessels using microwave digester (Milestone, ETHOS EZ). Required volume (1 ml) and weight (1 g) wastewater and bio-solids respectively were digested by adding 7 ml of HNO_3 (65%), 1 ml of H_2O_2 (30%). Temperature control microwave program (T_1= 200°C and T_2=110°C for 15 min at 45 bar pressure & 1200w power) was used to perform digestion of the samples. After digestion, the samples were cooled to room temperature and they were transferred to metal-free containers. The required number of aliquots of the samples was

made. They were used in estimating Ti concentration in the samples by ICP-MS.

4.3.1.2 Quantification of Ti in wastewater and sludge samples

Inductively coupled plasma – mass spectrometry (ICP-MS) is one of the promising analytical tools in detection and quantification of inorganic nanomaterials because of its elemental specificity, excellent resolution, and low detection limit. Therefore, total concentration of Ti in digested wastewater and sludge samples was determined by ICP-Quadrupole-MS (ICP-QMS; Agilent 7700 series, Agilent Technologies, Germany). The ICP-MS was equipped with a PEEK Mira Mist nebulizer and a PFA inert sample introduction kit with a sapphire injector (inner diameter 2.5 mm). Measurements were conducted at an RF power of 1,550 W and a carrier gas flow of 1.17–1.18 L/min. The samples were introduced directly into the ICP-MS system using the standard peristaltic pump with Tygon pump tubing (internal diameter of 1.02 mm), and ASX-520 autosampler. The multi-element standards supplied from Agilent Technologies' were used in calibrating standards for measuring Ti. A serial dilution of the stocks was performed to prepared concentrations of the standard Ti in 2% nitric acid to match the matrix of the samples (Fabricius et al., 2014). Concentrations of the standard solutions were chosen in the range where the concentration and counts per second are related linearly to obtain a calibration curve. Standardization of blank checks and calibration was carried out throughout the analysis. The samples obtained from the section 4.3.1.1 used for estimation of Ti at required dilutions of the same.

4.3.1.3 Characterization TiO_2 nanoparticles of by single particle-ICPMS

Characterization of TiO_2 nanoparticles was carried out by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as per the principle described by (Degueldre and Favarger, 2004). Nanoparticles in a given matrix can be analyzed by introducing samples at a low flow rate followed by their analysis using ICP-MS in time-resolved analysis (TRA) mode. It allows the detection of signals generated by a single particle during its vaporization and atomized in the plasma. Then, each signal measured can be correlated to the size and mass fraction of a unique nanoparticle. This method of NP characterization is called Single-Particle ICP-MS (SP-ICP-MS) analysis. The key feature of this analysis lies in the capacity of the ICP-MS to distinguish the data collected for individual nanoparticle present in a given matrix. For this reason, care must be applied in the sample dilution (particle number per volume) and selection of the integration time (Sannac et al. 2013). Therefore, analysis of Ti nanomaterial in water samples that were spiked with TiO_2 nanoparticles was determined by ICP-Quadrupole-MS (ICP-QMS; Agilent 7700 series, Agilent Technologies, Germany). The ICP-MS was equipped with a PEEK Mira Mist nebulizer and a PFA inert sample introduction kit with a sapphire injector (inner diameter 2.5 mm). Measurements were conducted at an RF power of 1,550 W and a carrier gas flow of 1.05 L/min. Nebuliser pump was set to 0.1 rps. Total acquisition time was set to 60 sec with an integration time of 10 milliseconds. Spray chamber temperature was set to 2 ^{0}C. Rutile TiO_2 nanoparticles were used as a standard to study the behavior of Ti nanoparticles in the water samples that were spiked with TiO_2. Nebuliser efficiency was standardized using rutile form of TiO_2 nanoparticles (<100 nm) procured from Sigma Aldrich as a reference standard. A stock solution

was prepared by adding the desired weight of Rutile TiO_2 nanoparticles to ultrapure water and sonicated for 30 minutes in a SONICA bath sonicator at a 40 kHz frequency. It was used as unknown to determine the sensitivity of ICP-MS measurements. A serial dilution of the stock was performed to obtain various concentrations of the standard Ti in Milli-Q water to match the matrix of the samples. The concentrations of Ti standards solution was chosen in the range where the concentration and counts per second are related linearly to obtain a linear calibration curve. Standardization of blank checks and calibration verification was carried out throughout the analysis. The samples were subjected to dilution by a factor $10^{3.}$ Analyses were performed in time-resolved analysis (TRA) mode using an integration time of 10 ms for all measurements.

4.3.1.4 Analysis of the data by sp-ICPMS

Time-resolved analytics of ICP-MS data was analyzed using a spreadsheet developed by the National Institute of Food Safety in the Netherlands (RIKILT). Briefly, the custom spreadsheet was used to obtain the distribution plot of the signal intensities. It allows the NP signals to be discriminated from the background (due to instrument noise and the signal from the dissolved component of the element in solution). The sensitivity of the ICP-MS (cps per µg/L) for Ti was calibrated using rutile nanoparticle (<100 nm) as standard thereby differentiating nanoparticle signals converted into the mass concentration of the element measured. The density of the TiO_2 (4.23 g/ml) was entered into the spreadsheet, allowing the volume of each NP to be calculated. Based on the assumption that the NPs are spherical, the cube root of the NP volume was used to calculate each

nanoparticle diameter. From this data, a size distribution and the median nanoparticle size of TiO_2 is calculated (Sannac et al. 2013).

4.4 Physicochemical characterization of r-TiO_2-ENP

Studying the behavior of engineered TiO_2 nanoparticles in a dispersion medium is crucial for the identification of environmentally critical properties those influence their behavior. Therefore, in the present study, stability of r-TiO_2-ENP dispersion is assessed in terms of size and charge (z-potential) of in ultrapure water.

4.4.1 Preparation of r-TiO_2-ENP suspension

r-TiO_2-ENP suspension was prepared in ultrapure water as described by Chowdhury et al., (2010). Concentrations of r-TiO_2-ENP used in this study were of 0.05, 0.1, 0.15, 0.2 and 0.25 mg/ml. The suspensions were subjected to probe (22 mm) sonication using vibra-cell (VCX-750, SONICS) for 30 min at 40% amplitude with 30 sec 'on' and 5 sec 'off' pulse to obtain homogeneous dispersion of r-TiO_2-ENP.

4.4.2 Electron microscopy studies of r-TiO_2-ENP suspension

Scanning electron microscopy: r-TiO_2-ENP suspension samples were mounted on the stubs using double-sided carbon conductivity tape. A thin layer of gold coating over the samples was done with the help of an automated sputter coater (JOEL, JFC-1600) for 3 min and observed under scanning electron microscope (JOEL-JSM 5600) at required magnifications (John and Lonnie, 1998).

Transmission electron microscopy: Dip preparation of nanoparticles was done as per floatation method of John and Lonnie (John and Lonnie, 1998). A drop of r-TiO_2-ENP suspension was placed on a piece of Parafilm and transferred to the carbon coated copper grid. After 10 min, an excess of water was gently drained with filter paper. After fixation, particles in the grid were stained with 2% uranyl acetate. The grid was observed under transmission electron microscope (Hitachi, H-7500) at required magnifications.

4.4.3 *Determination of size and charge of r-TiO_2-ENP*

Determination of size and zeta potential (charge) of r-TiO_2-ENP in suspension was performed using dynamic light scattering (Zetasizer, ZS90, Malvern, United Kingdom). The device is equipped with 4-mW He-Ne 633 nm laser and an electric field generator (for measuring charge). r-TiO_2-ENP suspension is prepared as described in 2.4.1 was vortexed [vortex mixer, MSW-308 (MAC, India)] to provide homogeneity to the sample. 1 ml of the homogenous sample was transferred to a polystyrene square cuvette (Malvern, United Kingdom) for measuring the size and 1 ml of the same sample was transferred to a folded capillary Zeta cell (Malvern, United Kingdom) for measuring zeta potential.

4.5 Evaluating the effect of electrolytes, dissolved organic matter (DOM) and pH on agglomeration of r-TiO_2-ENP

Formation of agglomerates was found to be one of the main factors, influencing the dispersion stability of engineered nanoparticles. Furthermore, particle agglomeration increases particle size, which in turn

enhances their sedimentation. Hence, it is essential to monitor the agglomeration behavior of nanomaterials in environmentally relevant conditions. Therefore, present study was designed to understand the agglomeration behavior in a multi-dimensional test matrix including environmentally relevant test parameters predominantly influencing nanoparticle agglomeration. These parameters include the presence of various cations and anions, organic matter, etc. The parameters which influenced agglomeration behavior and dispersion stability are an Ionic strength, pH, Concentration of dissolved organic matter (DOM), concentration, nature and surface chemistry, size and density of the particles, presence/absence of CO_2 in the surrounding medium. However, key parameters such as pH, the concentration of calcium ions and of DOM or natural organic matter (NOM) were chosen as most relevant parameters for assessing dispersion stability of r-TiO_2-ENP under natural conditions (OECD, 2014).

4.5.1 Preparation of nanoparticle stock dispersion

r-TiO_2-ENP (0.1 g) was pre-wetted in 1 ml deionized water and left for 24 h to ensure the proper interaction of material surface with water. After 24 h of pre-wetting, the wet paste is dispersed in 100 ml of milli-Q water, thus providing a total concentration of 1 g/L (1000 ppm). Dispersion is sonicated for 10 minutes at the power level of 40 W to ensure proper homogenization. Subsequently, size of the particles was determined by dynamic light scattering technique.

4.5.2 Agglomeration behavior of r-TiO_2-ENP in the presence of electrolyte

To study the agglomeration behavior of r-TiO_2-ENP dispersion in presence of electrolyte, $Ca(NO_3)_2$ was used under the tested pH or test dispersion containing natural organic matter (NOM). The aliquots of sonicated stock dispersion of r-TiO_2-ENP (2 ml) were taken into 50 ml tarson tubes and diluted with deionized water to 20 ml. Dilution of dispersion was made to minimize the contact of particles with electrolyte that added in the subsequent step. A stock solution of the electrolyte with a concentration of 100 mM was added to the previously diluted r-TiO_2-ENP dispersion l (20 ml) in amounts of 0, 0.4, and 4 ml to achieve the final concentrations of 0, 1, and 10 mM respectively. The samples were made with MQ water up to 40 mL.

4.5.3 Agglomeration behavior r-TiO_2-ENP in presence of DOM in the dispersions

4.5.3.1 Preparation of DOM stock solution

Prior to the observation of r-TiO_2-ENP agglomeration in presence of DOM, a stock solution of corresponding DOM was prepared and characterized. For these purposes, 2R101N Suwannee River DOM (SRDOM) was used in the study. The stock solution of DOM was prepared with the concentration of 1g/L (1000 ppm DOM). 250 mg of SRNOM was weighted and diluted in the 250 ml of MQ water, the resulted solution being kept in brown glass closed vessel. After adjusting pH (8.5-9), DOM solution was left under vigorous stirring for 24 hours. After that, pH was measured and adjusted to the same values (pH=8.5-9) if needed. The solution was filtrated using vacuum set-up,

consisting of a pump and bottle-top 0.2 μm PES filter. Thereafter, the solution of DOM was analyzed for the dissolved organic carbon (DOC) content. The resulted solution was kept at 4°C in brown-glass bottles avoiding exposure to the light. Prior to bringing the samples volumes to 40 ml, the necessary amount of SRNOM stock solution was added to the samples to achieve the concentration of DOC/DOM (10 ppm).

To study the influence of Electrolyte/DOM concentration on the particle agglomeration, samples containing 0, 1 and 10 mM $Ca(NO_3)_2$ with/without 10 ppm DOM were prepared. Suggested experimental design for investigation of Electrolyte/DOM influence on agglomeration of r-TiO_2-ENP nanoparticles is presented in the following Figure 3. 1

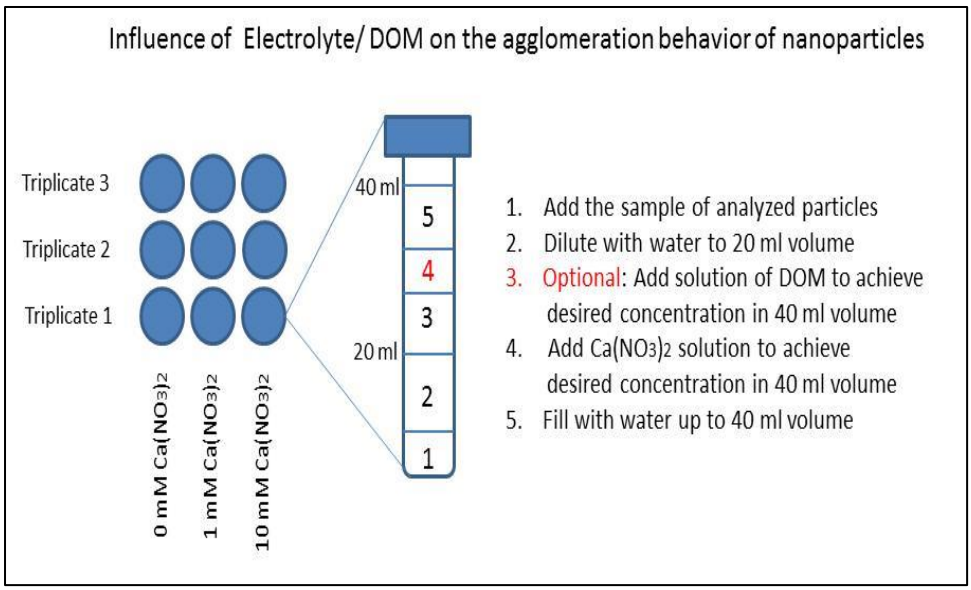

Figure 4.1 Experimental design for the investigation of electrolyte and DOC influence on agglomeration behavior of r-TiO_2-ENP.

4.5.4 *Influence of pH on the agglomeration behavior of r-TiO_2-ENP*

Prior to bringing the r-TiO_2-ENP sample volume to 40 ml, 100 mM $NaHCO_3$ solution was added to the experimental tubes in the volume of 2 ml to reach the concentration of 5 mM in the final volume of 40 ml. After volume adjustment, the pH was established at pH= 8.5 by adding 0.1 M solution of HCl and NaOH, respectively. The samples are equilibrated in the shaker for 24h. Afterwards, each sample underwent 30-second sonication at 40 W to ensure the homogeneous particle dispersion. When handling the samples with established pH, sample tubes were kept closed in-between the sampling procedures to avoid the influence of atmospheric carbonate on ionic strength of the samples. The pH of the dispersion was checked and adjusted if needed. Aliquots of samples were taken and diluted for further instrumental analysis, if required. To study the agglomeration of r-TiO_2-ENP in the media with established pH, $NaHCO_3$ was used as a buffering agent. Possible experimental design is presented in Figure 3.2

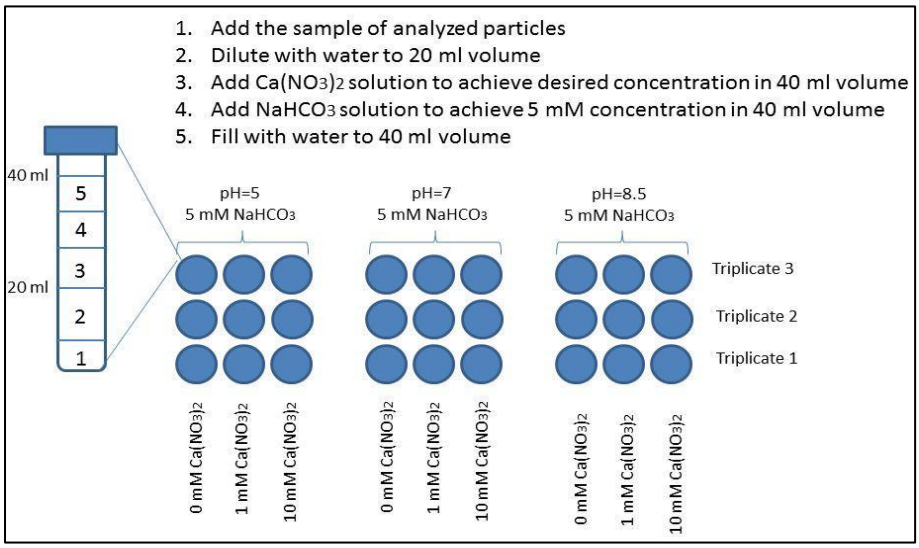

Figure 4.2 Experimental designs for the investigations of nanoparticle agglomeration behavior at fixed pH.

4.5.5 Sample Analysis

To check the dispersion stability of r-TiO_2-ENP, the remaining particle concentration in the top 0.5 cm volume of dispersion was determined. For a fully stable dispersion, this value will not change over time, while agglomeration and as a consequence settling will gradually reduce the concentration of particles in the top 0.5 cm volume of the sample. The aliquots of the supernatant of the samples were taken from the top 0.5 cm volume of dispersion and (if necessary) diluted for further analysis by ICP-OES method (OECD-2015). The aliquots are taken from the prepared samples every hour, starting hour 0 and finishing hour 6. Such frequency was found to be an optimal time to investigate dispersion stability of nanoparticles at the same time. Before the sampling at hour 6, samples underwent centrifugation at 1000 rpm for 2 minutes to enhance the particle sedimentation.

Aliquots were taken from the top layer of the sample (40 ml) but not directly at the air/water interface and not deeper than 1 cm. The aliquot of 0.5 ml was taken from the tubes. This volume was diluted into the mixture containing HNO_3:HF:H_2O in the ratio of 1:4:1 in a total volume of 10 ml. Resulted samples were vigorously shaken by hand after preparation and right before the analysis, to ensure their proper homogenization.

4.6 Toxicity assessment of r-TiO_2-ENP in earthworms

Elucidation of toxicity in environmentally relevant sentinels is lagging far behind *in vitro* studies that are conducted on human and other vertebrate cell lines. Hence evaluation of ENPs in environmental sentinel such as earthworm is imperative. Therefore, present study is originated to evaluate

toxicity of representative ENPs viz. metal oxide [Titanium dioxide (TiO2)], metal [Gold (Au)], polymeric nanoparticles such as polystyrene and polyamidoamine PAMAM dendrimers with unique size and charge. Out of the four nanoparticles viz. r-TiO_2-ENP, Au, PS and PAMAM dendrimers, used in this study, only r-TiO2-ENP showed toxicity endpoint i.e. mortality in earthworm by OECD-207 paper contact method. Hence further investigation was carried out on TiO_2. Additional data on screening of Gold (Au); Polystyrene and Dendrimers is presented in Appendix-1.

Direct contact test through a filter paper method (48 h) and artificial soil test for 48 h, 7 and 14 days exposure (OECD, 1984) were used to assess the toxicity of r-TiO_2-ENP in the earthworm.

4.6.1 Direct contact method

Filter paper contact method, Acute Toxicity Test No.207 (OECD, 1984) was adopted as described by (Van Leemput et al., 1989) and used to assess ecotoxicity of r-TiO_2-ENP in the earthworm. The method was modified is as follows: Prior to the experiments, earthworms were kept on a moist filter paper for three hours for depuration to clear their gut contents. One earthworm was housed in a petri dish, containing Whatman filter paper (grade 1), having a surface area of 68 cm^2. The petri dish was placed in a dark and the temperature was maintained at 20 ± 2 ^oC. To provide a moisture content of 35 ± 2%, the petri dish was intermittently sprinkled with distilled water whenever necessary. After 48 h, mortality of worms in each plate was recorded. Concentrations of r-TiO_2-ENP used in the experiments were 0.05, 0.1, 0.15, 0.2 and 0.25 mg /cm^2 that are equivalent to 0.05-0.25 mg/ml. For each concentration of r-TiO_2-ENP, 10 worms were exposed. And

the experiment was repeated three times for obtaining statistical significance. Controls were run in parallel with ultrapure water in place of r-TiO_2-ENP suspension.

4.6.2 Artificial soil test

The modified method of artificial soil test as per OECD-207 is as follows. The adult worms were collected and were further acclimatized for 48 h in artificial soil (evenly blended dry weight mixture of 68% No. 70 mesh silica sand, 20% kaolin clay and 10% moss peat, pH 6.0 ± 0.5) at 22 ± 2 °C prior to testing. Acute toxicity studies were conducted as per guidelines recommended by the Organization for Economic Cooperation and Development (OECD, 1984). The concentrations of r-TiO_2-ENP 0.05, 0.1, 0.2 and 0.4 mg/Kg of soil were used in the test. These concentrations were chosen with regard to the screening test concentrations that were used in direct contact method (section 4.6.1). It was aimed to evaluate the toxicity of r-TiO_2-ENP at equivalent concentrations that are below and above lethal concentrations that were documented during paper contact method. Ultrapure water was used for preparing r-TiO_2-ENP suspension. It was uniformly mixed with artificial soil (pH 6.0±0.5).

Glass containers (1 L capacity) were used to prepare the test arenas with 350 g of artificial soil (35% moisture) in three replicates. A batch of 20 adult earthworms (similar size and weight) that were acclimated in artificial soil under lab conditions were released into each test container and covered with perforated plastic film. The test conditions were maintained at 22 ± 2°C. The concentrations of r-TiO_2-ENP in soil at different concentrations were reconfirmed by X-ray Fluorescent analysis.

Controls were also run along with tests with the water (without r-TiO_2-ENP) to the artificial soil (35% moisture), and the mixture was divided into three control replicates as described above. The effect of TiO_2-ENP was monitored for 48 h, 7 and 14 days.

4.7 Bio-concentration studies

4.7.1 Quantification of titanium in earthworms

4.7.1.1 Microwave digestion of earthworm tissues

Earthworms that were exposed to sublethal concentration of TiO_2-ENP were subjected to acid digestion in closed Teflon vessels using microwave digester (Milestone, ETHOS EZ) as per (Fabricius et al., 2014). Approximately 0.5 mg of earthworm tissue was digested by adding 7 ml of HNO_3 (65%), 1 ml of H_2O_2 (30%). Temperature controlled microwave program (T_1= 200°C and T_2=110°C for 15 min at 45 bar pressure) was used to perform digestion of the tissue samples. After digestion, samples were cooled to room temperature and transferred to metal-free containers. Digested samples were subjected to appropriate dilutions in deionized water before ICP-MS analysis.

4.7.2 Measuring Ti concentration by ICP-MS

The total concentration of Ti in earthworm tissue digest was determined using ICP-Quadrupole-MS (ICP-QMS; Agilent 7700 series, Agilent Technologies, Germany) as per protocol described by Fabricius et al. (Fabricius et al., 2014). Measurements were conducted at a radio frequency (RF) power of 1,550 W and Ar as the carrier gas with a flow rate of 1.17–1.18 L/min. Rutile TiO_2 nanoparticles were used as the standard. A stock of

rutile TiO_2 nanoparticles (1 µg/ml) was prepared in ultrapure water and sonicated for 30 minutes in a bath sonicator (SONICA, India) at 40 kHz frequency. Serial dilution of the stock was performed to obtain desired concentrations of the standard Ti in 2% nitric acid so as to match the matrix used for the samples. Concentrations of Ti standards were chosen in the range between 25 and 400 ppb. The counts per second were related with Ti concentrations to obtain a linear calibration curve. Standardization of blanks and verification of calibration curves were carried out throughout the analysis. Scandium was used as internal standard to evaluate the fluctuations in plasma temperature.

4.8 Biochemical Estimations

4.8.1 Tissue Preparation and enzyme extraction

Live earthworms that were surviving after being exposed to various concentrations of r-TiO_2-ENP during filter paper test and artificial soil test were homogenized in 0.1 M phosphate buffer (pH 7.5) (10% w/v) using Potter-Elvehjam homogenizer which is equipped with a Teflon pestle. The homogenates were centrifuged at 5000×g for 10 min in Beckman (TLX-361544) centrifuge and the supernatant was further centrifuged at 5000×g for 10 minutes. Subsequently, supernatant from the second round of centrifugation was used to estimate the activity of antioxidant enzymes, acetylcholinesterase and lipid peroxidation. All enzyme preparations were carried out at 4°C. Protein was estimated by the method of Lowry et al (Lowry et al., 1951).

4.8.2 Estimation of Oxidative Stress Biomarkers

4.8.2.1 Antioxidant enzymes

4.8.2.2 Super Oxide dismutase (SOD)-(EC 1.15.1.1)

The specific activity of SOD enzyme was assayed by the method of Marklund and Marklund, (1974).

Principle: The assay is based on the principle that SOD catalyzes the dismutation of superoxide anion ($O_2^{\cdot -}$) to Hydrogen peroxide (H_2O_2) and oxygen. In this pursuit, specific activity was determined by the ability of SOD enzyme to inhibit the auto-oxidation of pyrogallol.

$$2O_2^{\cdot -} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

Procedure: Briefly, the assay mixture contained 50 mM Tris–HCl buffer (pH 8.4), containing 1 mM EDTA, 2.64 mM pyrogallol and 100 µL enzyme source in a final assay volume of 300 µL. The increase in absorbance was recorded immediately at 420 nm for 2 min. (kinetic mode) in a spectrophotometer using SoftMax Pro 6. One unit of SOD activity is defined as the amount of enzyme that causes 50% inhibition of pyrogallol auto-oxidation. The SOD activity was expressed in units/mg protein compared with the standard and control to report the specific activity in the samples.

4.8.2.3 Estimation of Catalase (EC 1.11.1.6)

Catalase activity was assayed by the method of Claiborne, (1985).

Principle: Catalase is a highly reactive enzyme, reacting with H_2O_2 to form water and molecular oxygen

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

Procedure: Briefly, the assay mixture consisted of 0.1 M phosphate buffer (pH 7.4), 0.019 mM hydrogen peroxide and 100 µL of enzyme source in a final volume of 3 mL. The enzyme activity was calculated using the extinction coefficient of H_2O_2 (0.0436 mM/cm) and expressed as micromoles of H_2O_2 consumed/min/mg protein.

4.8.2.4 Estimation of Glutathione Reductase (GR,)- EC 1.6.4.2)

GR activity was assayed by the method of Carlberg and Mannervik, (1975)

Principle: The oxidation of NADPH is followed spectrophotometrically at 340 nm.

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

Procedure: Briefly, the assay mixture contained 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.1 mM NADPH, 1 mM oxidized glutathione (GSSG) and 50 µL enzyme source in a final assay volume of 200 µL. The disappearance of NADPH was measured immediately at 340 nm for 2 min (kinetic mode) in a spectrophotometer using SoftMax Pro 5. The enzyme activity was expressed in nanomoles of NADPH oxidized/min/mg protein using the molar extinction coefficient of 6.22 mM/cm.

4.8.2.5 Estimation of lipid peroxidation

Lipid peroxidation (LPO) was determined by the thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), as the product formed due to peroxidation of lipids by the method of Utley et al., (1967) with some modifications as adopted by Fatima et al., (2000).

Principle: Malondialdehyde (MDA) produced peroxidation can react with thiobarbituric acid (TBA) reagent to form a pink colored product, which has an absorption maximum at 532 nm. The assay is calibrated with 1,1,3,3 tetramethoxypropane, which on hydrolysis produces malondialdehyde. The results are expressed in terms of the amount of malondialdehyde produced during the reaction.

Procedure: Briefly, 0.125 mL of homogenate and reaction mixture (10 mM $FeSO_4$, 200 mM ascorbate, 0.125 M KCl, 0.02 M Tris–HCl buffer, pH 7.4) was incubated for 30 min. at 37 °C, followed by 0.25 mL of 20% chilled TCA and 0.5 mL of 0.67% of TBA was added to the above mixture and boiled at 100°C for 10 min. After centrifugation, the absorbance of the supernatant was read at 532 nm to measure the TBARS in a spectrophotometer supported by SoftMax Pro 5. A standard curve was prepared using the commercially bought MDA (1, 1, 3, 3-Tetramethoxy propane, concentration ranging from 1-10 nanomoles) as a standard. The values are expressed as nanomoles of MDA formed/mg protein.

4.9 Acetylcholinesterase activity

The specific activity of AChE was determined as per the method of Ellman et al., (1961).

Principle: Acetylcholinesterase catalyzes the hydrolysis of acetylthiocholine, a sulfur analog the respective natural substrate, acetylcholine. Upon hydrolysis, the substrate analog produces acetate and thiocholine. Thiocholine in the presence of the highly reactive dithiobisnitro-benzoate

(DTNB) ion reacts to generate the yellow of the 5-thio-2-nitrobenzoate anion. The yellow color can be quantified by its absorbance at 412 nm.

Procedure: The assay consists of 2.8 mL of 0.1 M phosphate buffer, pH 7.2, 50 µL of 0.16 mM DTNB, 50 µL of protein, and 100 µL of 0.2 mM acetylthiocholineiodide (ACT. The reactions were carried out at 37 °C. The rate of production of thiocholine was quantified by measuring the reaction of the thiol with DTNB to produce the yellow anion (5-thio-2-nitrobenzoic acid). The reaction was recorded in a kinetic mode for 6 min. at 412 nm using a spectrophotometer (SpectraMAX Plus; Molecular Devices; supported by SOFTmax PRO-6.0). The specific activity of AChE was calculated as µmol/mg protein/min.

4.10 Proteomics

Earthworm homogenate was precipitated (using 50:50 Acetone: Ethanol with 0.1% acetic acid) and re-dissolved in a compatible buffer *viz.* ammonium bicarbonate buffer (10% w/v). The concentration of each sample was estimated using BCA assay in 20 ug of the samples. The samples were subjected to in-solution digestion as per Wiese et al (2007) accessed via: http://www.ccamp.res.in/sites/default/files/2011_08_05_ingel-insolution%20digest%20protocols.pdf - with additional alkylation and reduction. Digested peptides were reconstituted in -40µL of 2% ACN. 0.1% formic acid and 1 µL of the same was injected on to the column. Digested peptides were further subjected to 180-minute RPLC gradient, followed by the acquisition of the data on LTQ-Orbitrap-MS. The data was searched for

the identity on MASCOT as search engine using Swiss-Prot/TrEMBL databases and label-free quantification was performed using Progenesis software. List of proteins with their sequence generated through LC-MS/MS analysis were tabulated to propose the appropriate biomarkers of r-TiO_2-ENP.

4.11 Statistical analysis

The median lethal concentration (LC_{50}) of TiO_2-ENP has calculated Ldp Line software (Ehabsoft) that is based on Finney's calculation to compute 'probits' of the data supplied. After linearization of response curve by logarithmic transformation of concentrations, 95% confidence limits and slope function were calculated to provide a consistent presentation of the toxicity data. Chi-square analysis was used to elucidate the statistical significance at $p<0.01$.

Statistical significance of ICPMS data was assessed by their respective standard deviations and relative standard deviations. All measurements were conducted in triplicates. The significance of the data sets between treated and control experiments was analyzed with the help of Student's t-test at $p<0.05$.

Proteomic profiles of the earthworm, *Eisenia fetida* were assessed using ANOVA at $p<0.05$.

Chapter 5

Results and Discussion

5. Results and Discussion

5.1 Quantification of Ti in wastewater and sludge samples

The annual production of engineered TiO_2 nanoparticles (TNP) already reached around 6 million tons (Jovanović and Guzmán, 2014). Hence, release of TNP into the environment is inevitable during their production, transport, usage and final disposal (Bakshi et al., 2015; Soni et al., 2015; Sun et al., 2016). As a result, detection and characterization of TNP in environmental matrices have become essential to perform environmental risk assessments.

Studies based on exposure modeling revealed that predicted concentrations of TNP in wastewater effluents (0.7–16 µg/L) can be higher than that of predicted no-effect concentration (1 µg/L) (Gottschalk et al., 2009). Furthermore, toxicity potential of TNP was reported in the literature (Gottschalk et al., 2013; Li et al., 2016; S. Li et al., 2014; Shi et al., 2013). We also reported the ecotoxicity potential of TNP and associated confounding factors using earthworm as a sentinel (Siva and Sankar, 2014). Furthermore, occurrence of Ti in environmental matrices and its interaction with other metals including Zn and Cu has proven to induce toxicity in living systems (Cupi et al., 2015; Holden et al., 2014).

There exist knowledge gaps pertaining to the establishment of standard analytical methods to detect and characterize Ti and associated nanomaterials in the environment (Gottschalk and Nowack, 2013; Tourinho et al., 2012). Few studies attempted to elucidate occurrence of TNP in wastewater and sludge. However, these studies employed techniques such as

dynamic light scattering (Topuz et al., 2014) and atomic emission spectroscopy (Westerhoff et al., 2011) that could not simultaneously measure significant properties of nanoparticles such as size, concentration, and number (Pachapur et al., 2015).

Inductively coupled plasma mass spectrometry (ICP-MS) which facilitates precise quantification of specific element and characterization of metal based nanoparticles is gaining prominence. In recent times, operating ICP-MS in single particle mode (SP-ICP-MS) enabled rapid yet simultaneous analyses of elemental composition, number and size distribution of nanoparticles, such as TiO2 (Laborda et al., 2014; Proulx et al., 2016), Ag and Au (Lee et al., 2014; Yang et al., 2016), ZnO (Hadioui et al., 2015) in the environmental samples.

Therefore, the present study was designed to quantify the concentrations of (i) Ti in the supernatant and sludge fractions of influent sewage, aeration tank contents and treated effluent of activated sludge process using ICP-MS and (ii) TNP in the sludge fraction by SP-ICP-MS.

Concentration of Ti in the supernatant fraction of influent sewage, aeration tank contents and treated effluent was found to be 3.47±1.06, 2.15±0.6 and 0.71 ± 0.2 mg/L respectively, (detection limit=2.9 ppb, y=44.9x+70.38, R=0.99) (Fig. 1). Concentration of Ti in the influent sewage, reported in this study was found to be higher than the values reported in the literature, 3 mg/L (Kiser et al., 2009), 0.03 mg/L (Johnson et al. 2011), 1.8×10^{-3} mg/L (Khosravi et al., 2012). Gottschlak (2009) and Westerhoff et al. (2011)

predicted that the concentration of Ti in the influent sewage is in the range of 0.021mg/L to 1.233 mg/L. Likewise the measured concentrations are higher in magnitude than that of predicted concentrations of Ti in the environment (Neale et al., 2013).

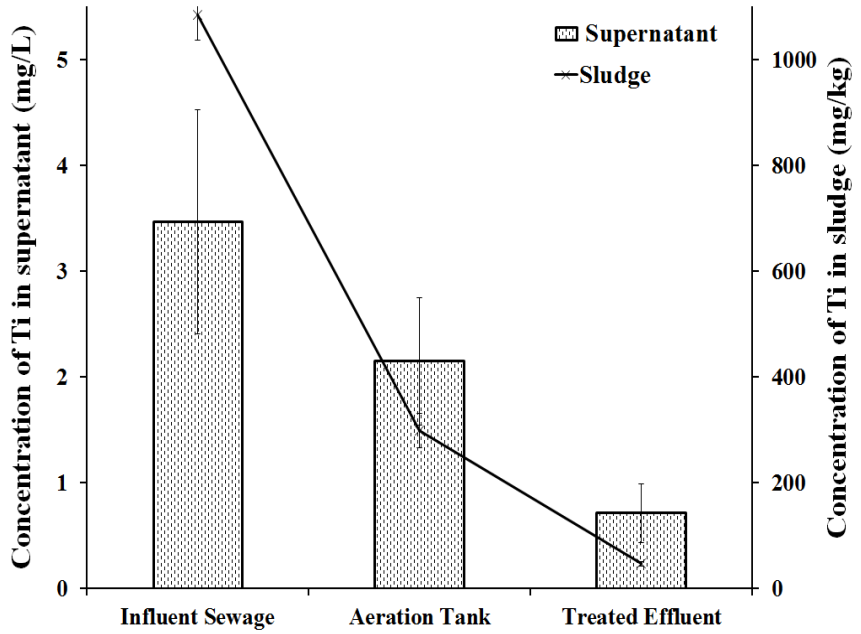

Figure 5.1 Concentration of Ti in supernatant (mg/L) and sludge (mg/kg) fractions of influent sewage, aeration tank, and treated effluent of a municipal sewage treatment plant. The data represent concentration± S.D.

Concentrations of Ti in sludge fraction of influent sewage, aeration tank contents, and treated effluent were found to be 1085±48, 298±32 and 47±4 mg/kg respectively (Fig. 1). Lowest concentration of Ti was found in the sludge sample obtained from treated effluent. Results indicated that the probability of Ti getting accumulated in sludge fraction is higher than being suspended in the supernatant. In the presence of high molecular weight organic compounds in the wastewater, TiO_2 undergoes heteroaggregation (Topuz et al. 2015). Therefore, from our results we surmise that presence of organic molecules could have facilitated agglomeration of Ti. Hence it could

have resulted in the observed highest standard deviation of Ti measured in the influent sewage samples. As it is evident that Ti can accumulate in sludge fraction of sewage, disposal of sludge at the end of sewage treatment can contribute to accumulation of Ti in soil, the ultimate sink of the terrestrial environment.

Modelling study done by Mueller and Nowack (2008) emphasized that predicted Ti concentrations in wastewater effluents (0.7-16 µg/L) are higher than their no effect concentrations (1 ug/L). Gottschalk et al. (2009) also predicted that environmental concentrations of Ti could range from 100 mg/kg to 802 mg/kg in sewage treatment plant sludge. Therefore, our results are in concordance with predicted concentrations of Ti in sewage sludge as reported in the above studies. Outcomes of the study contribute to demonstrate the distribution of Ti in various environmental compartments. Accumulation of Ti (10 mg/kg) in river sediments facilitates adsorption of various substances including phosphorous, arsenic etc. to the sediment (Luo et al. 2011). Adsorption capacity of Ti is directly proportional to its concentration. As the concentration of Ti reported in our study (47-1085 mg/kg) is much higher than that was reported by Luo et al (2011), so the adsorption capacity of Ti will be also be higher. Hence the outcomes of the study will pave a way to understand the possible interactions of Ti with other components in sewage samples. The study presents the first report that highlighted Ti distribution in supernatant and sludge fractions of municipal sewage. The results will be useful in monitoring Ti concentration in wastewater and biosolids, thereby promoting regulation of TiO2 in waste treatment and management.

In this study, SP-ICP-MS was used to characterize TNP in sewage sludge. TRA for influent sewage and aeration tank sludge fractions is shown in Fig. 2 (A1 and B1). The raw data was curated to eliminate the background signal (y=8035x+225695, R=0.98). The remaining intensities were converted into their corresponding particle size to obtain distribution plot of TNP in sludge samples [Fig. 2 (A2 and B2)]. From this size distribution plot, median particle size, particle number and mass concentration of TNP for sludge samples were calculated and presented in Table 1. Measured median particle size of TNP in the sludge fraction of influent sewage sample was 145 nm whereas this was almost half (71 nm) in aeration tank. The distribution of nanoparticle number in the influent sewage sludge fraction (4.8×10^{10} particles/kg) is higher than that of aeration tank contents (1.1×10^{8} particles/kg). TNP concentration determined from sludge fraction of influent sewage (13.6 mg/kg) is significantly higher than that of the aeration tank samples (3.3 mg/kg). Decrease in the size of TNP in aeration tank sludge fraction indicates that mechanical agitation of reactor contents by the sparged air could have resulted in dis-agglomeration of TiO2 from various organic and inorganic constituents of the sludge, forming smaller size/ aggregates of the particles. As the nanoparticles size is one of the critical factors that influence their distribution and concentration in a given matrix, interplay between sludge components and TNP in a given physicochemical condition would have resulted in their altered particle distribution and mass concentration.

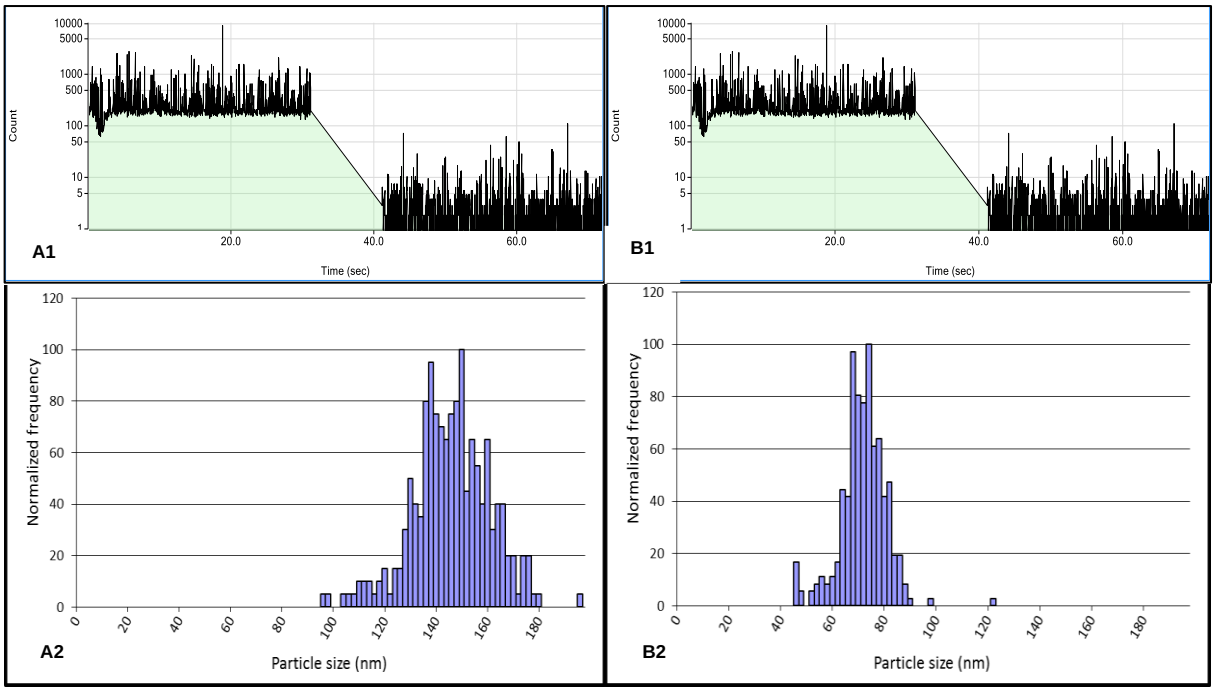

Figure 5.2 Time resolved analysis of SP-ICP-MS signals for the sludge fraction of influent sewage (A1) and aeration tank (B1). Corresponding particle size distribution in sludge obtained from influent sewage (A2) and aeration tank (B2).

Our results also indicate that the dwell time of 10ms (for influent sewage sludge fraction) and 3ms (for aeration tank sludge fraction) allows accurate characterization of TNP by SP-ICP-MS. Therefore, calibration of appropriate dwell time is the key factor in SP-ICP-MS analysis for precisely obtaining the chemical composition and mass concentration of the analyte. ^{47}Ti isotope analysis used in the study will definitely help in establishing the ratio of various stable isotopes of Ti i.e., ^{46}Ti to ^{50}Ti in sludge samples thereby understanding their potential to accumulate in the soil environment. Though results of the current study are limited to ^{47}Ti, it will definitely complement reports on other isotopes of Ti in sewage sludge. Compiling such data will help in understanding speciation of Ti in the given environmental sample.

Table 5.1 Characterization of sludge obtained from influent sewage and aeration tank of the municipal sewage treatment plant using SP-ICP-MS

Parameter	Influent sewage	Aeration tank
Concentration (particle number)	4.8×10^{10} particles/kg	1.1×10^{8} particles/kg
Concentration (mass)	13.6 mg/kg	3.3 mg/kg
Median particle size	145 nm	71 nm

Nebulizer efficiency (@ 0.1 rps) helped in minimizing the background noise caused by other constituents of the sample during measurements. TNP particle size distribution measured in the present study is within the estimated size limits of TNP reported by Lee et. al. (2014) that can be detected using SP-ICP-MS as well as the measured size limits reported by Kim et al. (2012) using analytical scanning electron microscopy. Hence, application of SP-ICP-MS in simultaneous determination of mass concentration, particle size and particle number of TNP from sewage sludge is the novelty of present study. Therefore, approach and outcomes of this study can serve as proxies in improvising ICP-MS based analytical methods to detect and characterize TNP. However, further investigations on reference nanomaterials that can be used in this regard for environmental monitoring of nanoparticles are warranted.

Size and functionalization of nanoparticles can also influence their distribution in effluents and biosolids (Guo et al. 2006; Brar et al. 2010). Therefore, widespread use of these functionalized nanomaterials in cosmetics and sunscreen lotions lead to the release of TNP and their byproducts into the municipal sewage treatment system. Moreover, TNP could pose environmental hazards even in low concentrations, owing to their

unique physicochemical properties such as surface area, size, and charge. Furthermore, as the usage of TNP is alarmingly increasing in various products, it is expected that in the near future, their presence in sewage, as well as sludge amended soils, also increase accordingly. Being metal based nanoparticles, TNP does not get biodegraded, hence get discharged out of the wastewater treatment systems along with the sludge and treated effluent and reach the environment (water and soil). Over time, TNP accumulates in the environment and could elicit toxicity in organisms that get exposed to them. Elsewhere we have also carried out a systemic study to unveil the toxicity of TNP in the earthworm, a soil sentinel. The studies have revealed that bioaccumulation of TNP in the worms led to their mortality by inducing oxidative stress (Siva and Sankar, 2014). Therefore, further investigations on TNP in the environment are imperative.

5.2 Determining Size and charge of rutile TiO_2-ENP (r- TiO_2-ENP)

Characterization of r-TiO_2-ENP in a dispersion medium is essential to understand the role of particle size and charge thereby agglomeration during toxicity assessment. Moreover, clear understanding of the relations between r-TiO_2-ENP toxicity and their physicochemical properties is not well documented. It is always speculated that the unanticipated toxicity profiles of nanoparticles are interrelated to intrinsic characteristics of nanoparticles in a given dispersion medium (von Moos and Slaveykova, 2014). Hence the present study was designed to understand the size and charge of r-TiO_2-ENP in ultrapure water. Outcomes of such studies will definitely aid in performing a comparative analysis of pristine behavior with that of complex media (e.g. electrolytes and natural organic matter) of nanoparticle

suspension. Hence, it can serve as a reference point for future studies to depict the toxicity of r-TiO_2-ENP and allied forms.

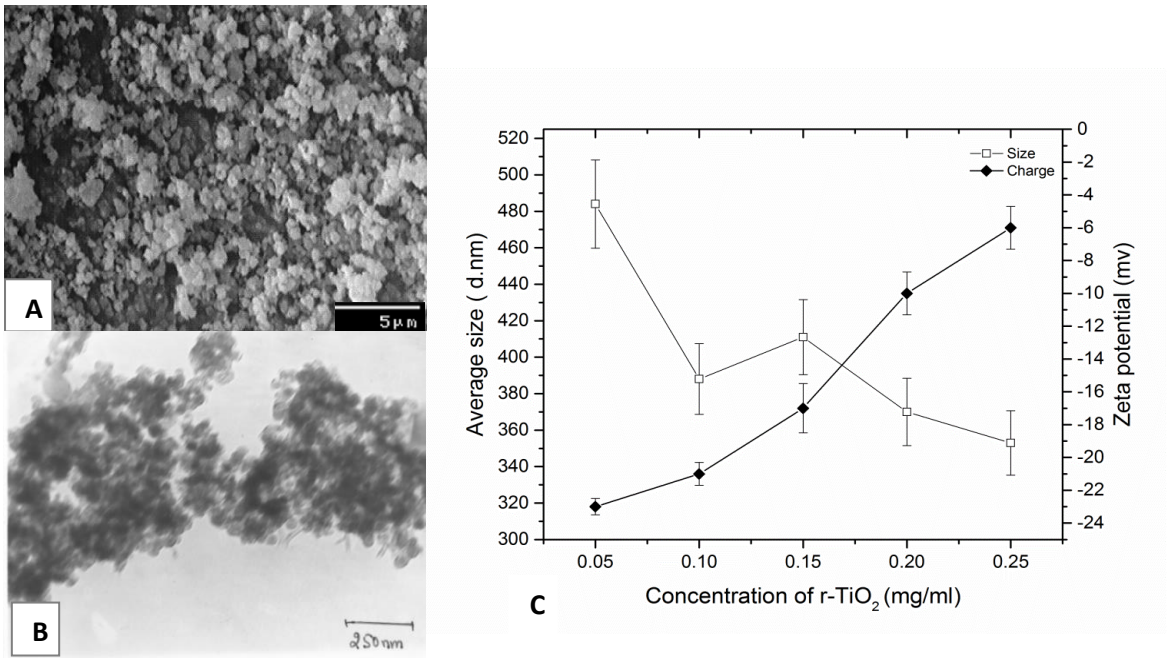

Figure 5.3 Scanning (A) & transmission (B) electron micrographs and dynamic light scattering measurements (C) of r-TiO_2–ENP. Data represent the mean size and charge ± S.D in (C)

Size distribution of r-TiO_2-ENP is presented in Figure-5.3 Scanning (A) and Transmission (B) electron micrographs. Particle size and corresponding zeta potential/charge (mV) of r-TiO_2-ENP are shown in Figure-5.3 (C). The results showed that primary particles of r-TiO_2-ENP underwent agglomeration in ultrapure water. In ultrapure water, variation of absolute value of zeta potential and hydrodynamic size of the particles is imperative (Kosmulski, 2009). It is evident that in ultrapure water, at a given mass concentration, the charge and size of the particle varies. The altered hydrodynamic size of r-TiO_2-ENP in the present study differs with previous reports (Buford et al., 2007; Sager et al., 2007) because of the difference in pH conditions (pH<6

and >8), crystal phase (anatase) and surface area of the TiO_2-ENP. Moreover, the particle size distribution with an average of 255 nm of r-TiO_2-ENP as indicated by electron micrographs [Figure 5.3 (A) and (B)] revealed that the agglomeration of these nanoparticles in ultrapure water is facilitated. Hence it was surmised that sedimentation of the nanoparticles at a given mass concentration could have occurred during aliquot preparation thereby altering the availability of nanoparticles in the exposure medium. However, similar kind of agglomeration and charge pattern of r-TiO_2-ENP was reported in cell culture media (Lankoff et al., 2012; Vevers and Jha, 2008) and natural aqueous matrices (Keller et al., 2010; Romanello and de Cortalezzi, 2013). In light of these observations, further studies to elucidate the effect of medium components, pH, ionic strength, surface charge of nanoparticles on the agglomeration of r-TiO_2-ENP that will assist in understanding their toxicity (Hotze et al., 2010; Zhou and Keller, 2010) are carried out.

5.3 Evaluating the effect of electrolytes, Dissolved Organic Matter (DOM) and pH on agglomeration of r- TiO_2-ENP.

The agglomeration behaviour of nanoparticle dispersions can influence their reactivity and distribution in the environment thereby leading to contamination (Waychunas et al., 2005; Zeng et al., 2009). Exposure assessment of nanoparticles needs info on agglomeration (Choi et al., 2007; Hoshino et al., 2004; Wu et al., 2010). Therefore, absolute characterization of nanoparticle dispersions becomes very important for promoting environmental applications and nanotoxicology investigations.

Insoluble or partially soluble nanoparticles in water are called as dispersion. Particles in the dispersion undergo agglomeration under different conditions of the dispersion medium. A natural force such as electrostatic interactions (attractive or repulsive), forces of Van der Waals attraction, and magnetic forces in-between the particles in the dispersion can control the particle interactions with the surrounding medium. Due to the forces acting in-between the colloidal particles, they can form (meta-) stable dispersions or undergo the processes of agglomeration. Van der Waals forces and lyophobic dispersion colloids cause agglomeration as long as no stabilizing, repulsing forces between particles are present. Hence such inherent property is very important to assess their toxic potential. A stable dispersion therefore always represents a non-equilibrium situation which is kinetically hindered to reach equilibrium in the agglomerated state. As the particle agglomeration increases particle size, it is essential to monitor the fate of nanomaterials under environmentally relevant conditions to understand their sedimentation behaviour. Therefore, a concept proposed by F. von der Kammer and checked for environmental relevance by Ottofuelling et al. (2) was used in assessing the agglomeration of r-TiO_2-ENP. To aid this phenomenon, a multi-dimensional test matrix resembling environmentally relevant test parameters that affect nanoparticle agglomeration was adopted. Furthermore, the parameters that influence particle agglomeration behaviour and dispersion including (i) ionic strength (ii) pH (iii) concentration of dissolved organic matter (DOM)/ Natural organic matter (NOM) were carried out.

Solution pH, ionic strength (IS), crystal phase, particle size, surface area of r-TiO_2-ENP influence hydrodynamic size, zeta potential and the degree of

agglomeration (Jiang et al., 2009; Loosli et al., 2015; Suttiponparnit et al., 2011). However, little is known about the agglomeration behavior of r-TiO_2-ENP under given conditions. Hence, a systematic study to divulge the behavior of r-TiO_2-ENP in aqueous suspensions is carried out in the study. The key parameters, such as concentration of calcium ions, DOM and pH were chosen as most relevant parameters for understanding the dispersion stability of the particles (Salminen et al., 1998). $Ca(NO_3)_2$ electrolyte was chosen because of both the dominant effect of multivalent cations (Ca^{2+}) on particle aggregation and their presence in relevant concentrations (compared to less effective monovalent ions and less abundant trivalent ions).

Outcomes of the present study revealed that aqueous suspensions with < 1 mM of Ca $(NO_3)_2$ could not cause any significant agglomeration of r-TiO_2-ENP in water. But 10 mM electrolyte, $Ca(NO_3)_2$ led to the significant agglomeration of r-TiO_2-ENP particles (Figure 5.4). In principle, the electrostatic and van der Waals forces could have controlled the TiO_2 aggregation process even for diverse morphologies (Liu et al., 2011a; Thio et al., 2011).

At 1 mM concentration of electrolyte, predominant electrostatic repulsion over the inter-particle interactions can cause aggregation at a relatively slow rate. As electrolyte concentration increased to 10 mM, the electric double layer (EDL) compression could have suppressed the electrostatic repulsion thereby accelerating the aggregation of nanoparticles (Zhou et al., 2013). In this pursuit, increase in the electrolyte concentration to 10 mM in aqueous suspensions has the potential to overcome the energy barrier. Hence, under

these conditions, Van der Waals attraction dominate and most collisions between TiO_2 nanoparticles and caused agglomeration of the particles.

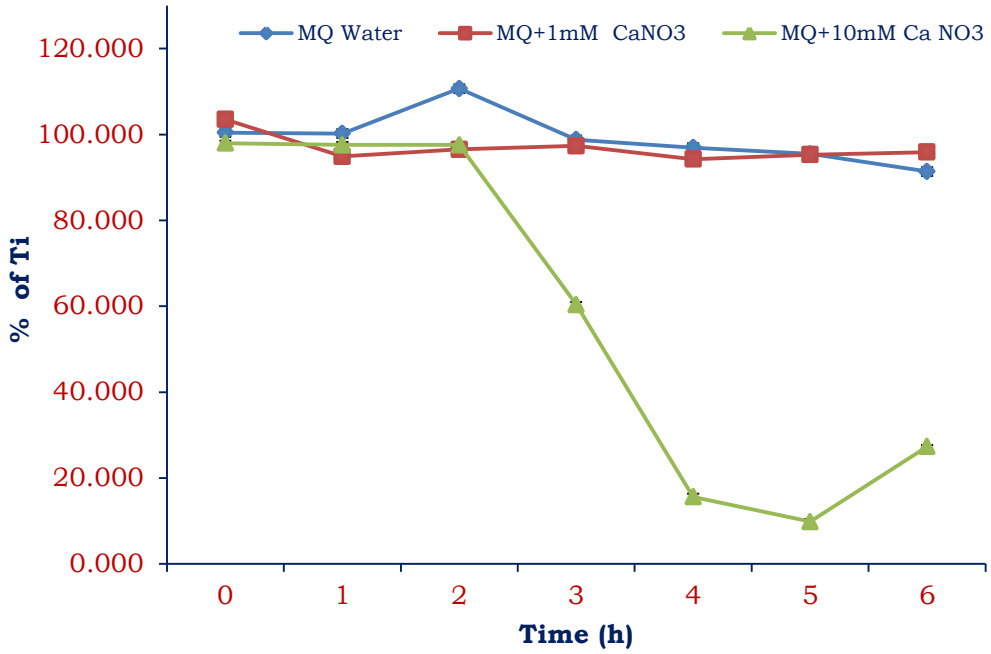

Figure 5.4 Agglomeration behavior of r-TiO_2-ENP in presence of 1mM $Ca(NO_3)_2$ and 10mM $Ca(NO_3)_2$. Data represents % of expected Ti concentration± S.D (Milli Q-water)

Therefore, present study revealed that Ca $(NO_3)_2$ concentration of 10 ppm will be useful as a critical coagulation concentration (CCC), can serve as an index to compare nanoparticle suspension stability in further experiments. Similar kind of results indicating the effect of monovalent cation of sodium (Na^+) on aggregation kinetics of anatase crystal phase of titanium were documented by (Loosli et al., 2015; Zhou et al., 2013) also explained the similar trends of agglomeration of TiO_2 nanoparticles in the presence of divalent cations including Ca^{+2} and Mg^{+2}.

Occurrence of DOM/ NOM at 10 mM influenced the agglomeration of r-TiO_2-ENP. Coexistence of electrolyte such as $Ca(NO_3)_2$ (1 ppm) along with the DOM at 10 ppm in water affected the agglomeration of r-TiO_2-ENP. However, the presence of DOM alone in ultrapure water has the potential to cause the agglomeration of r-TiO_2-ENP (Figure 5.5). Presence of DOM would have led to the masking of surface of r-TiO_2-ENP particles. Subsequently, a single humic acid macromolecule that is present in DOM could have facilitated the aggregation of nanoparticles. Therefore, the "bridging effect" of DOM and humic acid can facilitate the agglomeration of the nanoparticles (Chen et al., 2006; Zhou and Keller, 2010).

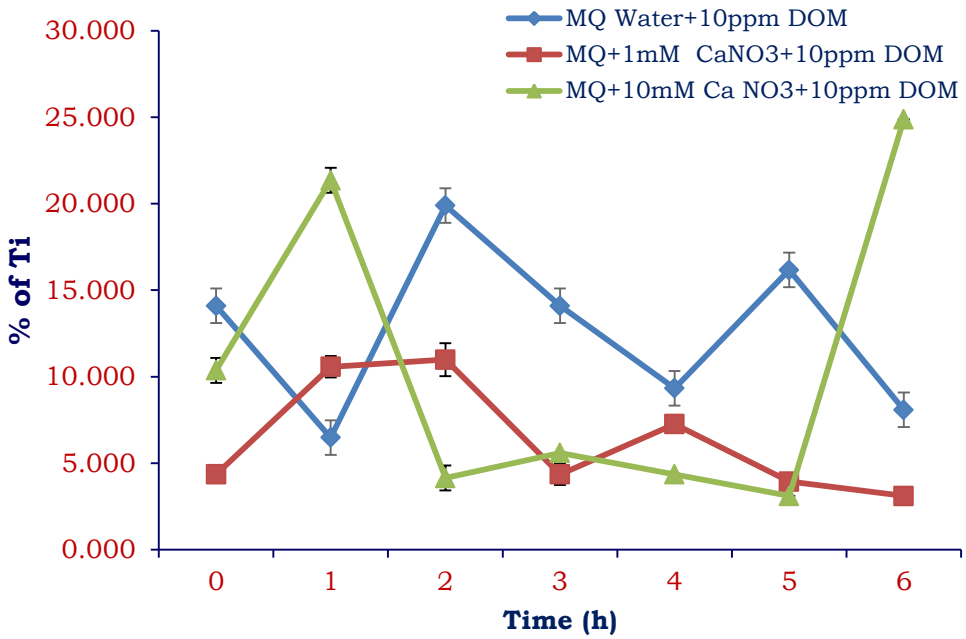

Figure 5.5 Agglomeration behavior of r-TiO_2_ENP in presence of 1mM $Ca(NO_3)_2$ + 10 ppm DOM and 10mM $Ca(NO_3)_2$ +10 ppm DOM. Data represents % of expected Ti concentration± S.D

Moreover, as the divalent cations (Ca^{+2}) can also contribute to the "bridging effect", role of divalent cations present in the medium in inducing agglomeration of the particles is imperative (Liu et al., 2011b). Similar kind of results demonstrated the role of bridging effect on agglomeration of silica nanoparticles (Abe et al., 2011). As outcomes of the present study are first of their kind, comparisons of these results with the other reports is not straight forward.

Effect of pH (8.5) on the agglomeration of TiO_2-ENP was evaluated in the present study (Figure 5.6). pH of the water used in the present study was selected based on fact that TiO_2 nanoparticles are unstable at this pH value. In this pursuit, in order to understand the effect of pH-8.5 on r-TiO_2-ENP, present study utilised $NaHCO_3$ (5mM) as buffer in the experiment. Because the smaller concentrations of this agent (1 mM) are insufficient to impart significant buffering capacity, while the higher concentrations (10 mM) can enhance agglomeration and particle sedimentation.

Change in the pH of the solution leads to variation in the surface charge (zeta potential) and hydrodynamic size of nanoparticles. For mineral oxides including TiO_2 when dispersed in water, ionic strength on the surface controls their charge in the absence of preferential adsorption of soluble ions in solution (Morrison and Ross 2002). Lowering the pH facilitate positive surface charge whereas increasing pH leads to negative surface on these nanoparticles in water dispersion.

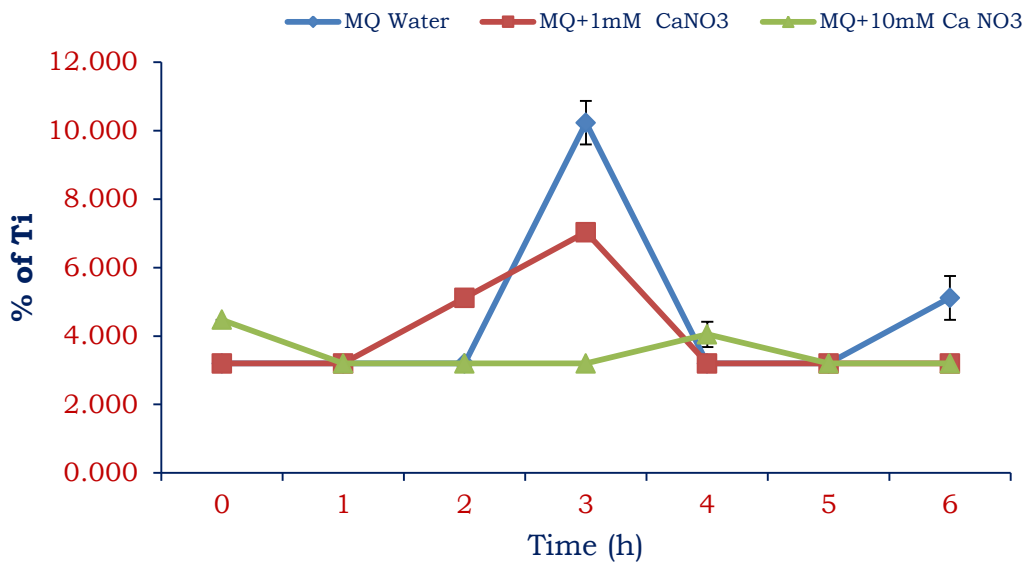

Figure 5.6 Agglomeration behavior of r-TiO_2-ENP at pH-8.5 in presence of 1mM $Ca(NO_3)_2$ and 10mM $Ca(NO_3)_2$. Data represents % of expected Ti concentration± S.D

Furthermore, ionic strength of the dispersion medium influences the hydrodynamic diameter of the particles by changing both zeta potential and thickness of electrical double layer. Higher concentrations of ionic strength (10 mM) caused by electrolyte facilitate the formation of smaller electrical double layer thickness, weaker electrostatic repulsive force, and subsequently larger hydrodynamic size of the particles in a dispersion medium (Suttiponparnit et al., 2011). Hence, pH-8.5 at 10 ppm of $Ca(NO_3)_2$ in the present study might have led to the increase in agglomeration of large size particles. Furthermore, occurrence of divalent cations (Ca^{+2}) increases the hydrodynamic diameter of the particles by lowering surface charge of the particles (Suttiponparnit et al., 2011). Therefore, divalent cations supplemented from $Ca(NO_3)_2$ could have facilitated the decrease in surface charge of the particles. However, even in the absence of divalent cations, agglomerations of particles were observed in the present study. This might

have resulted because of the dissolution of atmospheric CO_2 in the dispersion during the course of experiment. At pH-8.5 which is greater than isoelectric pH could have led to the charge inversion of r-TiO_2-ENP. Hence the increase in affinity between Ca^{+2} and negative surface area of r-TiO_2-ENP resulted in larger agglomerates due to the dispersion instability. Similar kinds of results were reported by (Loosli et al., 2015) in evaluating the effect of $CaCl_2$ on the dispersion stability of anatase crystal phase in ultrapure water.

5.4 Toxicity assessment of r-TiO_2-ENP in earthworm

Data pertaining to the toxicological evaluation of engineered nanoparticles (ENPs) in sentinels of environmental relevance is meagre. Despite the progress in nanotoxicology, advancement in assessing the potential of ENPs to induce toxicity in sentinels raised more hypotheses than true answers. Therefore, knowledge of fundamental aspects of environmental risks associated with ENPs is to be substantiated in several key areas of toxicity assessment.

Toxicological assessment and associated end points in soil sentinels were not documented for representative nanoparticles viz. Titanium dioxide (TiO2); Gold (Au); Polystyrene and Dendrimers. Hence toxicity potential of these nanoparticles was screened by OECD-207 paper contact method. Out of the four nanoparticles *viz.* r-TiO_2-ENP, Au, PS and PAMAM dendrimers, used in this study, only r-TiO2-ENP showed toxicity endpoint i.e. mortality in earthworm. Toxicity screening of Gold (Au); Polystyrene and Dendrimers

are presented in Appendix-1. Further studies that were carried out for evaluating toxicity of r-TiO_2-ENP are as follows:

5.4.1 Paper contact method

Physicochemical properties such as particle size, agglomeration state, surface charge etc., alter the fate and behavior of nanoparticles that are introduced into any biological (test) medium. Hence evaluating such properties during toxicity evaluation of nanoparticles is imperative (Powers et al., 2007). As the cellulosic materials are the best substrates (Cheng et al., 2011) to obtain significant distribution of nanoparticles, filter paper was chosen as the test medium for toxicity evaluation r-TiO_2-ENP. Furthermore, the techniques that have evaluated nanoparticles toxicity using paper contact method are not well documented in nanotoxicology. Therefore, present study was designed to understand the applicability of paper contact method to elucidate the effect of r-TiO_2ENP concentrations (0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2) on earthworms that are exposed to different 48 h on filter paper. The concentrations were chosen based on a range finding test conducted to bracket the LC_{50} of r-TiO_2-ENP on earthworm by filter paper test. Despite the agglomeration, r-TiO_2ENP affects the mortality of earthworm during the study. LC_{50} TiO_2-ENP in earthworm by filter paper contact test is presented in table 4.2.

Table 5.2 Effect of r-TiO_2-ENP (mg/cm^2) in relation to the mortality rate, regression equation, LC_{50} values (mg/cm^2) and Chi-square (χ^2) for 48 h against adult earthworm, *Eisenia fetida* by paper contact method (n=20)

Nanoparticle type	Acute toxicity range* *(95% confidence limit)* *(X^2=7.9; (p<0.05); slope=3.8±0.31)*		LC_{50} (mg/cm^2)
	Lower (mg/cm^2)	Upper (mg/cm^2)	
r-TiO_2-ENP	0.10	0.15	0.13 ± 0.01

LC	Concentration (mg/cm^2)	Lower limit (mg/cm^2)	Upper limit (mg/cm^2)
50	0.133	0.10	0.15
95	0.3522	0.30	0.63
10	0.05	0.021	0.06

Based on the range finding test, median lethal concentration of r-TiO_2-ENP was determined (Table-5.2). Increased surface area of nanoparticles can increase their reactivity. In order to understand the toxic potential of r-TiO_2-ENP even at low concentrations thereby subtle effects of nanoparticles on mortality, concentration windows were chosen at a range of 0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2. Though the window of the concentrations was small, the test was intended to document any significant effects of r-TiO_2-ENP at lower concentrations. Results indicated that 0.133 mg/cm^2 can kill 50% of the earthworm population when exposed through skin contact.

Moreover, the particles size and charge of r-TiO_2-ENP used in the present study were found to be inversely related [Figure 5.3 (C)]. Therefore, the % mortality of worms would have been resulted because of the interplay of

these parameters at various concentration of r-TiO_2ENP on filter paper. Furthermore, altered charge could have led to the variation in the amount of Ti being taken by earthworms through the skin contact. Similar trends in aggregation transition of anatase TiO_2-ENP from 1 mg/mL to 10 mg/L were reported by Canas et al., (2011). The study also elucidated toxicity of TiO_2 anatase nanoparticles in earthworm *Eisenia fetida.* It affirms that agglomerated primary particles can lead to the variation in the available concentration (%Ti) thereby altering the mode of toxicity in earthworm.

Hence, the phenomenon of agglomeration is interdependent on particle size, and charge. It is also surmised that agglomeration can influence nanoparticle uptake by earthworm. In this regard, results of current study are in fine tune with (Gilbert et al., 2009; Waychunas et al., 2005; Zeng et al., 2009) and response of the test organism upon exposure to TiO_2 nanoparticles possessing crystal phases other than rutile (Hoshino et al., 2004; Lockman et al., 2004; Wu et al., 2010).

5.4.2 Artificial Soil test

The concentrations of r-TiO_2-ENP 0.05, 0.1, 0.2 and 0.4 mg/Kg of soil were used in the test. These concentrations were chosen based on the results that were obtained in direct contact method. It was aimed to evaluate the toxicity of r-TiO_2-ENP at concentrations that are below (0.05, 0.1 mg/kg) and above (0.2 and 0.4 mg/kg) lethal concentration (0.13 mg/kg) that were documented during paper contact method. However, the nominal concentrations were subjected to x-ray fluorescent analysis to ascertain

absolute concentrations. Results indicated that the nominal concentrations (measured concentrations) are 0.05 (0.049 ± 0.01), 0.1 (0.102 ± 0.017), 0.2 (0.193 ± 0.015), 0.4 (0.39 ± 0.022) mg/kg. Results indicated that there was no significance difference between the nominal and observed concentrations. However, concentration range selected for soil artificial test is not sufficient enough to induce mortality. Though a study conducted by C. W. Hu et al., (2010) revealed similar kind of observations, the size of the particle (10-20 nm) used in the study differs from the size (100 nm) and range of concentrations (0.05, 0.1, 0.2 and 0.4 mg/kg) of r-TiO_2-ENP used in the present study (100 nm). Though, 0.133 mg/cm^2 can kill 50% of the earthworm population when exposed through skin contact, equivalent concentrations that were chosen based on such study could not cause/ induce mortality in earthworm during soil exposure. Nevertheless, outcomes of the study can be used as starting point for further studies to assess the effect of r-TiO_2-ENP or similar kind of nanoparticles using OECD-207 guidelines.

TiO_2-ENP cause toxicity to terrestrial invertebrates only at concentrations ≥1 gkg^{-1} (C. W. Hu et al., 2010). It is also evident from the present study. Thus, standard toxicity tests employing earthworms in r-TiO_2-ENP toxicity assessment provide information only on insensitive endpoints such as mortality, at concentrations ≥1 g kg^{-1}. Hence there is a need to evaluate the toxicity of r-TiO_2-ENP with regard to their bio concentration thereby effect on more sophisticated endpoints such as biochemical markers including the milieu of trace elements, antioxidant enzymes and molecular indicators (e.g. Proteins).

5.5 Bioconcentration of Ti in earthworm

Dermal uptake of metals can be directly related to the concentration of the metal that is available in the exposure medium. As the complex nature of soil and its other properties can influence the oral route uptake of metals, soil exposure of the nanoparticles cannot directly be explained (Vijver et al., 2003). When dermal uptake is the only route along which metals are accumulated, exposure and effects of testing material may indeed be well explained from soluble concentrations in the relevant media of exposure. Uptake of metals by from exposure medium via skin (dermal) and by ingestion (oral) is very important to delineate their bio- concentration potential in earthworm. Therefore, present study used the dermal uptake of r-TiO2-ENP to elucidate its potential to undergo bioconcentration on the skin of earthworm. Bioconcentration of r-TiO_2-ENP at 0.05 to 0.25 mg/cm^2 was used to understand the lethal and sub lethal effects of r-TiO_2-ENP in earthworm. Bioconcentration potential of r-TiO_2-ENP was evaluated for 48 h through paper contact method (Figure 5.7). Results indicated that 5, 13, 21, 35, 61 ng/g of r-TiO_2-ENP accumulated in the earthworm tissue at 0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2 respectively. Thus concentration dependent accumulation of r-TiO_2-ENP would have resulted in the mortality of the earthworm. However, earlier study conducted by Canas et al., (2011) reported that anatase TiO_2-ENP could induce mortality in earthworm, even at high concentrations (5000 and 10000 mg/L). Furthermore, concentration of Ti in tissues of an organism may differ according to the size and surface charge of the TiO_2-ENP when their shape is the same (E. J. Park et al., 2014). In contrast, despite agglomeration of r-TiO_2-ENP, bioconcentration of the particles increased on earthworm skin. This could be attributed to the factors that crystal type i.e. rutile used in the present study and anatase

reported elsewhere. Furthermore, the *z*-average of the particles used in the present study (353 nm) is less when compared to the other study conducted by Canas et al. (2011) which has showed no results on Z- average.

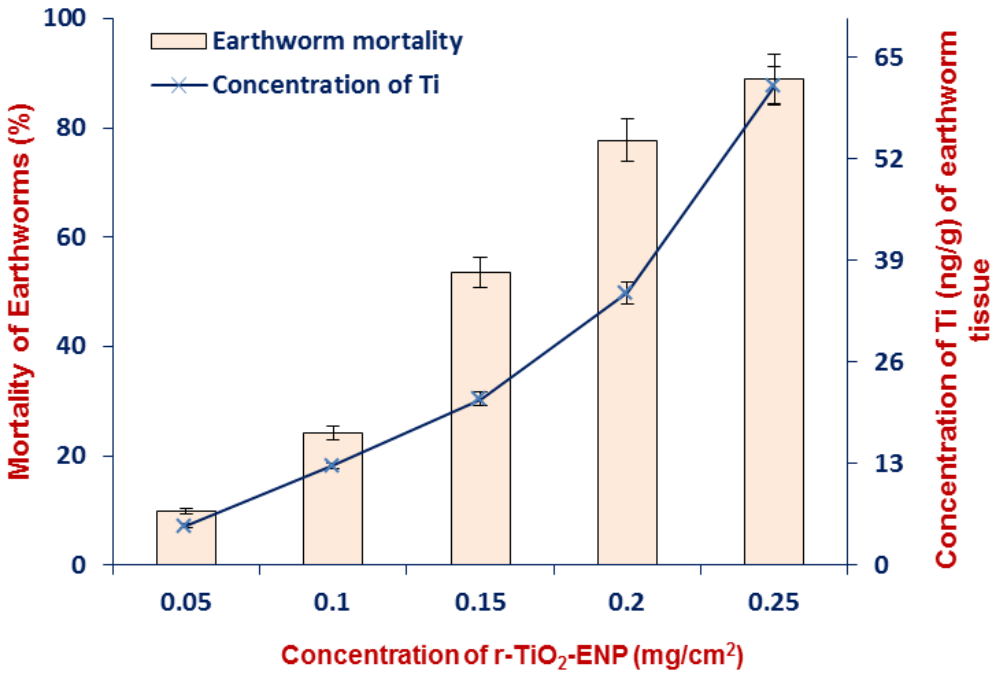

Figure 5.7 Mortality (%) and bioconcentration of Ti in earthworms exposed to r-TiO_2-ENP. Data represent mean concentration ± S.D (*n=3; p<0.05*)

The mode of concentration of Ti on earthworm skin could also be attributed to the charge (-23, -21,-17, -10, -6, mV) of r-TiO_2-ENP at respective concentrations 0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2. Thus charge at various concentrations of r-TiO_2-ENP might have altered the uptake of the particles. These observations suggest that uptake of agglomerated r-TiO_2-

ENP and subsequent accumulation in earthworm is affected by size and charge during dermal exposure through filter paper medium. Thus it is evident from the study that type of the particle, size and mode of exposure influence the toxicity. To the best of knowledge, this is the first of its kind in reporting the accumulation of r-TiO_2-ENP in earthworm through dermal contact. However, further studies are warranted to understand depuration of r-TiO_2ENP in earthworm to delineate its bio-concentration factor in earthworm. Such studies will definitely aid in advancing the science of nanoecotoxicology studies thereby providing complete understanding of bioaccumulation mechanisms of r-TiO_2-ENP and similar kind of materials in environmental sentinel organisms.

5.6 Assessing levels of trace elements in earthworm tissue

Recent studies indicated the potential of nanoparticles to alter the homeostasis of electrolytes and trace elements (Benetti et al., 2014). In this regard, present study was conducted to understand the effect of Ti bioconcentration on trace element and electrolytes in earthworm. The effect of Ti bioconcentration on the levels of trace elements was measured at upper limit of median lethal concentration (0.15 mg/cm^2) and sub lethal concentrations (0.05 and 0.10 mg/cm^2) of r-TiO_2-ENP. Results indicated the dose dependent effect of Ti concentration led to the accumulation of electrolytes (Na, Mg, K and Ca) (Figure-5.8) and trace elements (Mn, Cu and Fe) Figure 5.9.

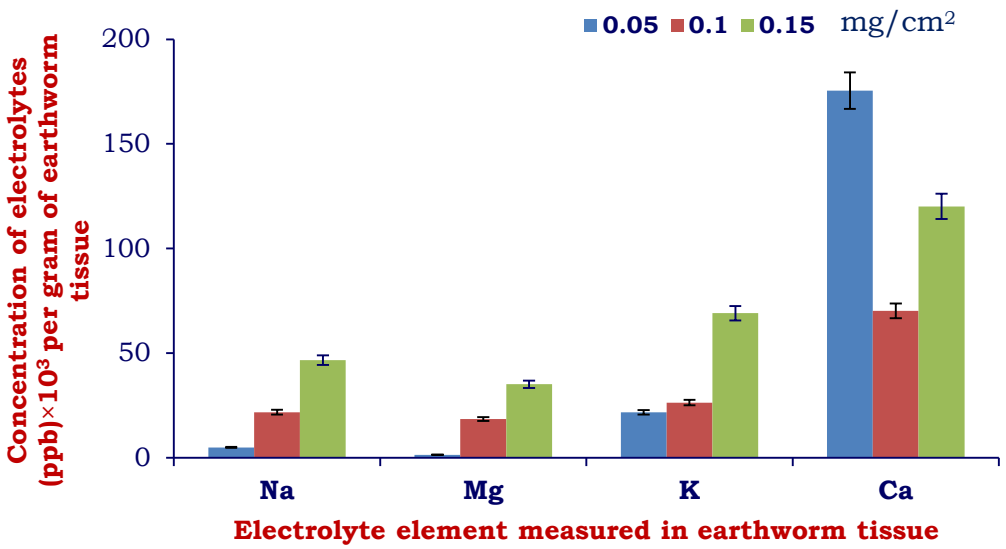

Figure 5.8 Concentration of Na, Mg, K and Ca in earthworm tissue exposed to 0.05, 0.1 and 0.15 mg/cm^2 of r-TiO_2-ENP. The data represent concentration ± RSD. Data is normalized with respect to control (*p<0.05)*

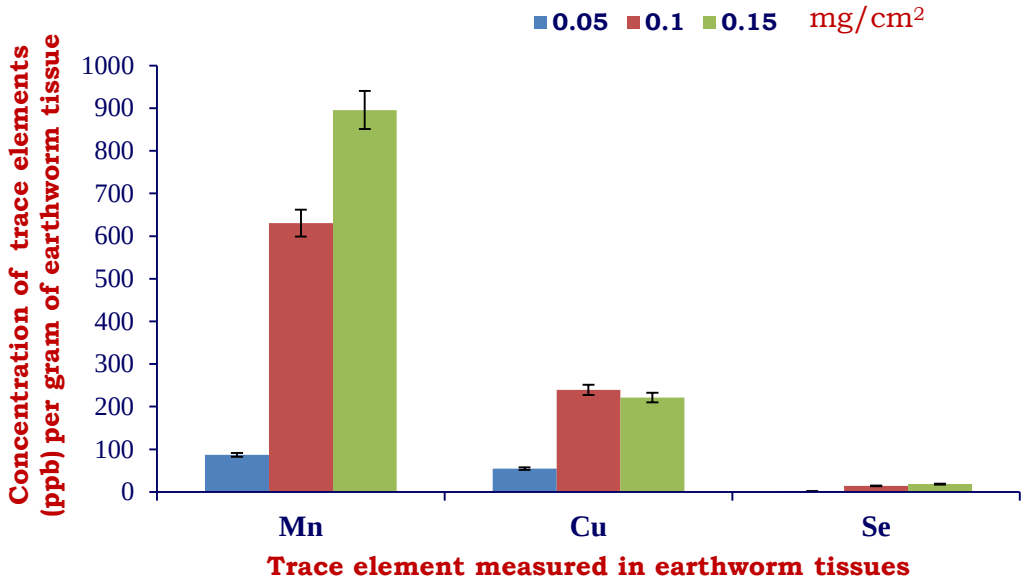

Figure 5.9 Concentration of Mn, Cu, and Se in earthworm tissue exposed to 0.05, 0.1 and 0.15mg/cm^2 of r-TiO_2-ENP. Data represent concentration ± RSD. Data is normalized with respect to control *(p<0.05)*

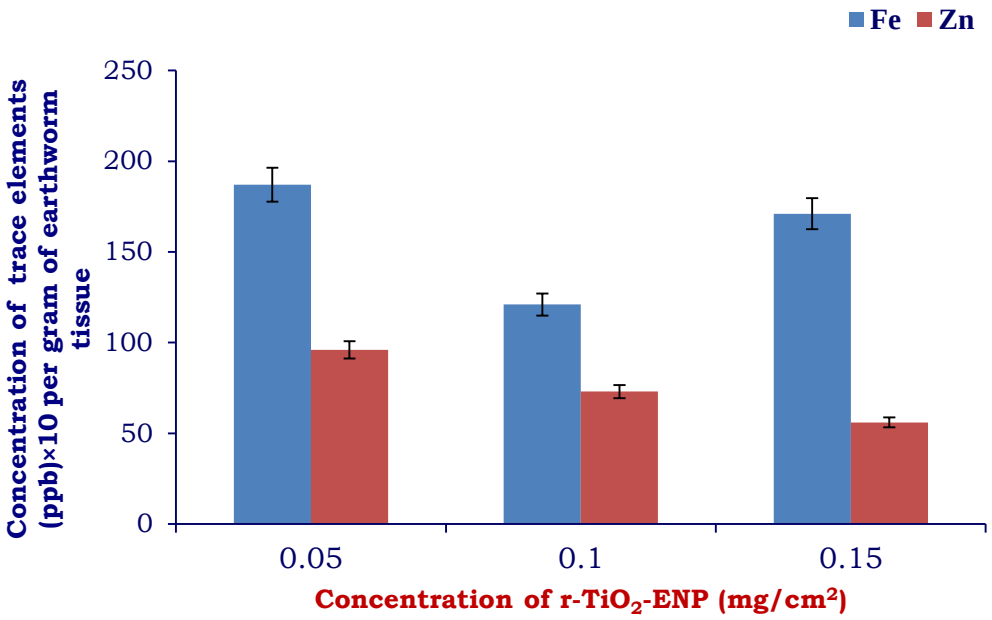

Figure 5.10 Concentration of Fe and Zn in earthworm tissue exposed to 0.05, 0.1 and 0.15mg/cm^2 of r-TiO_2-ENP. The data represent concentration ± RSD. Data is normalized with respect to control *($p<0.05$)*

The order of accumulation of electrolyte Ca was found to be greater than potassium whereas the accumulation of Na and Mg do not differ significantly. Among the trace elements Mn, Cu, Fe and Zn were found to accumulate significantly. Overall, it was observed that the accumulation of r-TiO_2-ENP to altered the homeostasis of electrolytes and trace elements. From these observations, it is speculated that unstable oxidation state (+2) of Ti in TiO_2, it could have acted as an amphoteric oxide which behaved as an acid and as a base (Park et al., 2014). Thus it should have triggered the dissociation of TiO_2 under the influence of redox response thereby allowing, the substitution of Ti by other ions in binding sites (Bal et al., 2013; Cyert and Philpott, 2013). Furthermore, bio- concentration of metallic ions in cytoplasm have the potential to alter the concentration trace elements including Mn, Zn and Cu (the main components of superoxide dismutase) Fe

(co factor of catalase and amphoteric oxide as Ti), Co and Al (amphoteric oxides) in living system (Park et al., 2014). Similar types of results were documented by (Valant et al., 2012) in describing the effect of TiO_2 nanoparticles on the digestive gland membrane of terrestrial isopod, *Porcellio scaber.* Thus, present study will assist in predicting the possible effects of Ti based nanoparticles on various essential elements in cells or other sentinel species.

5.7 Biochemical responses: Activity of antioxidant enzymes, lipid peroxidation, choline esterase function and proteomic profiles

Subtle responses to low-dose exposures have the potential to alter biochemical pathways in an organism when compared to their high-dose exposures. Patterns of molecular responses at lower exposure concentrations and corresponding higher doses also vary in terms of molecular responses (Andersen and Krewski, 2009; Klaper et al., 2014). Therefore, assessing the highly temporal oxidative stress responses in evaluating the effects of nanoparticles which are considered as acutely not toxic is important. However, metallic nanomaterials are toxic at lower doses. This could be attributed to their dissolution/ availability in exposure environments rather than by the nanomaterial itself. Surprisingly, there exist is a significant knowledge gap in this pursuit. Oxidative stress responses to toxic agent are highly temporal. They have potential to undergo dissipation quickly thereby altering the role of inflammatory mediators in a time dependent manner. In this regard, literature demonstrated that nanomaterials can cause oxidative stress and inflammation in the organisms. Moreover, such effect can subside for 24 h -72 h of time period. Such phenomenon in response to rutile TiO_2 nanoparticles was demonstrated in mouse lung cells. (Ambrosone et al.,

2012; Husain et al., 2013). However, such studies with respect to TiO_2-ENP are not well documented in various sentinels including earthworm. With regard to these facts, present study is aimed at providing a snapshot of the oxidative stress responses in earthworm exposed to low doses of r-TiO_2-ENP.

Conversion of TiO_2 into ionic titanium can mediate generation of hydroxyl radicals via Fenton reaction (Tengvall et al., 1989). Therefore, it can act as a pro-oxidant to cause oxidative damage in the cells or living systems. Recent investigations concluded that exposure to TiO_2-ENP may lead to imbalance in oxidative environment of the cells in the organisms (Khalil, 2015a; Menard et al., 2011; Su et al., 2014) adverse impact on physiology of the cells (Ratnasekhar et al., 2015; Srpčič et al., 2015) and peroxidation of lipids in the membrane (Su et al., 2014). In this context, several endogenous antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT) can scavenge the reactive oxygens species (ROS) to mitigate their adverse effects on cellular constituents and thereby lipid peroxidation. Pro-oxidant effects of nanoparticles and other pollutants can produce malondialdehyde (MDA) as end product of lipid peroxidation. It is considered as an indicator of oxidative damage caused by pollutant stress. Therefore, antioxidant enzymes, levels of lipid peroxidation can serve as indicators of nanoparticle toxicity (Z. Li et al., 2014). Therefore, monitoring specific activities of these enzymes can help in understanding the effect of ROS that are produced during adverse effects of pollutants on cells. GR activity can help in surmising the redox state of a cell or an organism in a given environmental stress by reducing disulfide glutathione. Hence, the present study aimed at measuring the activities of SOD, CAT and GR. Measuring the

specific activities of these enzymes that help in speculating the possible oxidative stress pathway in earthworm during its exposure to r-TiO_2-ENP. Specific activities of antioxidant enzymes SOD (A), CAT (B), GR (C) and LPO (D) measured in dermal contact and soil exposure for 48h are presented in Figure 5.11 and 5.12 respectively.

5.7.1 Antioxidant enzyme activity and lipid peroxidation during dermal contact (48 h)

Specific activity of SOD observed in earthworm during direct contact exposure is presented in Figure 5.11 (A). SOD activity increased from 0.05 mg/cm^2 (3.3 ± 0.32 U/mg) to 0.25 mg/cm^2 (18.5 ± 0.85 U/mg). It indicates that the SOD is induced during dermal exposure of earthworm to r-TiO_2ENP in a concentration dependent manner significantly. However, the activity of SOD in the study might be related to the accumulation of r-TiO_2ENP during exposure. Thus the percent of Ti accumulated in earthworms have led to the increase in activity of SOD. Furthermore, SOD has potential to scavenge free radicals as a substrate and is responsible for catalyzing the dismutation of the superoxide radical O_2• to H_2O_2. Hence, it is speculated that r-TiO_2-ENP has the potential to cause an increase in the level of H_2O_2, which must be scavenged by CAT.

Activity of catalase CAT is decreased in earthworm exposed to r-TiO_2-ENP when compared to the control [Figure 5.11 (B)]. The decrease was found to be significant at concentrations above 0.15 mg/cm^2. TiO_2-ENP has the potential to bind to catalase by the electrostatic and hydrogen bonding. TiO_2-

EN induce catalase activity at a lower concentration and inhibition at a higher concentrations (Zhang et al., 2014). Moreover, electron transferring potential of CAT can enhance its biological activity (Chelikani et al., 2004). In this context, results of the present study are fine tune with the facts about CAT activity during TiO_2-ENP toxicity. Furthermore, as the accumulation of Ti was proportional to the concentration, availability of Ti might have facilitated the impairment of electron transfer efficiency of CAT thereby decreasing its activity over control at higher concentrations (Tehrani et al., 2013). Hence it resulted in the inhibition of CAT activity.

Results indicate the pro-oxidant potential of Ti to produce free radicals such as $OH^{-\cdot}$ Our results are in fine tune with the activity profile of CAT reported by (Zhang et al., 2014). Outcomes of current study are in agreement with the effects of TiO_2-ENP on the activity of CAT in earthworm exposed to TiO_2 in 0.5 g/kg of the soil (C W Hu et al., 2010), leaves of *Cucumis sativus L.* (Servin et al., 2013) and spinach chloroplasts (Ferre-Huguet et al., 2008). Further studies are needed to ascertain size dependent effect of TiO_2-ENP on conformational changes of catalase.

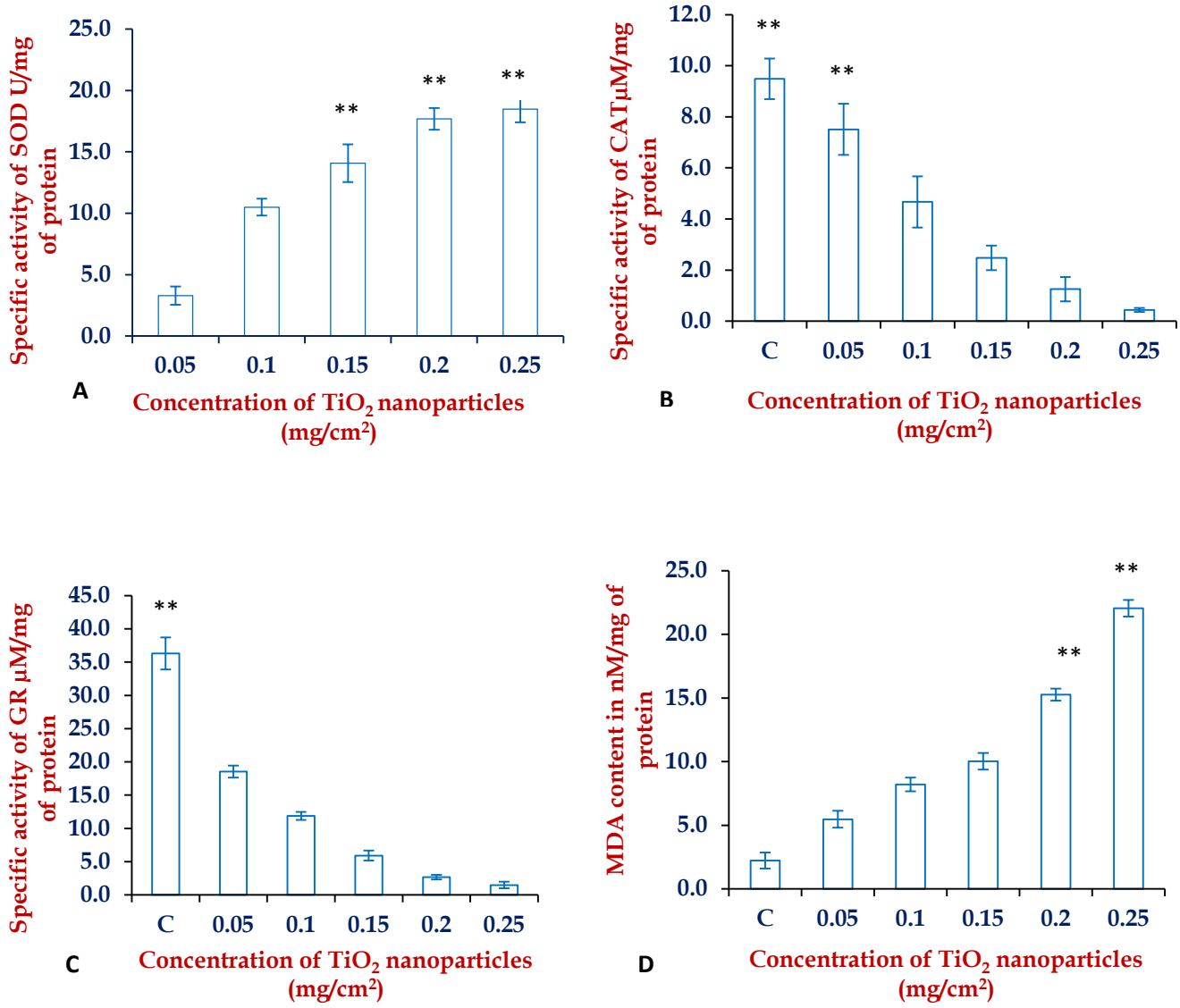

Figure 5.11 Specific activities of antioxidant enzymes SOD (A), CAT (B), GR (C) and LPO (D) in earthworm, *Eisenia fetida* exposed to r-TiO_2-ENP after 48h by filter paper method. The final data represents mean ± S.D (n=3, p<0.05). SOD specific activity (A) is presented over control.

Activity of GR is presented in [Figure 5.11 (C)]. r-TiO_2-ENP inhibited glutathione reductase in earthworm in a dose dependent manner. Significant inhibition of GR can be related to the fact that the accumulation of Ti in

earthworm is directly proportional to the concentration of r-TiO_2-ENP (reported earlier in section 5.5). GSH exhibits high affinity for metals, forming thermodynamically stable but kinetically labile mercaptides with metals (Wang and Ballatori, 1998). As a result, the impaired interconversion GSSG to GSH thereby diminishing the free radicals scavenging ability of earthworm exposed to r-TiO_2-ENP is possible. Thus it resulted in the inhibition of GR thereby leading to oxidative stress in earthworm. The reduction in the activity of GR was observed in mice exposed to TiO_2ENP (Hu et al., 2011; Ma et al., 2010).

Induction of LPO in terms of MDA in earthworm exposed to TiO_2ENP [Figure 5.11 (D)] indicated the potential of r-TiO_2-ENP to cause damage of lipids. In general, GSH formed as a result of GR activity can form adducts with hydro peroxides thereby leading to the generation of free radicals (Wang and Ballatori, 1998). As a result, induction of LPO might have resulted because of the inhibition of catalase, and glutathione reductase. Moreover, internalization of TiO_2 nanoparticles can lead to the elevated levels of intracellular ROS thereby ameliorating the lipid peroxidation in the cells (Dubey et al., 2015). Hence accumulation of Ti could have triggered lipid peroxidation in earthworm tissues by generating ROS. Therefore, it is surmised that elevated levels of lipid peroxidation might be due to the severe oxidative stress caused by r-TiO_2-ENP in earthworm. It was also affirmed that TiO_2-ENP resulted in severe oxidative stress by inhibiting the activities of GR and CAT antioxidant enzymes in earthworm during dermal exposure.

5.7.2 Antioxidant enzyme activity and lipid peroxidation during soil exposure (48 h)

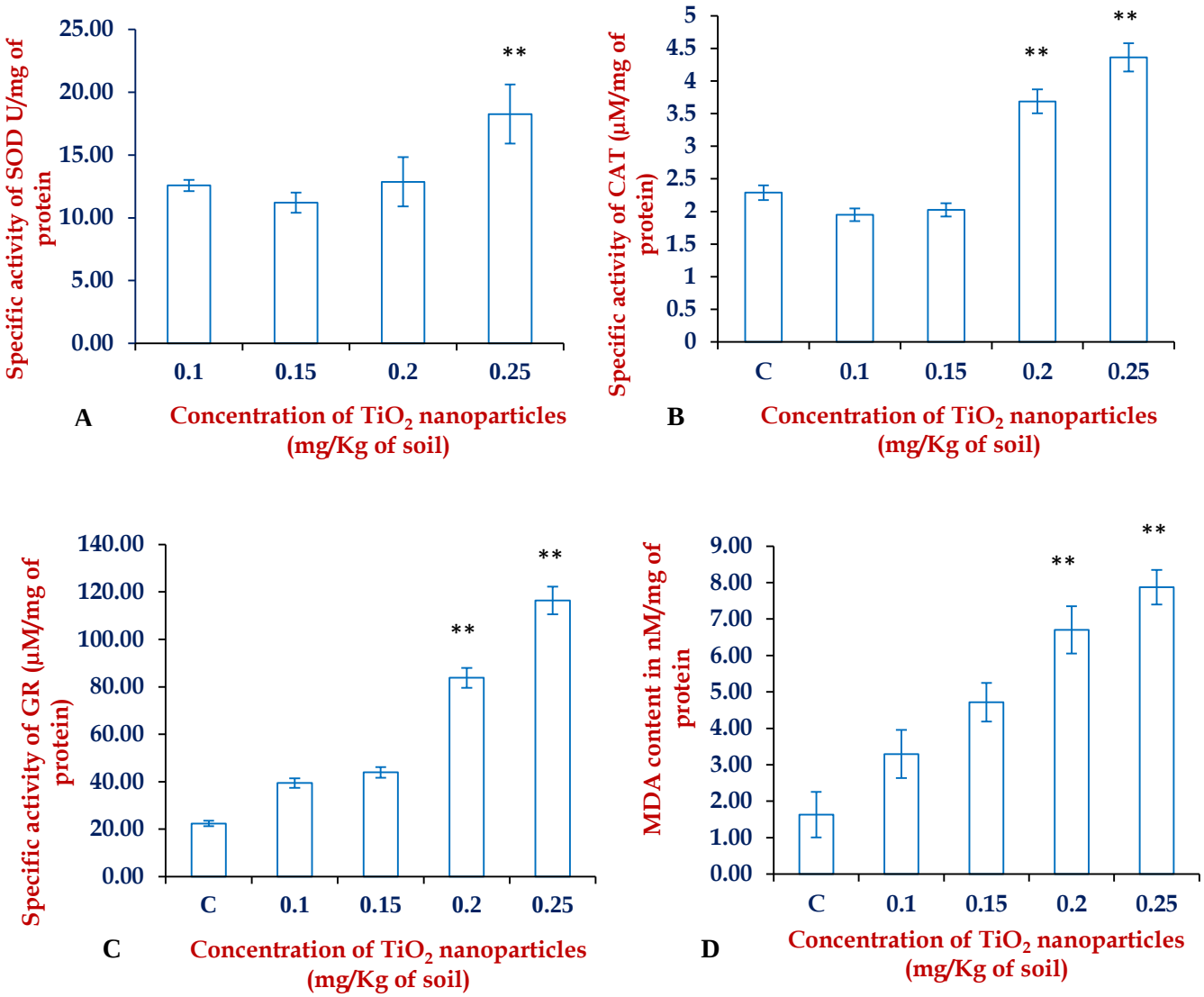

Figure 5.12 Specific activities of antioxidant enzymes *SOD (A), CAT (B), GR (C) and LPO (D) in earthworm, *Eisenia fetida* exposed to r-TiO_2-ENP after 48h by artificial soil method. The final data represents mean ± S.D (n=3, p<0.05). SOD specific activity (A) is presented over control.

SOD activity was induced in earthworms in soil exposure method when compared to control. But there was no significant differences were observed among the treatments at 0.1-0.2 mg/kg. However, at 0.025 mg/kg, there is a

significant induction of SOD. It indicates that the concentrations of r-TiO_2-ENP that are greater than LC_{50} could induce SOD activity. It is also evident that the range of concentrations 0.1-0.2 mg/ kg was not sufficient to induce the changes in SOD activity. However, 0.25 mg/kg could induce the activity of SOD. Hence even the effect of small window of concentrations of r-TiO_2-ENP on earthworm can be demonstrated through soil exposure for 48h. Though the experimental setup and sentinel used is different, similar kind of results was documented by (Xia et al., 2015) in marine micro algae *Nitzschia closterium* exposed to r-TiO_2-ENP. Furthermore, the range of concentrations used and specific activity of SOD reported in the study will help in demonstrating the effect of r-TiO_2-ENP in future studies.

Soil exposure of earthworm resulted in the induction of CAT [Figure 5.12(B)]. TiO_2-ENP has the potential to bind to catalase by the electrostatic and hydrogen bonding. TiO_2-ENP induce catalase activity at a lower concentration and inhibition at a higher concentrations (Zhang et al., 2014). However, these results contradict with that of direct contact exposure of r-TiO_2-ENP. This could be attributed to the fact that the low dissolution/ non-availability of r-TiO_2-ENP below 0.15 mg/kg in the soil. It is also interesting to know that activity of CAT in the tested concentrations would help in establishing a fact that the range of concentrations with small window range could assist in identifying the differences in oxidative responses of earthworm through dermal and soil exposure for 48 h.

GR activity was induced in earthworms during soil exposure [Figure 5.12 (C)]. It indicates the potential of Ti to alter the glutathione cycle thereby causing oxidative stress above 0.2 mg/kg of r-TiO_2-ENP. It is also interesting note that the GR activity observed during filter paper exposure are quite contradicting with the soil exposure. Once again it can be attributed to the availability of r-TiO_2-ENP in the soil medium at a given concentration to alter the function of GSH cycle during metal detoxification. However, further studies are warranted to understand the effect of r-TiO_2-ENP through ingestion and dermal uptake to their low dose-chronic exposures. In this regard, outcomes of the present study help in providing baseline data to design such experiments. It also assists in underlining the signatures of GR activity at non-lethal concentrations/ low doses of r-TiO_2-ENP in earthworm.

Induction of LPO in terms of MDA in earthworm exposed to r-TiO_2-ENP [Figure 5.12 (D)] indicated their potential to damage of lipids. In general, GSH formed as a result of GR activity can form adducts with hydro peroxides thereby leading to the generation of free radicals (Wang and Ballatori, 1998). As a result, induction of LPO might have resulted because of the activation of SOD, CAT and GR. Moreover, internalization of TiO_2 nanoparticles can lead to the elevated levels of intracellular ROS thereby ameliorating the lipid peroxidation in the cells (Dubey et al., 2015). Hence accumulation of Ti could have triggered lipid peroxidation in earthworm tissues by generating ROS. Therefore, it is surmised that elevated levels of antioxidant enzyme is observed to combat the ROS damage thereby mitigating the effects of lipid peroxidation. Thus, oxidative stress responses

caused due to r-TiO_2-ENP via soil and dermal exposures in earthworm help in predicting the role of these markers in evaluating toxicity.

In conclusion, r-TiO_2-ENP can cause oxidative stress in earthworm (Iavicoli et al., 2011; Shi et al., 2013). Moreover, altered trace element levels could have contributed to the cascading response of superoxide dismutase; catalase and glutathione reductase. Induction of these parameters can serve as important tool to understand the basis of oxidative stress induced by low doses of r-TiO_2-ENP at short term exposure in earthworm.

5.7.3 Antioxidant enzyme activity and lipid peroxidation during soil exposure for 7 and 14 days

TiO_2-ENP has the potential to induce oxidative stress in earthworm at a concentrations ≥1g/kg of the soil. As per the literature, type of the crystal and size of the particle will differ in eliciting toxicity in sentinel organisms. Furthermore, till date, studies have conducted toxicity assessment of anatase (32 nm) (Canas et al., 2011), rutile (10-20 nm)(C. W. Hu et al., 2010) in earthworm at concentrations greater than 1 g/kg of the soil. In fact, the environmentally realistic concentrations range from 3-13 mg/kg in biosolids from sewage treatment plant (section 5.1) with median size of 70-145 nm. Though there exist a limitation in terms of techniques that differentiate various crystal phases in given environmental sample, emerging science in this pursuit revealed that global scale nano TiO_2 (rutile form) has much wider applications especially in consumer products in comparison with the anatase form. Hence it has much potential to get released into the environment. Furthermore, humic acid present as part of soil can reduce the the toxicity of TiO_2 nanoparticles during their toxicity assessment (Yang et

al., 2013). In light of these facts, present study attempted to understand the toxicity of r-TiO_2-ENP in soil exposure medium and subsequent antioxidant enzyme responses at non-lethal concentrations ranging from 0.05 to 04 mg/kg in earthworm.

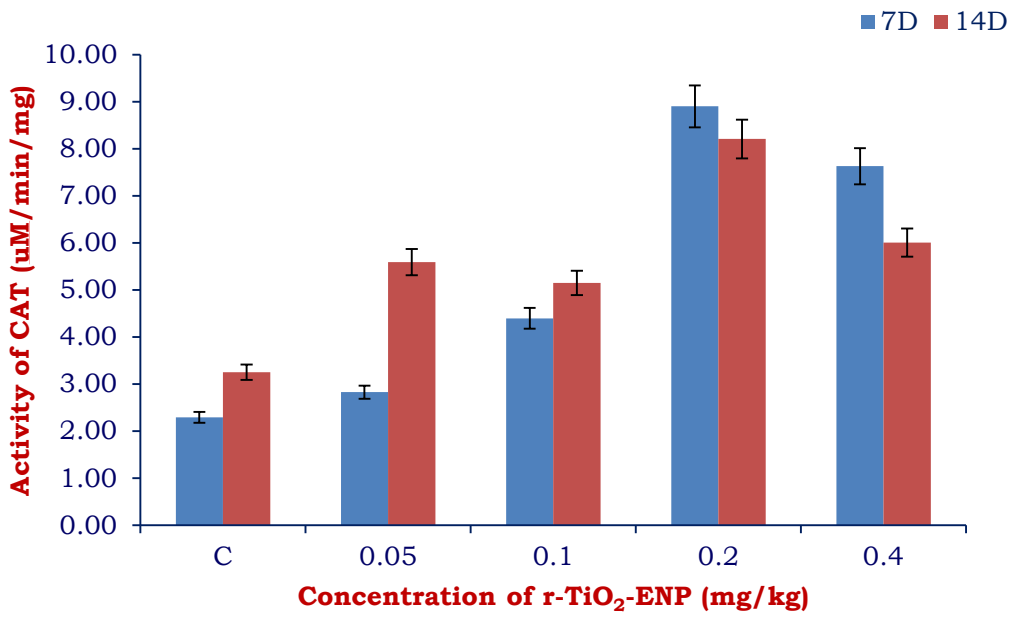

Figure 5.13 Specific activity of CAT in earthworm, *Eisenia fetida* exposed to r-TiO_2-ENP in artificial soil method for 7 and 14 days. The final data represents mean ± S.D (n=5, $p<0.05$)

Soil exposure of earthworm resulted in the induction of CAT (Figure 5.13). TiO_2-ENP has the potential to bind to catalase by the electrostatic and hydrogen bonding. TiO_2-ENP induce catalase activity at a lower concentration and inhibition at a higher concentrations (Zhang et al., 2014). However, results indicated that soil exposure of r-TiO_2-ENP elicit the activity of CAT. This could be attributed to the fact that the low dissolution/ non-availability of r-TiO_2-ENP at 0.05 and 01 mg/kg in the soil. Moreover, the

difference between the activity of CAT for 7 and 14 days at chosen concentrations was found to be very less. This indicates that CAT can provide more insights into the oxidative stress mechanisms thereby acting as a marker of oxidative stress at non-lethal concentrations.

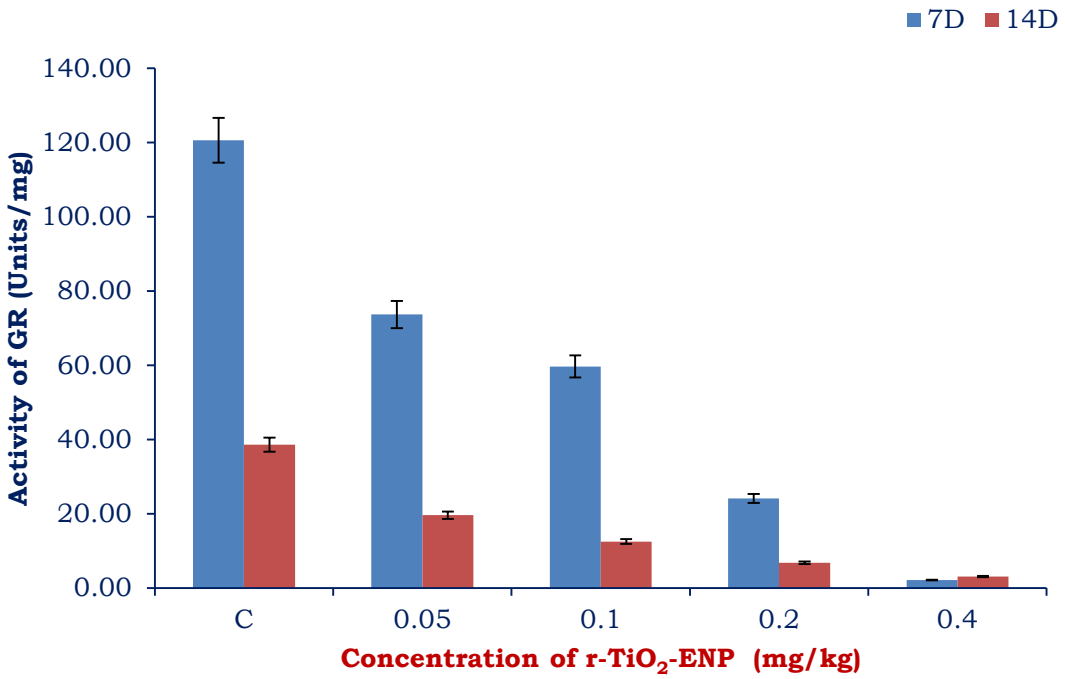

Figure 5.14 Specific activity of GR in earthworm, *Eisenia fetida* exposed to r-TiO_2-ENP in artificial soil method for 7 and 14 days. The final data represents mean ± S.D (n=5, $p<0.05$)

Activity of GR is presented in (Figure 5.14). r-TiO_2-ENP inhibited glutathione reductase in earthworm in a dose dependent manner. Activity of GR for 7 and 14 days decreased in a dose dependent manner. Decreased activity of GR at 14 days is more pronounced than that of 14 days. It is evident from the results that antioxidant profiles at lower exposure periods will differ from the long duration exposure under same conditions of the experiment. GSH exhibits high affinity for metals, forming thermodynamically stable but kinetically labile mercaptides with metals (Wang and Ballatori, 1998). As a

result, the impaired interconversion GSSG to GSH thereby diminishing the free radicals scavenging ability of earthworm exposed to r-TiO_2-ENP is possible. Similar kind of results were documented during the mice exposure to TiO_2ENP (Hu et al., 2011; Ma et al., 2010).

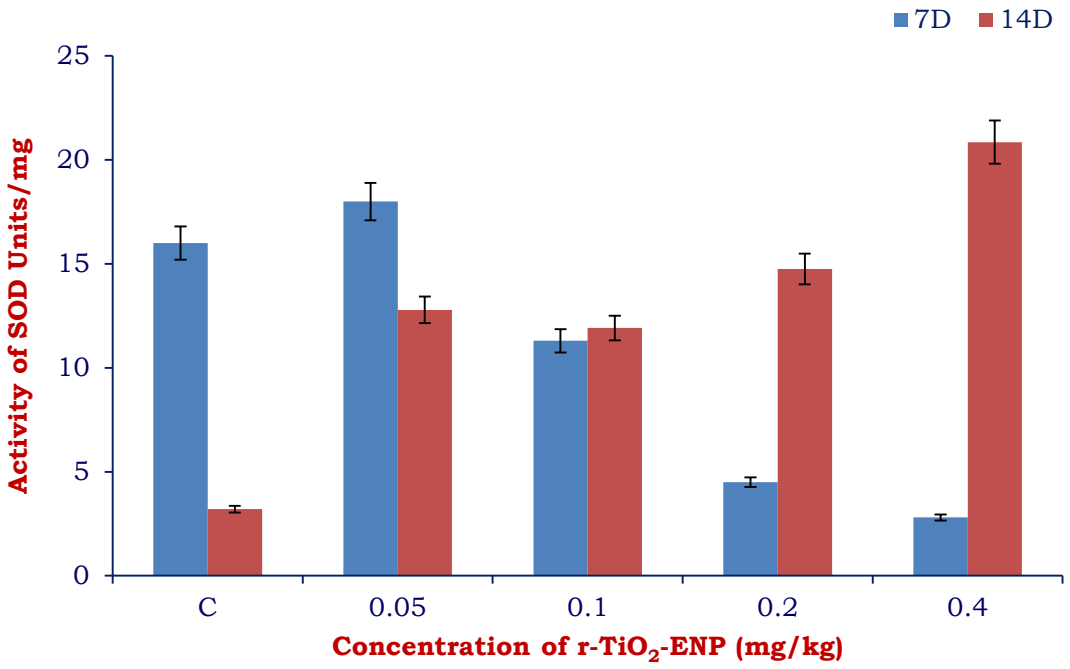

Figure 5.15 Specific activity of SOD in earthworm, *Eisenia fetida* exposed to r-TiO_2-ENP in artificial soil method for 7 and 14 days. The final data represents mean ± S.D (n=5, $p<0.05$).

SOD activity in earthworms exposed to r-TiO_2-ENP is presented in Figure 5.15. Results indicated dose dependent activation of SOD activity. However, activities at 0.1 mg/kg to 0.4 mg/kg of r-TiO_2-ENP were found to be more significant in 14 days exposure. Similar kind of results were observed when earthworms were exposed to sub-lethal concentrations of anatase nanoparticles (21 nm) (Khalil, 2015b). On the other hand, SOD activity for 7 days was found to be decreased significantly at concentrations > 0.1 mg/kg. These observations indicate that the mode of exposure and duration are very

important in divulging the SOD activity during r-TiO_2-ENP toxicity. Though the window of concentrations chosen in the study did not result in mortality, response of SOD observed in the study will assist in explaining the toxicity of low doses of r-TiO_2-ENP. The results also revealed that the SOD response does not differ significantly between 7 and 14 days of exposure to r-TiO_2-ENP. Hence, evaluation of temporal responses such as antioxidant enzymes at lower doses for short term exposure can provide more insights into the toxicity mechanisms of r-TiO_2-ENP.

Induction of LPO in terms of MDA in earthworm exposed to r-TiO_2-ENP (Figure 5.16) indicated their potential to damage lipids. Increase in lipid peroxidation was found to be dose dependent. Lipid peroxidation was found to be more significant at 14 days when compared to 7 days. Hence it is evident from the results that despite the activation of antioxidant enzymes at higher concentrations, peroxidation of lipids is observed. Therefore, it is surmised that r-TiO_2-ENP have the potential to induce oxidative stress even at non-lethal concentrations.

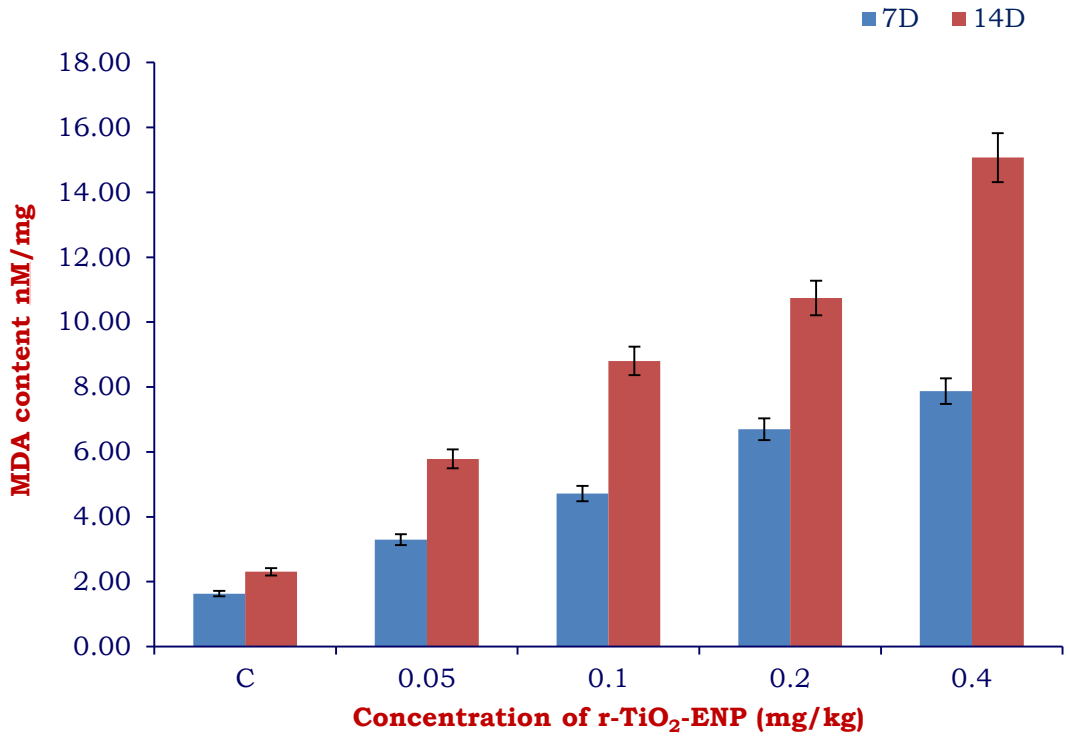

Figure 5.16 Induction of LPO-MDA (nM/mg) in *Eisenia fetida* exposed to r-TiO_2-ENP in artificial soil method for 7 and 14 days. The final data represents mean ± S.D (n=5, $p<0.05$)

5.7.4 Acetylcholinesterase activity during soil exposure

Acetylcholinesterase (AChE) is an enzyme that degrades the neurotransmitter acetylcholine in cholinergic synapses of the nervous system (Čolović et al., 2015). The important role of AChE activity in pollution monitoring and its biochemical significance has been characterized in macroinvertebrates (Essawy et al., 2009; Rault et al., 2007; Rickwood and Galloway, 2004). Toxicity of anatase TiO_2-ENP is evaluated in a common earthworm to Egypt, *Pheretima hawayana*. However, such studies are confined to regional species. Moreover, assessing AChE in *Eisenia fetida, commonly used earthworm species* for r-TiO_2-ENP will provide important baseline data for neurotoxicity. Therefore, measuring the altered levels of

AChE activity which can serve as a marker to understand the effect of r-TiO_2-ENP toxicity is carried out in the present study.

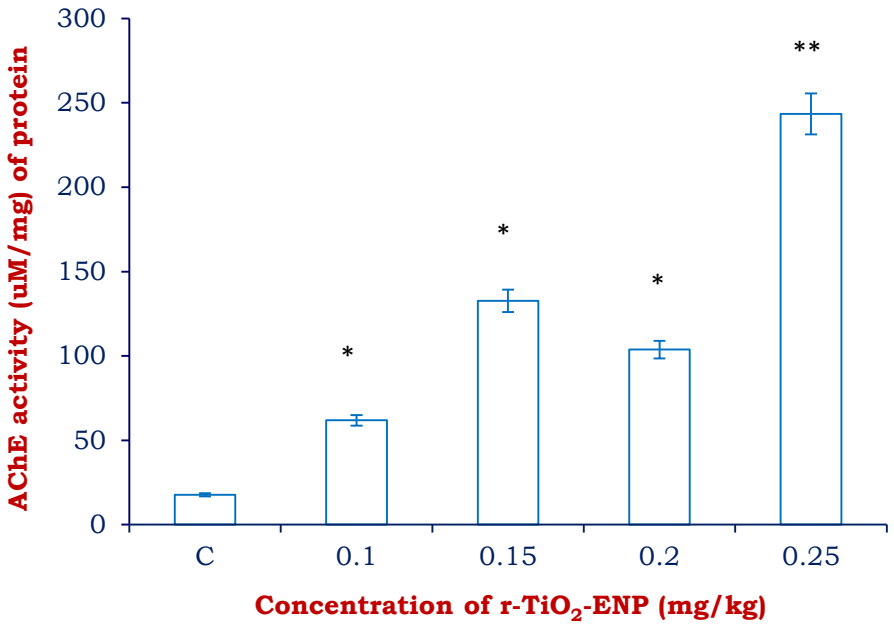

Figure 5.17 Specific activity of acetylcholinesterase (AChE) in earthworm, *Eisenia fetida* exposed to r-TiO_2-ENP after 48h by artificial soil method. The final data represents mean ± S.D (n=3, p<0.05). *specific activity is presented over control

In the present study, the levels of AChE were induced in earthworms exposed to r-TiO_2-ENP via soil exposure for 48 h (Figure 5.17). Activation of AChE in earthworm is directly proportional to dose r-TiO_2-ENP. Furthermore, these nanoparticles alter the homeostasis of Mg, Ca and Na elements (reported in the section 5.5). Thus, altered homeostasis of trace elements can be linked to the possible inhibition of Mg/Ca ATPase's thereby promoting the activity of AChE. Moreover, altered K and Na levels could have contributed the differences in electrochemical gradients leading to the disturbance in neuronal transmission (Olasagasti et al., 2014). Similar kind of results were observed in a study conducted by Khalil,(2015). However,

the particle type and size (anatase and 21 nm) as well as species of earthworm differ from the current study.

AChE activity in earthworms exposed to r-TiO_2-ENP via soil exposure for 7 and 14 days is presented in Figure 5.18.

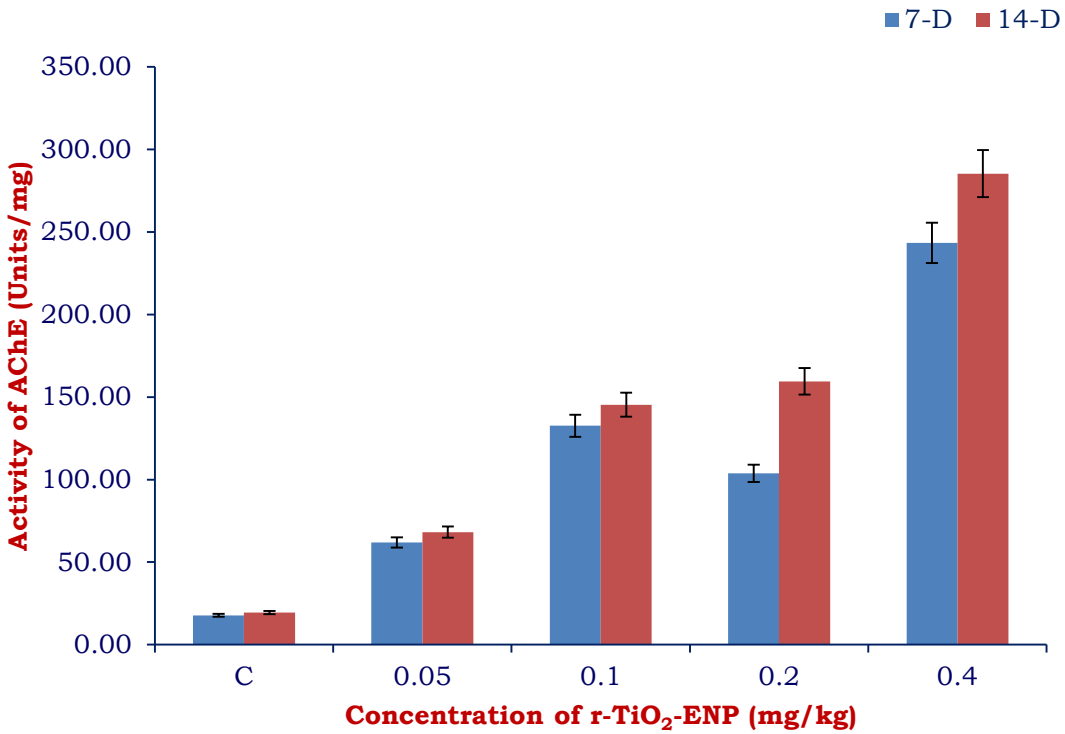

Figure 5.18 Specific activity of acetylcholinesterase (AChE) in earthworm, *Eisenia fetida* exposed to r-TiO_2-ENP after 7 and 14 days by artificial soil method. The final data represents mean ± S.D (n=3, $p<0.05$)

Results indicate that prolonged exposure to r-TiO_2-ENP induces AChE activity in earthworm in a dose dependent manner. It also provides an evidence of response to r-TiO_2-ENP during the course of experiment. As on date, only mortality is used as an endpoint of acute toxicity test. In this pursuit, AChE activity can serve as a more sensitive marker to understand the toxicity of nanoparticles including r-TiO_2-ENP. However, outcomes of the

present study are in agreement with fact that the increase in exposure duration can increase the activity of AChE in earthworm reported elsewhere (Khalil, 2015b). But the concentration regime (10 to 100ug/ kg), nanoparticle (anatase, 21 nm) differ from the present study. Thus the outcomes of the study contribute to the understanding and designing toxicity assessment methods for divulging neurotoxicity in soil sentinels. Therefore, results can serve as proxy for further studies that are warranted to demonstrate the accumulation of r-TiO_2-ENP in earthworm and subsequent effects on neuronal transmission.

5.8 Proteomic Profiles of earthworms exposed to r-TiO_2-ENP

Studying the expression of genes at the protein level is called as Proteomics (Wang et al., 2010). Such studies provide not only direct information on the protein level but also activity profiles of the relevant proteins (Naaby-Hansen et al., 2001). Hence proteomic approaches can be used to determine molecular responses with regard to toxicological effects of contaminants or stressors (Dowling and Sheehan, 2006; Lemos et al., 2010).Furthermore, differential expression of genes or proteins in a sentinel can serve as an indication of the specific perturbation in biochemical pathways due to the toxic insult on the organism (Andersen and Krewski, 2009; Lushchak, 2011). Initiatives such as ToxCast program by US EPA pave a way to explore the potential of endpoints assessed during *in vitro* cellular assays with the omics profiles including proteomic endpoints to predict whole organism effects.

But these efforts needs data pertaining to type of assay and number of toxicological pathways explored for modelling metabolic pathways. Moreover,

studies that address the impact of nanomaterials are also emerging in this regard for exploring the extrapolation of selected biomarkers such as oxidative stress into high-throughput nanomaterial testing as a key indicator of toxicity. However, investigations on proteomics of earthworms in nanotoxicology lags far behind the other species (Wang et al., 2010). Similarly, protein profiling of earthworms using proteomic approaches following r-TiO_2-ENP exposure is first of its kind in nanotoxicology. Hence the present study is carried out to elucidate the proteomic profiles of earthworm *Eisenia fetida* exposed to r-TiO_2-ENP.

From the results, it was observed that 22 proteins that were identified through label free quantitation, met the requirements of 1 fold change (either decrease or increase) greater than 1 ($p<0.05$) using Orbitrap Analysis. Relative quantitation of the non-conflicting peptides was carried out. Sequence coverage of the peptides and proteins was assessed from MASCOT analysis that matched 100% with databases against *Eisenia fetida.* Fold change in the proteins is presented in terms of average normalized abundancies. These are presented as the proteins that are up-regulated, down regulated during the exposure of worms to LC_{10} (0.05 mg/cm^2) and the upper limit LC_{50} (0.15 mg/cm^2) of r-TiO_2-ENP. Analysis of these protein profiles is provided in Table 5.3 and Table 5.4. Corresponding normalized abundancies of these proteins is presented in Figures 5.18, 5.19 and 5.20.

Table 5.3 Analysis of upregulated proteins in *E. fetida* exposed to r-TiO_2-ENP

Accession	Peptides (Quantitation)	Score	ANOVA (p)*	Fold	Description	Function
319433534	49 (33)	187.12	0.38	1.19	heat shock protein 70	**ATP binding and stress response**
354620989	23 (12)	109.42	0.49	1.4	catalase	**Protects from toxicity of H_2O_2**
46250715	21 (15)	101.57	0.55	1.16	annetocin receptor	**Cell signaling**
83944656	19 (17)	79.06	0.08	1.54	beta-adrenergic receptor kinase 1-4	**Kinase activity**
60735079	51 (46)	143.35	0.58	1.32	Valosin containing protein-2	**ATP binding and stress response**
148807788	20 (18)	53.98	0.91	1.07	Phytochelatin synthase	**Metal detoxification**
83944648	5 (4)	59.63	0.58	1.31	ubiquitin	**Protein degradation and relocation**
167599376	9 (7)	20.54	0.62	1.42	Coactosin-like protein	**Actin binding**
83944654	6 (5)	14.25	0.55	1.55	pyruvate carboxylase	**ATP binding and stress response**
2274816	5 (3)	12.66	0.33	1.55	lysenin-related protein	**Hemolysis and ion transport**
83944638	1 (1)	1.97	0.22	1.99	HSP60	**ATP binding and stress response**
2547055	15 (14)	57.01	0.56	1.15	lombricine kinase	**ATP binding and stress response**
377552755	3 (1)	17.54	0.78	1.28	cytochrome c oxidase subunit II, partial (mitochondrion)	**Electron transport and metal ion binding**
83944650	7 (7)	17.5	0.08	3.05	protein kinase C1	**ATP binding and stress response**
260181579	3 (2)	6.38	0.98	1.47	pi-class glutathione S-transferase	**Detoxification by conjugation**
66361087	3 (1)	3.24	0.33	4.38	Chain A, Crystal Structure Of Earthworm Fibrinolytic Enzyme Component B: A Novel, Glycosylated Two-chained Trypsin	**Fibrinolysis and hydrolysis of proteins**

From the Table 5.3, it is evident that upregulation of proteins pertaining to the ATP binding, electron transport, and conjugation/ detoxification of metal ions suggest that the respiration chain reaction in mitochondria may be compromised as a result of r-TiO_2-ENP exposure. Subsequently, perturbations in energy metabolism by elimination or reduction of ATP generation in mitochondria might have resulted by r-TiO_2-ENP exposure. Similar kind of results were reported in earthworms exposed to Roxarsone (Guo et al., 2015). Hence induction of such responses can contribute to the survival of earthworms during toxic insult. TiO_2-ENP acute exposure results in upregulation of a battery of efflux pumps and nutrient transporters. This is evident from the results that r-TiO_2-ENP induced activity of (i) heat shock protein 70 (ii) beta-adrenergic receptor kinase 1-4 (iii) valosin containing protein-2 (iv) pyruvate carboxylase (v) HSP60 (vi) lombricine kinase (vii) cytochrome c oxidase subunit II and (viii) protein kinase C1. Subsequently, they lead to the overproduction of reactive oxygen species and impair the redox repair systems without inducing mortality. In this pursuit, outcomes of the present study are in agreement with the studies conducted by (Dorier et al., 2015) in gut epithelial cells. Furthermore this is supported by the induction of pi-class glutathione S-transferase whsoe induction enable the transferase reaction during toxicity induced by r-TiO_2-ENP.

HSP70 is involved in protection of the cells from various stressors (Padmini and Usha Rani, 2008). From the study, it is evident that r-TiO_2-ENP induces/ activates the pathways related to HSP70. Thus it could aid in survival of worms during the toxic insult of r-TiO_2-ENP. Annetocin, an oxytocin-related peptide in the earthworm *Eisenia fetida* (Oumi et al., 1996)

affects behavior of worms such as rotatory movements, body-shape, and secretion of mucous from the clitellum. Thus induction of such protein also provides the strong evidence of interaction r-TiO_2-ENP with epidermal layers of the worm during the study. Induction of phytochelatin synthase, an enzyme involved in detoxification of heavy metals indicates the effect of r-TiO_2-ENP on production of phytochelatin in earthworms (Brulle et al., 2007). Normalized abundance of these proteins is presented in Figures 5.18, 5.19 and 5.20.

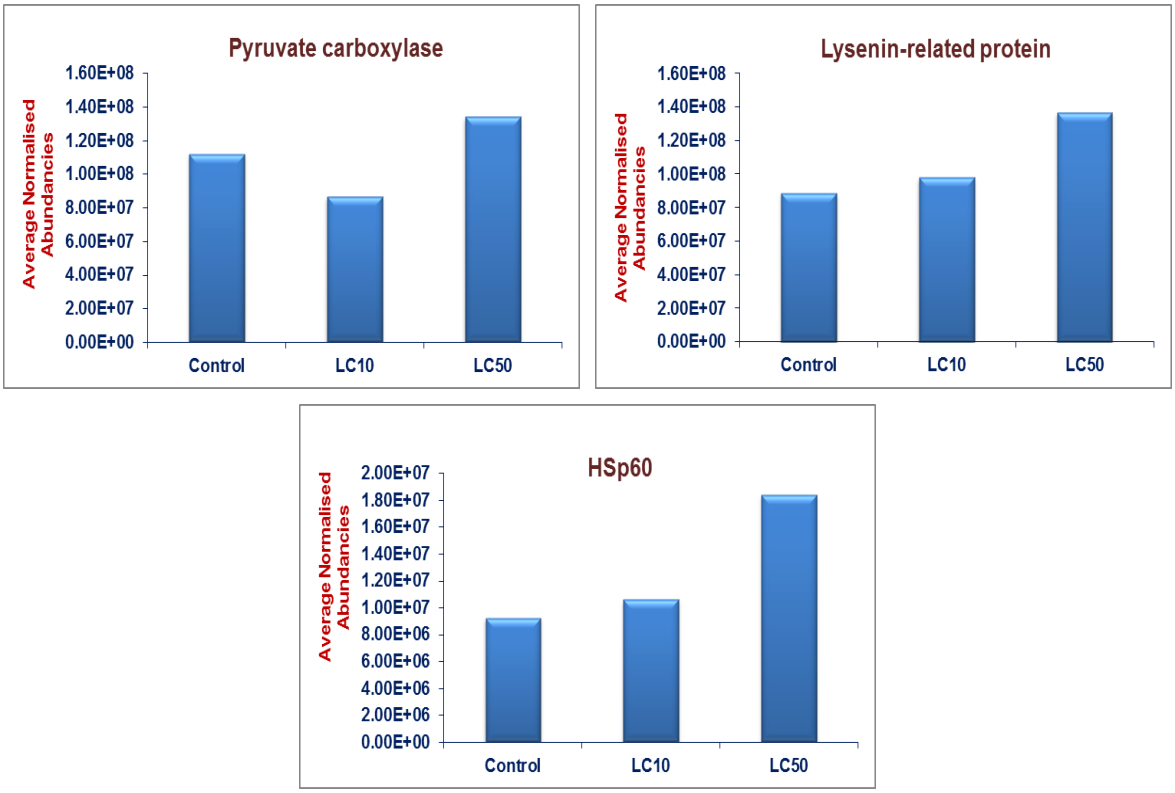

Figure 5.19 Upregulated proteins in *Eisenia fetida* exposed to r-TiO_2-ENP at upper limit of LC_{50} (0.15 mg/cm^2) for 48 h by direct contact method. The final data represents mean ± S.D (n=3, p<0.05).

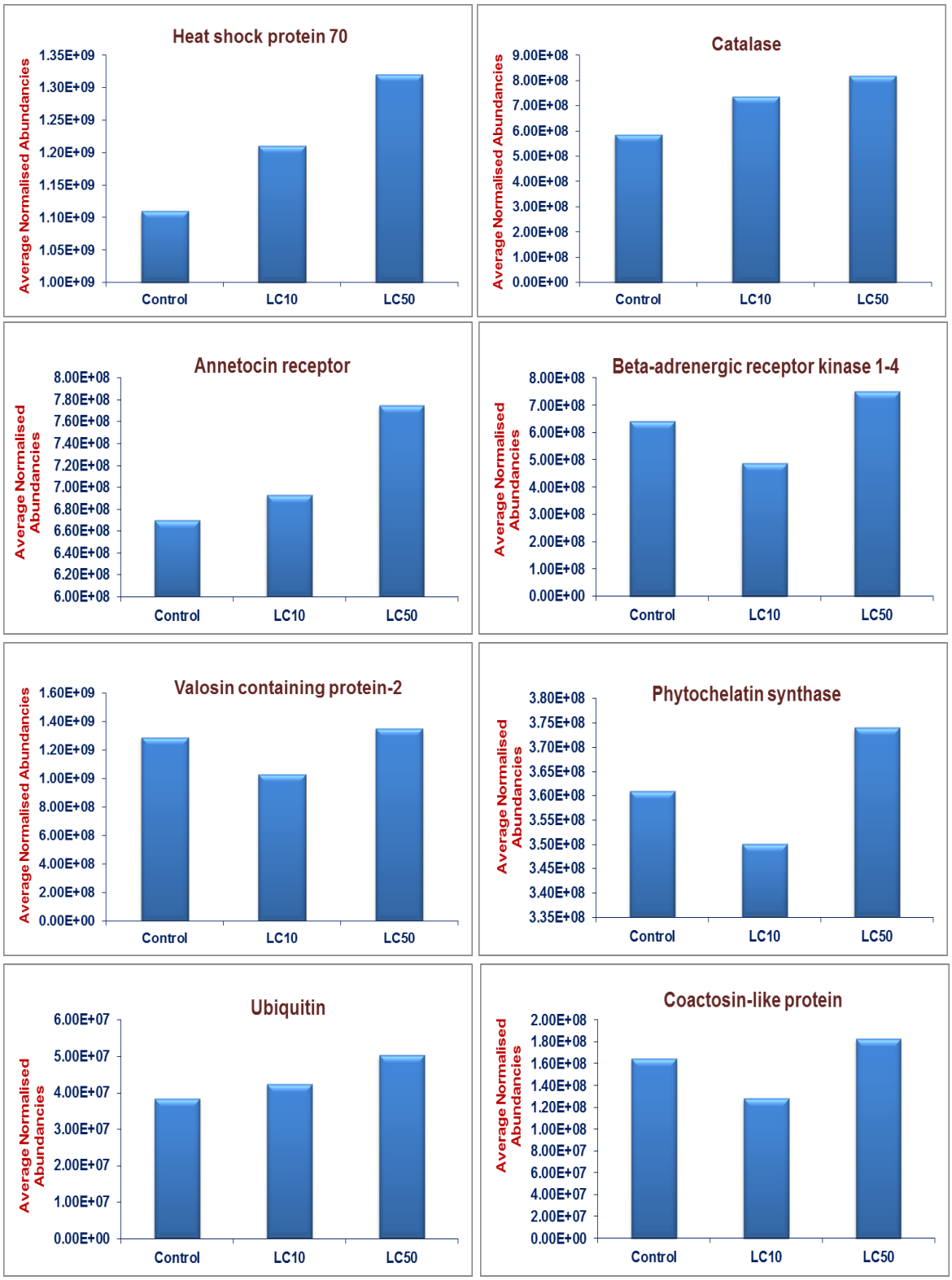

Figure 5.20 Upregulated proteins in *Eisenia fetida* exposed to r-TiO_2-ENP at upper limit of LC_{50} (0.15 mg/cm^2) for 48 h by direct contact method. The final data represents mean ± S.D (n=3, p<0.05).

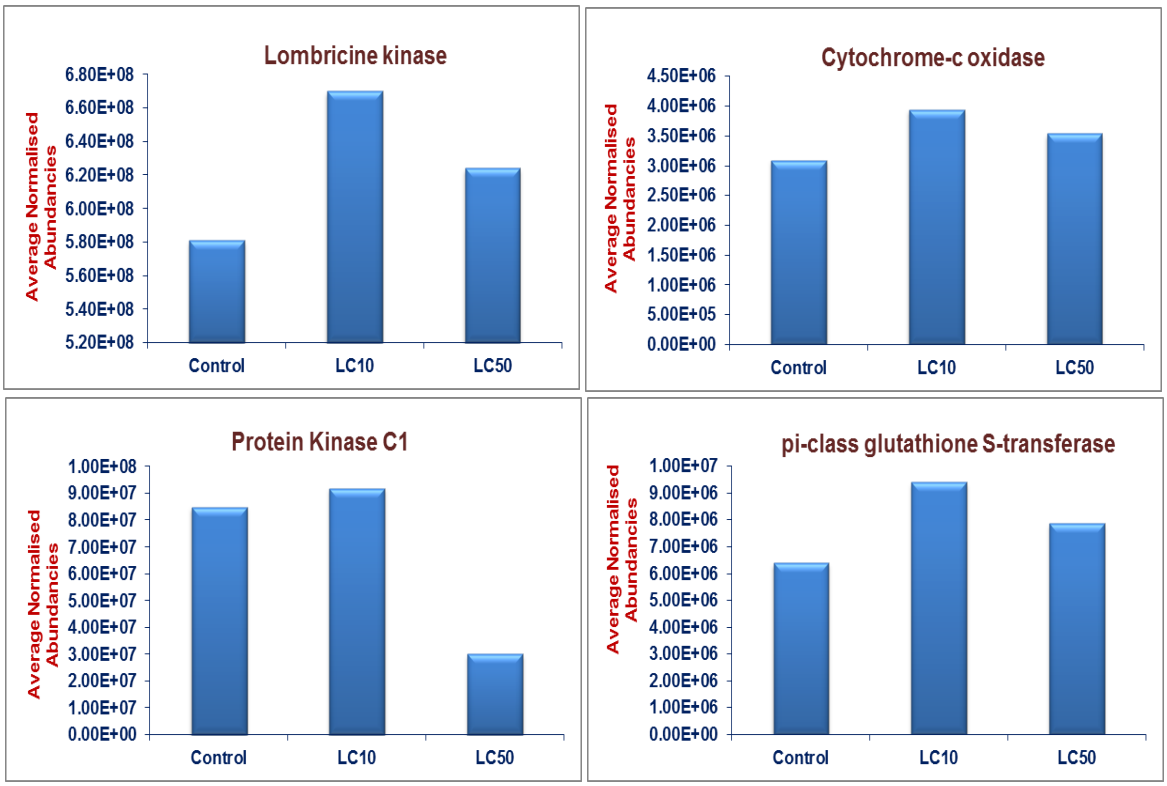

Figure 5.21 Upregulated proteins in *Eisenia fetida* exposed to r-TiO_2-ENP at LC_{10} (0.05 mg/cm^2) for 48 h by direct contact method. The final data represents mean ± S.D (n=3, p<0.05)

Upregulation of pi-class glutathione s-transferase (pi-GST) indicate the role of GSH in in the formation of protein and lipid adducts formed during r-TiO_2-ENP exposure. However, role of pi-GST is well established with regard to their ability to participate in conjugation during phase-II metabolism of detoxification of toxicants. It has potential to protect the cells from the oxidative damage caused by peroxides (LaCourse et al., 2009). Thus the study affirms the decrease in glutathione reductase activity observed during dermal route and soil exposure for 48 h. Induction of cytochrome-c-oxidase

and protein kinase c1 also contribute to the mitigation of toxic insult caused by r-TiO_2-ENP.

Table 5.4 Analysis of downregulated proteins in *E. fetida* exposed to r-TiO_2-ENP

Accession	Peptides (Quantitation)	Score	ANOVA (p)*	Fold	Description	Function
60735077	31 (24)	143.36	0.92	1.06	valosin containing protein-1	**ATP binding and Hydrolase activity**
1339877	20 (17)	119.15	0.94	1.12	precursor protein of EEP-2	**Augment Contraction of the gut**
60735081	28 (24)	76.63	0.74	1.13	cell division control protein 6	**Cell division and DNA replication initiation**
83944644	11 (2)	71.75	0.36	1.46	catalase	**Protects from toxicity of H_2O_2**
6647579	23 (21)	70.4	0.61	1.66	Cadmium-metallothionein	**Metal ion binding**
269930118	5 (4)	24.11	0.71	1.35	cyclophilin A	**Protein folding**

Metallothionein (MT) plays an important role in protection against the damages due to metabolic regulation and oxidants (Coyle et al., 2002). The nano-TiO_2 has the potential to compete with other metal ions including copper with sulfhydryl group thereby inhibits the detoxification by metallothioneins (Fan et al., 2011). Therefore, it is postulated that Ti from nanoparticles can exhibit the competitive inhibition with other metal ions in the milieu of physiology.

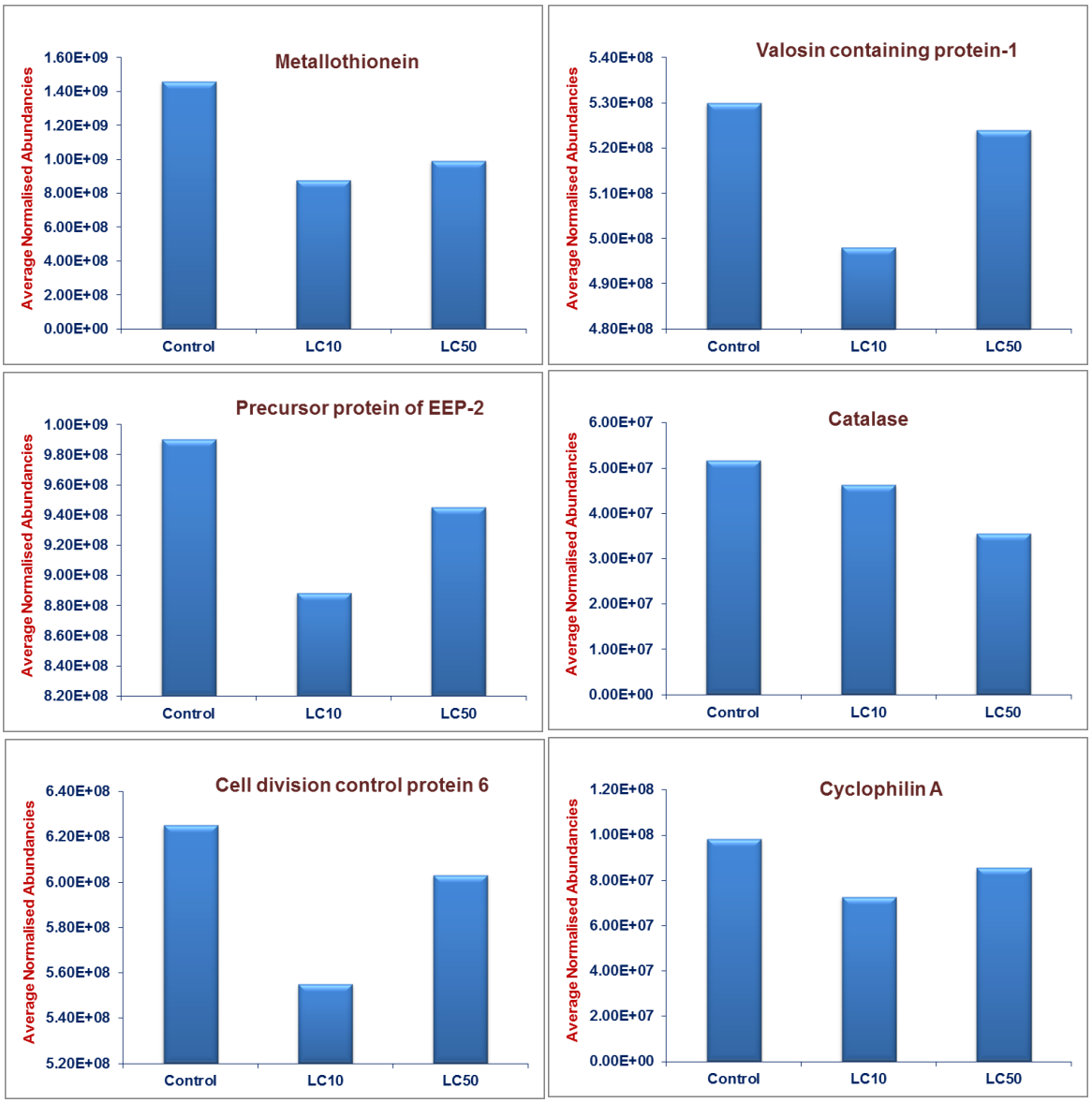

Figure 5.22 Downregulated proteins in *Eisenia fetida* exposed to r-TiO_2-ENP at LC_{10} (0.05 mg/cm²) and upper limit of LC_{50} (0.15 mg/cm²) when compared to control for 48 h by direct contact method. The final data represents mean ± S.D (n=3, p<0.05).

Inhibition of ubiquitously expressed protein such as valosin-containing protein (VCP) belonging to the ATPases protein family (Ju et al., 2009) indicates the possible effect of r-TiO_2-ENP on these process. From these observations, it is also evident that r-TiO_2-ENP have the potential to inhibit

the process of autophagy. Precursor protein EEP-2 is involved in the repetitive contraction of the gut in *E. fetida.* Coordinated and combined effect of VCP-1 and ATP-ases protein family (Ju et al., 2009) indicates the possible effect of r-TiO_2-ENP various transporters systems in the organism. Cyclophilin-A inhibition indicates the isomerization of proteins thereby folding. Cell division control protein inhibition also indicates the inhibition of DNA replication during r-TiO_2-ENP exposure.

Proteomic approach using label free quantitation revealed that 22 proteins are significantly expressed *($p<0.05$)* in the earthworm during acute toxicity of r-TiO_2-ENP. It was observed that 16 proteins were upregulated whereas 6 proteins were down regulated when compared to the respective control. Study underlines the role of various proteins that affect stress-responsive system and behavior upon exposure to r-TiO_2-ENP in earthworms. This is especially true with regard to the upregulation of (i) heat shock protein 70 (ii) beta-adrenergic receptor kinase 1-4 (iii) Valosin containing protein-2 (iv) pyruvate carboxylase (v) HSP60 (vi) lombricine kinase (vii) cytochrome c oxidase subunit II and (viii) protein kinase C1 which are indicate the significance of mitochondrial structures during metabolism obstruction. Besides these proteins, study also identified other proteins to paraphrase new biomarkers to assess toxicity of r-TiO_2-ENP in future research. Moreover, to the best of our knowledge, this study is the first of its kind in highlighting the analysis on proteomic profiles of the earthworm during r-TiO_2-ENP exposure. The study will provide more insights for developing earthworms as early warning indicators or sentinels for assessing environmental and health hazards of nanoparticles such as r-TiO_2-ENP.

Findings in the present study also assist in better understanding of the mechanism of r-TiO_2-ENP toxicity and survival mechanisms employed by earthworms.

Chapter 6

Conclusions

6. Conclusions

Nanotoxicology/ nanoecotoxicology is emerging as a domain of toxicological sciences. Though there is a significant rise in the number of publications with regard to nanotoxicology, still there is a lack of information pertaining to specific implications of nanoparticles due to their engineered properties including their size, charge and agglomeration. Despite good progress, several challenges including the dosimetry, the validation of *in vitro* studies for toxicity testing are imperative in this field. But nanotoxicologists' have already understood the fact that "not all nanomaterials are created equal". Hence a small difference in nanoparticle properties can elicit a different biological response. Therefore, there is a need for a systematic characterization of nanoparticles along with standardized and validated procedures for evaluating their toxicity. Hence careful analysis of ENPs that are having a diverse chemical composition, crystal structure and primary size is carried out in the present study with regard to rutile titanium dioxide engineered nanoparticles (r-TiO_2-ENP).

Till date, relevant risk assessment and safety guidelines for Titanium dioxide nanoparticles (TNP) are not well documented. In this context, appropriate analytical methods for detection and characterization of TNP under realistic environmental concentrations are needed. Present research is the first of its kind conducted on municipal sewage sludge to report the concentrations of Ti using ICP-MS and Titaniumdioxide nanoparticles (TNP) using SP-ICP-MS. Occurrence of TNP in environmental samples could serve as a tracer material for detection and characterization of other nanomaterials with

similar size and aggregation properties, thereby assisting in their risk assessment. The study also contributes towards finding the ratio of stable isotopes of ^{47}Ti in sewage, and their potential to accumulate in the soil environment. Results of the study will contribute primarily to quantifying the concentration of TNP using SP-ICP-MS and ultimately for assessing the environmental risk posed by them. Thus, present study contributes to the advancement of analytical methods such as SP-ICP-MS to corroborate the occurrence of titanium nanoparticles in environmental matrices including wastewater and sludge samples.

Interdependence of nanoparticle properties and mass concentration is one of the limitations to adjudicate the appropriateness of current guidelines in toxicity testing. Hence physicochemical characterisation carried out in the study revealed that r-TiO_2-ENP undergoes agglomeration in an ionic free environments such as ultrapure water and milli-Q-water. r-TiO_2-ENP forms agglomerates of primary particles (100 nm) is established with the help the particle size distribution with an average of 255 nm of TiO_2-ENP as indicated by electron micrographs Hence z-average of size (341 to 480 nm) and charge/zeta potential (-6 to -23 mV) of r-TiO_2-ENP at different concentrations *viz.* 0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2 (equivalent to 0.05, 0.1, 0.15, 0.2 and 0.25 mg/ml) reported in the present study will assist in designing of further experiments in ecotoxicology. Till date, in nanotoxicology studies, selection and standardization of metrics for nanoparticle/ nanomaterial dose has been considered as a matter of further research. However, there is no consensus on standard metrics agreed upon for toxicological evaluation of nanoparticles. Therefore, dosimetry of r-TiO2-ENP employed in present study (Dermal exposure: 0.05, 0.1, 0.15, 0.2 and

0.25 mg/cm^2) can serve as an initial point to arrive at the metrics of TiO_2-ENP toxicity in future.

Presence of electrolyte such as Ca $(NO_3)_2$ at a concentration >10 ppm in aqueous suspensions cause significant agglomeration of r-TiO_2-ENP in water. Simultaneous occurrence of electrolytes (1 ppm) and natural organic matter/ dissolved organic matter (DOM) (10 ppm) in water cause the agglomeration of r-TiO_2-ENP. However, the presence of DOM alone in ultrapure water also leads to the agglomeration of r-TiO_2-ENP. At pH-8.5, an unbuffered system with 5mM $NaHCO_3$ (to buffer the experimental conditions) could cause agglomeration of r-TiO_2-ENP. In conclusion, the differential behavior of r-TiO_2-ENP documented in the present study will help in comprehending the behavior of these nanoparticles in toxicity testing as well as in environmental media.

Though several investigations reported TiO_2 nanoparticle toxicity (*in vivo* and *in vitro*), there is a lack of information on r-TiO_2-ENP properties in divulging their toxicity mechanisms. Moreover, adjudging the suitability of current conventional approaches is one of the major challenges for evaluating toxicity of these nanoparticles on environment due to the lack of information in this regard. In this context, the current work is perhaps the first systematic study to unveil the toxicity of r-TiO_2-ENP nanoparticles by OECD-207 guidelines. Median lethal concentration (LC_{50}) of r-TiO_2-ENP in earthworm, *Eisenia fetida* was found to be 0.13 ± 0.09 mg/cm^2 through skin / dermal contact. However, exposure of r-TiO_2-ENP via soil exposure did not show mortality of earthworm for 48 h, 7 days and 14 days.

Moreover, route of administration and dose dependent bioconcentration of r-TiO_2-ENP reported in the present study aid in understanding depuration studies in upcoming research with regard to bioaccumulation of nanoparticles in the earthworm, *Eisenia fetida*. Furthermore, study also provide cues for assessing the effect of r-TiO_2-ENP on the homeostasis of electrolytes (Na, Mg, Ca and K) and trace elements (Mn, Cu and Fe) even at above (0.2 and 0.25 mg/cm^2) and below (0.05, 0.1 mg/cm^2) lethal concentrations (0.13mg/cm^2) through dermal exposure. The study contributes crucial information with regard to the development of microcosm studies for evaluating the toxicity of nanoparticles.

Toxicity of nanomaterials at lower doses may not always result in the mortality. Hence mortality may not be the right indicator to adjudge toxic effect of nanoparticles which are considered as acutely not toxic. Therefore, assessing the highly temporal oxidative stress responses such as antioxidant enzymes can serve as a sensitive method in this regard. Surprisingly, there exist is a significant knowledge gap in this pursuit with regard to r-TiO_2-ENP. Hence present study provides the role of antioxidant enzymes viz. Superoxide dismutase (SOD), Catalase (CAT) and Glutathione reductase (GR) and lipid peroxidation (LPO).

- **Dermal exposure** of earthworm to r-TiO_2-ENP for 48 (at concentrations-0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2) causes an **increase** in specific activity of **SOD** and **decrease** in the specific activities of **GR and CAT**.
- **Soil exposure** of earthworm to r-TiO_2-ENP for 48 (at concentrations-0.1, 0.15, 0.2 and 0.25 mg/kg) causes **increase** in specific activities

of **SOD**, **GR and CAT**. The specific activity of **acetylcholinesterase (AChE)** also **increasead** at these concentrations.

- **Soil exposure** of earthworm to r-TiO_2-ENP for 7 days (at concentrations- 0.05, 0.1, 0.2 and 0.4 mg/kg) causes an **increase** in specific activities of **CAT**, and decrease in the activities of **SOD and GR**. Specific activity of **acetylcholinesterase (AChE)** also **increasead** at these concentrations.
- **Soil exposure** of earthworm to r-TiO_2-ENP for 14 days (at concentrations- 0.05, 0.1, 0.2 and 0.4 mg/kg) causes an **increase** in specific activities of **SOD** and **CAT**, and decrease in the activities of **GR**. The specific activity of **acetylcholinesterase (AChE)** also **increasead** at these concentrations.
- **Lipid peroxidation increased all the experiments indicate the prevalence of oxidative stress.**

Thus r-TiO_2-ENP could act as strong pro-oxidants to alter the activities of antioxidant enzymes and lipid peroxidation. The study concludes that increase activity of SOD and decrease in CAT and GR activity can serve as sensitive markers of oxidative stress caused by lethal concentrations of r-TiO_2-ENP for 48h through dermal exposure in earthworm, *Eisenia fetida.* Whereas an increase in the activity of SOD and CAT, decrease in GR activity in earthworm, *Eisenia fetida* can serve as sensitive markers of oxidative stress caused by non-lethal concentrations of r-TiO_2-ENP for 48h, 7 days and 14 days of soil exposure in earthworm, *Eisenia fetida.* r-TiO_2-ENP caused lipid peroxidation in earthworm Therefore, it is surmised that elevated levels of antioxidant enzyme is observed to combat the effect ROS damage. Thus, oxidative stress responses caused due to r-TiO_2-ENP via soil

and dermal exposures in earthworm, *Eisenia fetida* help in predicting the role of these markers in evaluating toxicity of nanoparticles. Moreover, comprehending the altered homeostasis of trace elements and electrolytes along with cascading response of SOD, CAT and GR can serve as an important tool to understand the basis of oxidative stress induced by low doses of r-TiO_2-ENP at short term exposure in earthworm.

Studies documenting the effect of r-TiO2-ENP effect on AChE in soil sentinel organisms including earthworm *Eisenia fetida* are scarce. In this pursuit, activation of AChE during r-TiO2-ENP exposure in *Eisenia fetida* contributes to the understanding the role of AChE during acute toxicity of nanoparticles. Moreover, linking altered homeostasis of trace elements and electrolytes with the increased activity of AChE assist in divulging the effect of r-TiO2-ENP in neurotoxicity.

The study underlines the role of various proteins that affect stress-responsive system and behavior upon exposure to r-TiO_2-ENP in earthworms. This is especially true with regard to the upregulation of (i) heat shock protein 70 (ii) beta-adrenergic receptor kinase 1-4 (iii) Valosin containing protein-2 (iv) pyruvate carboxylase (v) HSp60 (vi) lombricine kinase (vii) cytochrome c oxidase subunit II and (viii) protein kinase C1 which are indicate the significance of mitochondrial structures during metabolism obstruction. Besides these proteins, study also identified other proteins to paraphrase new biomarkers to assess toxicity of r-TiO_2-ENP in future research. Moreover, to the best of our knowledge, this study is the first of its kind in highlighting the analysis of proteomic profiles of the

earthworm during r-TiO2-ENP exposure. The study will provide more insights for developing earthworms as early warning indicators or sentinels for assessing environmental and health hazards of nanoparticles such as r-TiO2-ENP. Findings in the present study also assist in better understanding of the mechanism of r-TiO2-ENP toxicity and survival mechanisms employed by earthworms.

Present study aids in the advancement of analytical methods such as SP-ICP-MS to detect and characterize nanoparticles in environmental compartments including wastewater and sludge samples. Interpretations of the study can serve as cues to design a comprehensive approach in evaluating toxicity of nanoparticles using earthworm sentinel, *Eisenia fetida*. In order to elucidate the toxicity of metal oxides which are considered as acutely non-toxic such as Titanium dioxide, there is a need for paraphrasing sub-lethal responses to understand their long term effects. Therefore, present recommends that besides assessing mortality, fecundity/ reproductive behavior as endpoints of toxicity, there is need for considering (i) physicochemical characterization (ii) bioconcentration and (iii) sub-lethal sensitive biomarkers including antioxidant responses and proteomic responses, is important in comprehending the toxicity of nanoparticles at non-lethal/ lower exposure doses. Thus outcomes of the study will be useful in advancing the science ecotoxicology thereby hazards for developing a safety assessment paradigm in the era of nanotechnology.

In summary, the advent of nanotechnology paved marvelous opportunities in different fields including environmental cleanup, medicines, foods, electronics, and personal care products. Till date, effects of nanoparticles on soil sentinels are meagre. However, efforts to address the same are being made, including the present thesis. Though the present thesis has shed some light on environmental monitoring of Ti using SP-ICPMS, possible toxicological effects of TiO_2-ENP (rutile crystal phase) in earthworms, it warrants the future research on the following aspects:

a. Analytics that can distinguish sources (anthropogenic or natural) of TiO_2-ENP in the soil environment and their realistic environmental concentrations.
b. Design and validation of test methods to prepare stable suspensions of TiO_2-ENP.
c. Bioavailability of TiO_2-ENP and proportions of various crystal phases viz. brooklite, anatase and rutile of the same in the soil environment
d. Biotransformation and hence the interaction of TiO_2-ENP in various environmental compartments.

It is believed that the outcomes of the current study will pave the way to assess the hazards due to the release of TiO_2-ENPs into the environment which will help for the establishment and the implementation of effective, protective regulatory policies. It also will provide the data pertaining to biomarkers in earthworm during the acute toxicity of engineered nanoparticles.

Chapter 7

Specific Contributions

7. Specific Contributions

Steady growth of nanotechnology and extensive use of engineered nanoparticles (ENP) in personal care products, pharmaceutics, and biomedical applications has raised concerns about their implications on environment human health. However, recent toxicological studies in this regard raised more questions than answers. There exists paucity of data in regard to toxicity evaluation of metal oxide nanoparticles including rutile crystal phase of Titanium dioxide engineered nanoparticles (r-TiO2-ENP) in invertebrates. Hence, in the light of development of an invertebrate based toxicity assessment and biomarkers, earthworm, *Eisenia fetida* is chosen in the present study to evaluate toxicity of r-TiO2-ENP. Besides toxicity evaluation, study sheds light on advancing the detection and characterization of Ti and TiO2 nanoparticles in environmental matrices using single-particle ICP-MS.

Present study contributes to the

(i) Advancement of analytical methods such as SP-ICP-MS to detect and characterize nanoparticles in environmental compartments.

(ii) Interpretations of the study can serve as cues to design a comprehensive approach for developing earthworms as early warning indicators or sentinels for assessing hazards and toxicity of engineered nanoparticles such as TiO_2-ENP.

(iii) Besides antioxidant enzyme, acetylcholinesterase responses and proteomics to r- TiO_2-ENP help in the transition of conventional

biomarkers such as mortality into a mechanism-based risk assessment and hazard ranking of nanoparticles.

(iv) Results of the study will be useful in documenting ontology of r-TiO_2-ENP in nanoparticle databases that facilitate computational modeling and development of predictive nanotoxicolgy. As a consequence, the study will be useful in advancing the science of nanoecotoxicology for developing a safety assessment paradigm in the era of nanotechnology.

It is believed that the outcomes of the current study will pave way to assess the hazards of the release of TiO_2-ENPs into the environment which will help for the establishment and the implementation of effective, protective regulatory policies. It also will provide the data pertaining to biomarkers in earthworm during the acute toxicity of engineered nanoparticles.

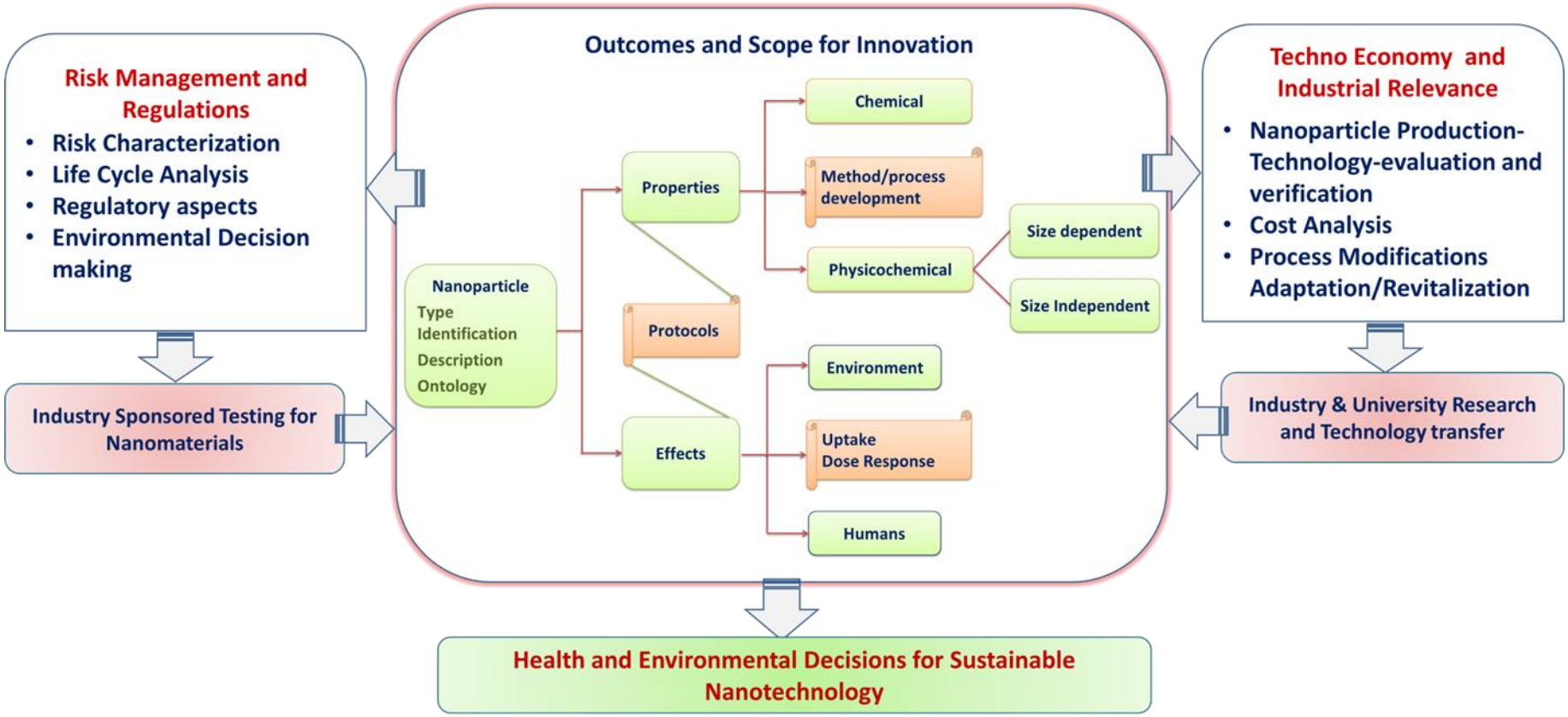

Figure 7.1 Contributions of the current work to new paradigms in toxicological assessment of TiO_2 nanoparticles

Chapter 8

Future Scope of the Work

8. Future Scope of the work

Innovations with the advent of nanotechnology paved marvelous opportunities in different fields including environmental cleanup, medicines, foods, electronics, and personal care products. Till date, effects of nanoparticles on soil sentinels are meagre. However, efforts to address the same are being made, including the present thesis. Though the present thesis has shed some light on (i) advancing the science analytics of ICPMS for environmental monitoring of Ti (ii) Physicochemical characterization of r-TiO_2-ENP in terms of size, charge and agglomeration (iii) Toxicological evaluation of r-TiO_2-ENP in earthworm, *Eisenia fetida*, way forward in this regard is depicted below:

a. Parameters that are employed in SP-ICP-MS for detection and characterization enable the design and optimization of sample preparation, identification and characterization of reference materials and corresponding isotopes in analytical chemistry. However, such methods will pave a way to develop analytical methods which enable the differentiation of anthropogenic or natural sources of metal oxide/ metal based nanoparticles in various compartments of environment including the soil. Thus it will facilitate determining the realistic environmental concentrations of nanoparticles.

b. Approach/ methods adapted for physicochemical characterization of r-TiO_2-ENP using dynamic light scattering and ICP-OES methods can be used as starting point for validating non-invasive methods based on the principles of X-ray-fluorescence and Flow-cytometer.

c. There is a much need to develop simple and effective tools that will allow analysis of agglomeration behavior of engineered nanoparticles in various exposure media that are employed in toxicity testing.

d. Till date, in nanotoxicology studies, selection and standardization of metrics for nanoparticle/ nanomaterial have been considered as a matter of further research. Conventional dosing approaches include particles number, mass and surface area of nanoparticles. However, most of the studies employ dosimetry as the mass of nanoparticles per volume concentration such as mg/ml. Though most of the nanotoxicity studies emphasize that equivalent, exposed surface area is the most appropriate metric for comparing biological effects of nanoparticles. However, there is no consensus on standard metric agreed upon in toxicological evaluation of nanoparticles. Therefore, dosimetry of r-TiO_2-ENP employed in present study can serve as an initial point to conclude the metrics of nanoparticle toxicity in future screening studies. This information is available as the nanotoxicology field continues to develop.

e. The saga of decade research in nanoecotoxicology still needs copious amount information to comprehend the mechanism of nanoparticle toxicity. Bioconcentration studies conducted on r-TiO_2-ENP using ICP-MS helps in designing experiments to divulge (i) bioaccumulation thereby interactions of engineered nanoparticles with cells or cellular components and (ii) to measure ions of metal/metal oxide engineered nanoparticles.

f. Measuring reactive oxygen species during r-TiO_2-ENP toxicity will help in understanding the oxidative stress in more comprehensive manner.

g. In toxicology, the significance of biomarkers is well established in depicting the interaction of a toxicant and biological system. In order to predict the possible toxic effects of emerging nanoparticles, there is a need for systems toxicology which enables the integration of biomarker responses to understand the biological pathways perturbed by engineered nanoparticles. Hence data generated as part of proteomics in this study has much potential to contribute develop a mechanism-based risk assessment approaches for nanomaterials. Furthermore, mechanism based high-throughput screening methods are to be developed for hazard ranking of engineered nanoparticles.

h. Physicochemical properties (size, charge and agglomeration) of r-TiO_2-ENP, a method of toxicity testing (Dermal or soil exposure), dosimetry used and lethal and sub lethal concentrations, biochemical responses including antioxidant enzymes, lipid peroxidation and proteomic profiles will be useful in developing an ontology of r-TiO_2-ENP in future nanoparticle databases. It underlines future scope of the present study for developing databases for engineered nanoparticles which can assist computational modeling studies in predictive nanotoxicolgy.

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Appendices

APPENDIX-1

In this study, size and charge of TiO_2, Au, Polystyrene and Polyamidoamine (PAMAM) dendrimers' suspension in Milli-Q-water were determined using dynamic light scattering technique and the results are presented below:

Table A1 Size and charge of nanoparticles

Nanoparticle	Size (d.nm) ± S.D	Charge (mV) ± S.D
Titanium dioxide (rutile) (r-TiO_2)	341± 17	-18 ± 3.2
Gold (Au)	71± 9.5	-45 ± 8.2
Polystyrene (PS)	111± 11.5	386 ± 15
PAMAM dendrimers (Generation 0)	-64.9 ± 5.8	-27.5 ± 3.2

Table A2 Mortality assessment of nanoparticles using OECD-207 paper contact method

Out of the four nanoparticles viz. Titanium dioxide, gold, polystyrene and PAMAM dendrimers, titanium dioxide nanoparticles showed toxicity in earthworm by OECD-207 paper contact method.

Nanoparticle	Concentrations screened for toxicity assessment	Mortality after 48 h
Titanium dioxide (rutile) (r-TiO_2)	0.05, 0.1, 0.15, 0.2, 0.25 mg/cm^2	**Observed**
Gold (Au)	2.5, 5, 10, 20, 40 µg/cm^2	**Not observed**
Polystyrene*	100, 200, 400, 800 and 1000 µg/cm^2	**Not observed**
PAMAM dendrimers* (Generation 0)	125, 250, 500, 1000, 2000 µg/cm^2	**Not observed**

**Results are published in Journal of Pollution Research (attached in list of reprints)*

APPENDIX-2

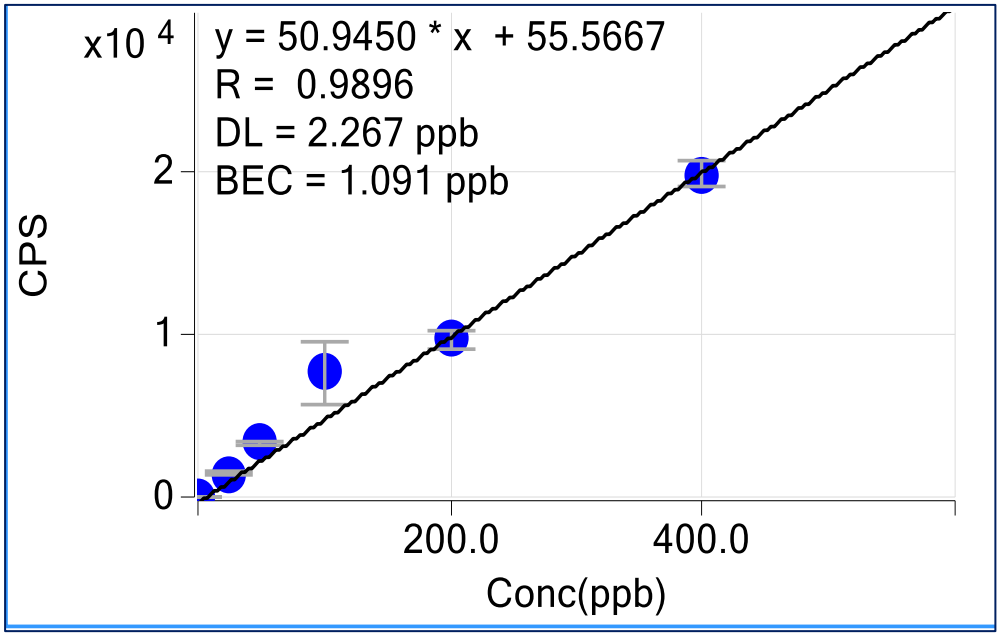

Figure-A1 Standard graph for Ti in ICPMS analysis 100 nm (rutile) nanoparticles of size <100 nm

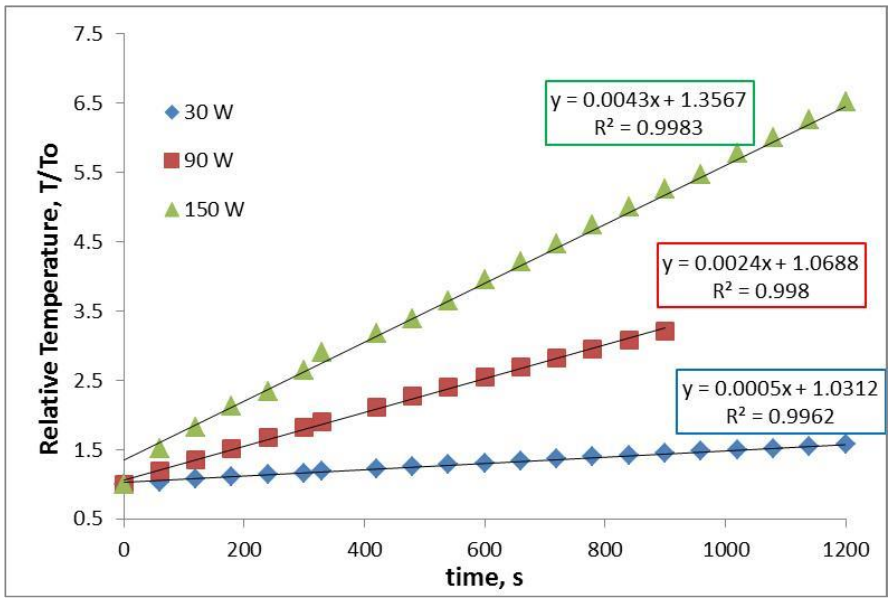

Figure-A2 Standard Graph for optimizing sonication time and watts as per test guidelines of OECD to understand agglomeration behavior of r-TiO_2-ENP particles

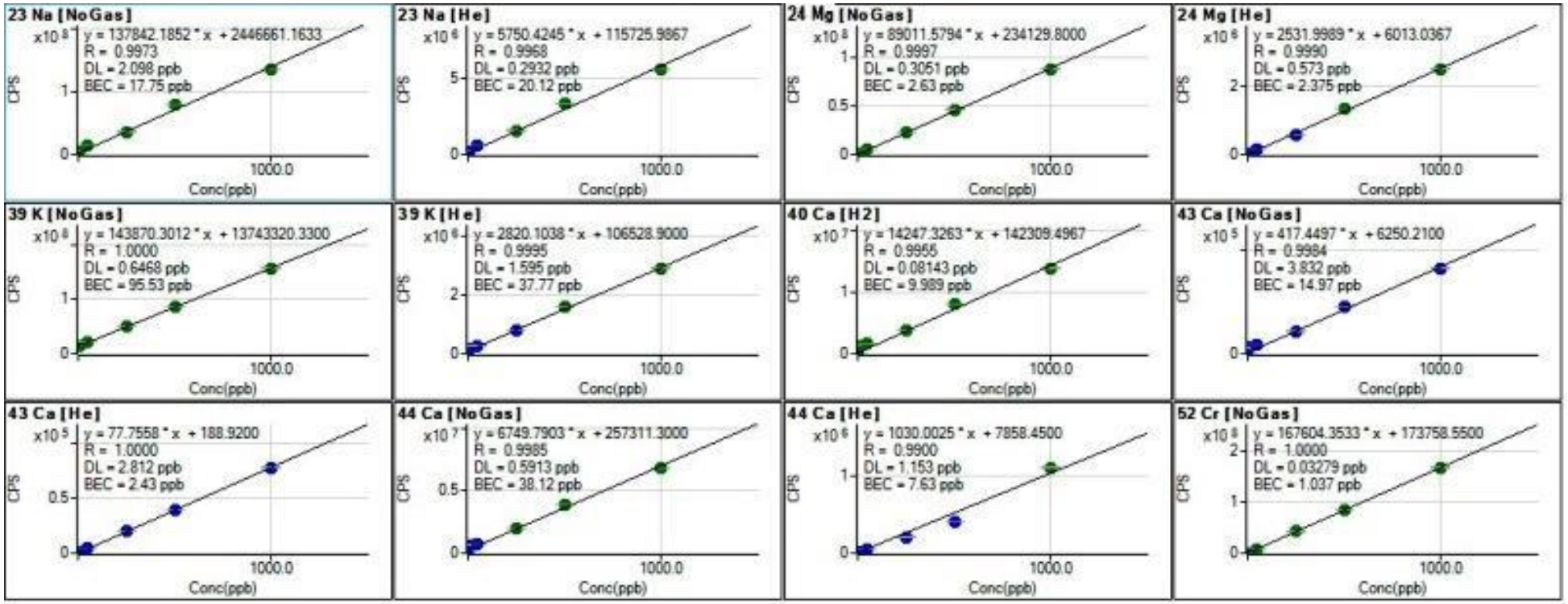

Figure-A3 Standard Graphs of analysis of trace elements obtained during ICPMS

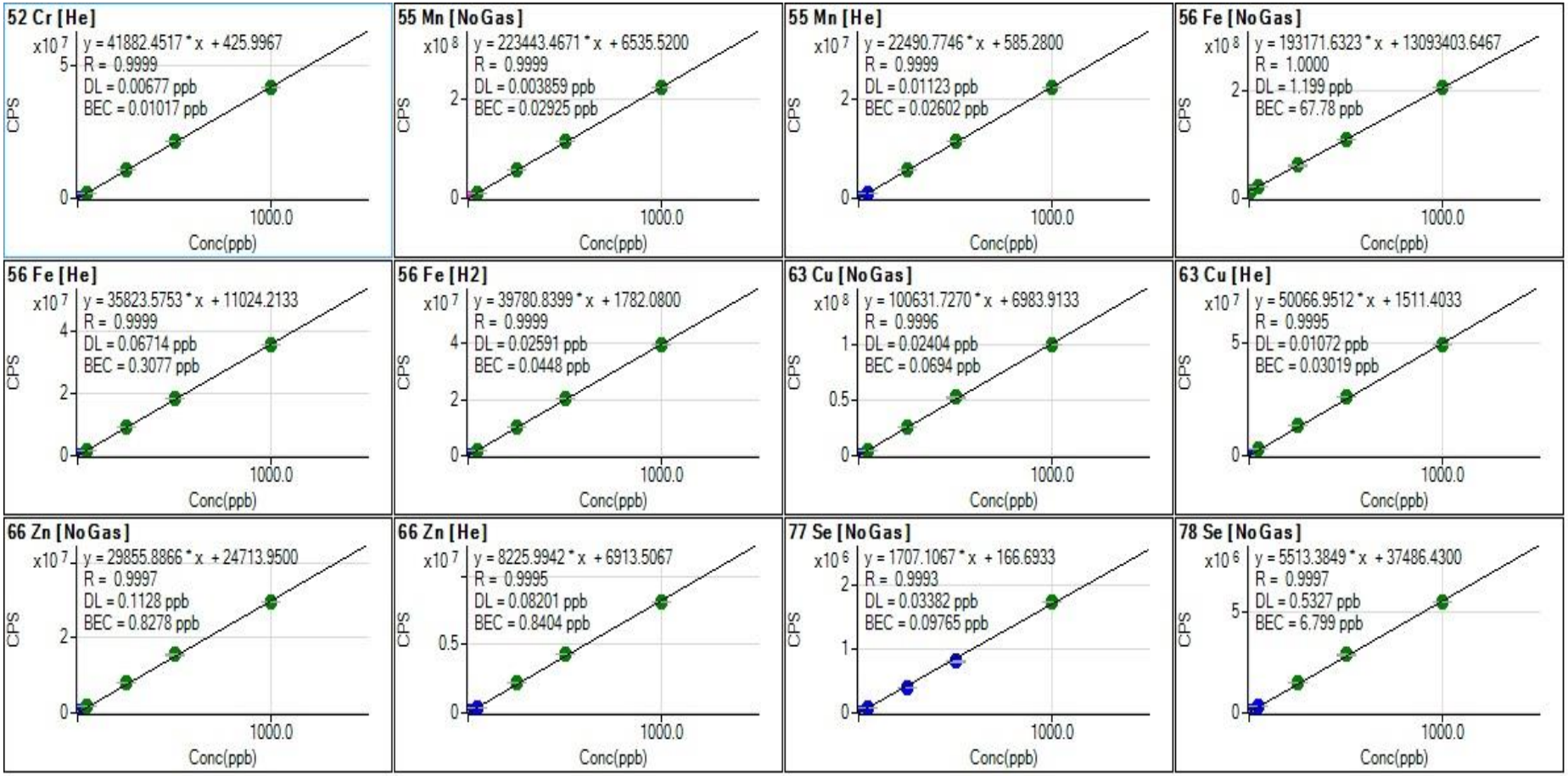

Figure-A4 Standard Graphs of analysis of trace elements obtained during ICPMS

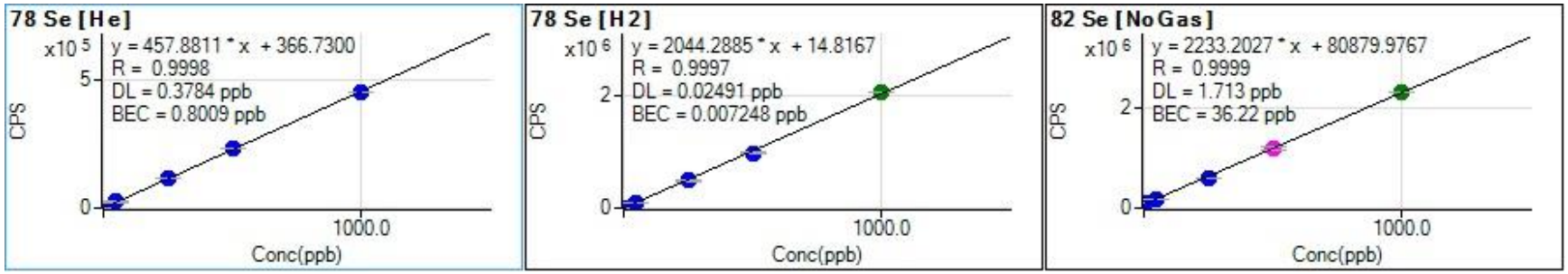

Figure-A5 Standard Graphs of analysis of trace elements obtained during ICPMS

List of Publications

List of Publications

I. Publications in Peer-Reviewed Journals

1) **B. Siva Prasad**, P. Sankar Ganesh, K. Lagan, T. Vishaka, S. S. Vutukuru and S. K. Sarkar. Evaluation of Antioxidant Enzyme Responses in Earthworms Exposed to Polystyrene Nanoparticles and PAMAM Dendrimers, Pollution Research, 2017, 36 (2), 313-319.

2) **Siva Prasad Bitragunta**, Sankar Ganesh Palani, Anil Gopala, Santosh Kumar Sarkar, Venu Gopal Reddy Kandukuri, Detection of TiO_2 nanoparticles in municipal sewage treatment plant and their characterization using single particle ICPMS, 2017, Bulletin of Environmental Contamination and Toxicology, 2017, 98(5), 595-600.

3) **Siva Prasad Bitragunta**, Sankar Ganesh Palani, Nanowaste and environment: Current understanding and way forward. Society of Material Chemistry Bulletin, 2016, 7(1), 15-20.

4) Ch. Surekha , NRR Neelapu, **B. Siva Prasad**, P. Sankar Ganesh, Induction of defense enzymes and phenolic content by Trichoderma viride in Vigna mungo infested with infested with Fusarium oxysporum and Alternaria alternata, International Journal of Agricultural Science and Research, 2014, 4 (4), 31-40.

5) Ch. Surekha , NRR Neelapu, G. Kamala, **B. Siva Prasad**, P. Sankar Ganesh , Efficacy of Trichoderma viride to induce disease resistance and antioxidant responses in legume Vigna mungo infested by Fusarium oxysporum and Alternaria alternata, International Journal of Agricultural Science and Research, 2013, 3 (2), 285-294.

6) **Siva Prasad Bitragunta**, Ashwini Sri Hari, Sankar Ganesh Palani and Santosh Kumar Sarkar. Comprehensive toxicological evaluation of rutile TiO2 engineered nanoparticles in earthworm: Physicochemical characterization, LC50 determination, bioaccumulation assessment and oxidative stress measurement, Environmental Toxicology and Chemistry (Under Revision).

II. Inter Lab Validation of Test Guidelines of OECD

Siva Prasad B and Sankar Ganesh P. Report-Round Robin Test for Evaluating Test Guidelines (TG) on Agglomeration Behaviour of TiO_2 (NM105) Nanomaterial in Different Aquatic Media. OECD-2015.

III. Book

Assessment of Titanium dioxide nanoparticle toxicity in earthworms, Ashwini Sri Hari, **Siva Prasad Bitragunta**, Sankar Ganesh Palani. ISBN: 978-3-659-55283-0, Lambert Academic Publishing, 2014.

IV. Book Chapter

Bitragunta S.P., Menon S.A., Ganesh P.S. (2018) Recent Advances in Toxicology of Gold Nanoparticles. In: Hussain C. (eds) Handbook of Environmental Materials Management. Springer, Cham.

B. Siva Prasad, Ashwini Sri Hari, and P. Sankar Ganesh. Should we say NO to NaNO? Preliminary study to corroborate occurrence of nanoparticles in treated wastewater samples. ISBN: 81-90293-11-6, Trans-Knowledge Book Company, 2015.

V. Abstracts presented in international conferences abroad

a. **B. Siva Prasad**, A. Menon, S.K. Sarkar, P. Sankar Ganesh. Assessing Sub-lethal Effects of Gold Nanoparticles on Oxidative Stress and Neurotoxicity in Earthworm, *Eisenia fetida*, Society of Environmental Toxicology and Chemistry Asia/Pacific 2016 Conference, National University of Singapore, September 2016.

b. **Siva Prasad Bitragunta** and Sankar Ganesh Palani. Integrated 'Omics' of Coelomic Fluid: An Attempt to Understand Phenomics of TiO_2 Nanoparticle Toxicity, The Toxicologist, 150 (1), PS-1501, 117, 2016.

c. **Siva Prasad Bitragunta**, Santosh Kumar Sarkar and Sankar Ganesh Palani, Silver nanoparticle toxicity in a marine gastropod *Telescopium telescopium,* a potential biomonitor of tropical intertidal mangrove sediments" was published in the proceedings of SETAC North America 36th Annual Meeting-2015 at Salt Lake City, Utah

d. **Siva Prasad Bitragunta** and Sankar Ganesh Palani, 2015. Effect of TiO_2 nanoparticles on trace element homeostasis: An inquiry into metallomics in earthworm, *Eisenia foetida*. Proceedings of 4th Young Environmental Scientists Meeting-2015, Petnica Science Centre, Belgrade, Serbia, Europe.

e. **Siva Prasad Bitragunta** and Sankar Ganesh Palani, 2014. Confounding factors and future challenges to delineate ecotoxicity of TiO_2 nanoparticles. 9th SETAC Asia/Pacific Conference, 14-17 September-2014, Adelaide, Australia.

f. B. Siva Prasad, S. Ashwini, P. Sankar Ganesh. Physicochemical Characterization and Ecotoxicological Evaluation of TiO_2 nanoparticles in Earthworm *Eisenia foetida*. 53rd Annual meeting, Society of Toxicology, 23-27 March- 2014. Phoenix, Arizona. USA

VI. Abstracts presented in national conferences

a. **B. Siva Prasad**, R. Anvesh, Debkumar Chakraborthy, P. Sankar Ganesh. Contemporary challenges posed by emerging contaminants on bioprocessing of wastewater: Implications of engineered nanomaterials: National Seminar on Advances in Bioprocess Engineering, Hyderabad, November 2016.

b. **B. Siva Prasad**, S. Aarathi Menon, P. Sankar Ganesh. Toxicology in the era of nanotechnology, (Lead Lecture) National Conference in Research Advances in Biotechnology, October, 2016.

c. **B. Siva Prasad**, S. Aarathi Menon, P. Sankar Ganesh. Evaluating the effects of TiO2 nanoparticles on cholinergic and antioxidant defensive systems in earthworm, *Eisenia fetida*, National Conference in Research Advances in Biotechnology, October, 2016.

d. **B. Siva Prasad** and P. Sankar Ganesh, Investigating Silver Nanoparticle Toxicity in Horn Snail: A Potential Biomonitoring System of Intertidal Sediments in Sundarbans" during 35th Annual Meeting of Society of Toxicology (India) 19-21 November, 2015 at Hyderabad.

e. **B. Siva Prasad**, Asma Ahmed, P. Sankar Ganesh. 'Potential applications of enzymes derived from termite gut microflora for biochemical degradation of lignocellulosic waste and concomitant biofuel production': proceedings of Innovations in Chemical Engineering (ICE) - 2013, 15-16 November-2013. BITS Pilani, Hyderabad, India.

f. **B. Siva Prasad,** Ashwini Sri Hari, P. Sankar Ganesh, 2013, 'Development of Environmental Biomarkers to Monitor Endocrine Disrupting Chemicals in Water and Wastewater': proceedings of 2nd National Conference on Sustainable Water Resources Planning, Management and Impact of Climate Change, 5-6 April-2013. BITS Pilani, Hyderabad., India.

VII. Full length papers presented in national conferences

a. **B. Siva Prasad**, P. Sankar Ganesh. An integrated analytical approach to monitor TiO_2 nanoparticles in sewage water: An early warning for safety assessment of engineered nanomaterials in aquatic environment. Submitted to 102nd Indian Congress held in Jan-2015

b. **B. Siva Prasad**, Ashwini Sri Hari and P. SanKar Ganesh. Should we say NO to NaNO? Preliminary study to corroborate occurrence of nanoparticles in treated wastewater samples. National conference on technology, policy and community, Small Experiments in Sustainability, 14-15 March- 2014. BITS-Pilani, Hyderabad, India.

c. **B. Siva Prasad**, Mathews M. John,, M. Atul Govardhan, Kannan Ramaswamy, P. Sankar Ganesh, 2012, 'Rapid assessment of persistent organic pollutant, Sulfamethoxazole in aquatic ecosystems, using surface Plasmon resonance based biosensors': National Conference on conservation and management of wetland ecosystems, LAKE 2012, Mahatma Gandhi University,6-8 November-2012. Kottayam, India.

d. **B. Siva Prasad**, P. Sankar Ganesh, 2012, 'Flipped Teaching: Insight, Interpretation and Feasibility in the Indian Scenario': proceedings of ISTE 42nd Annual Convention, 20-22 December-2012. Hyderabad, India.

Scholastic Distinctions and Awards of the Scholar/ Candidate

2016

- **Graduate Student Travel Award**-Society of Toxicology (SOT)-USA.
- **International Travel Award**- Association of Scientists of Indian Origin (ASIO)-SOT

2015

- **Young Scientist Award** from Society of Toxicology-India.
- **Society of Environmental Toxicology and Chemistry (SETAC)-Travel grant** to attend **4th Young Environmental Scientist** (YES) at Serbia
- **Graduate Student-Representative** SETAC-Global Science Committee

2014

- **Best Abstract Award** from Association of Scientists of Indian Origin (ASIO) - Society of Toxicology, USA

Brief Biography of the Supervisor

Prof. P. Sankar Ganesh, Associate Professor of Biological Sciences, has been at Birla Institute of Technology and Science, Pilani, Hyderabad Campus, India since 2009. Dr. Ganesh completed post-doctoral training at Institut National des Sciences Appliques (INSA) de Toulouse, France. He obtained his PhD in Environmental Science and Engineering from Pondicherry (Central) University, India. He has been member of elite academic societies and associations including Society of Environmental Toxicology and Chemistry, Society of Toxicology, International Solid Waste Association and National Solid Waste Association of India. Beginning his professional career in 1998, Dr. Ganesh has little more than 20 years of academic and industry experience in the field of Environmental Science and Engineering. Dr. Ganesh has been involved in several projects both at national and international levels. He has been session chairman at various conferences. He has been the Editor-in-Chief, Journal of Biosciences and in the panel of reviewers of selected journals including Toxicology Mechanisms and Methods, Fuel and Malaysian Journal of Microbiology. He has published a book, three book chapters and over 50 articles in reputed journals and conference proceedings. Dr. Ganesh is also holding the position of Associate Dean, International Programmes & Collaboration at BITS Pilani, Hyderabad Campus.

Brief Biography of the Candidate

Mr. B. Siva Prasad, Doctoral Candidate (Environmental Nanotoxicology) in Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Hyderabad Campus, India. His research topic is nano (eco) toxicology and metabolomics to delineate environmental implications of nanomaterials. His research area integrates the principles of ecotoxicology and metabolomics to delineate implications of nanomaterials on the environment. He pursued post-graduate studies research in Environmental Biotechnology (University First Rank with Gold Medal) from Jawaharlal Nehru Technological University, Hyderabad and has more than 12 years of experience in academia and research. Till date, he published one book, a book chapter and around 19 publications in various conferences of National & International repute and peer-reviewed journals. He received Young Scientist Award-2015 from Society of Toxicology (STOX)-India. He also received Best Abstract Award-2014, International Travel Award-2016 from Association of Scientist of Indian Origin, Society of Toxicology (SOT), USA. He received several travel awards from International Society of Environmental Toxicology and chemistry (SETAC) and SOT. His other interdisciplinary research interests include nanomaterial waste management, bioindicators of climate change, in vitro toxicology, and emerging contaminants in the environment including endocrine disruptors in various environmental compartments.

Brief Biography of the Supervisor

Prof. P. Sankar Ganesh, Associate Professor of Biological Sciences, has been at Birla Institute of Technology and Science, Pilani, Hyderabad Campus, India since 2009. Dr. Ganesh completed post-doctoral training at Institut National des Sciences Appliques (INSA) de Toulouse, France. He obtained his PhD in Environmental Science and Engineering from Pondicherry (Central) University, India. He has been member of elite academic societies and associations including Society of Environmental Toxicology and Chemistry, Society of Toxicology, International Solid Waste Association and National Solid Waste Association of India. Beginning his professional career in 1998, Dr. Ganesh has little more than 20 years of academic and industry experience in the field of Environmental Science and Engineering. Dr. Ganesh has been involved in several projects both at national and international levels. He has been session chairman at various conferences. He has been the Editor-in-Chief, Journal of Biosciences and in the panel of reviewers of selected journals including Toxicology Mechanisms and Methods, Fuel and Malaysian Journal of Microbiology. He has published a book, three book chapters and over 50 articles in reputed journals and conference proceedings. Dr. Ganesh is also holding the position of Associate Dean, International Programmes & Collaboration at BITS Pilani, Hyderabad Campus.

Brief Biography of the Candidate

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Reprints of Publications

Bull Environ Contam Toxicol
DOI 10.1007/s00128-017-2031-8

Detection of TiO_2 Nanoparticles in Municipal Sewage Treatment Plant and Their Characterization Using Single Particle ICP-MS

Siva Prasad Bitragunta[1] · Sankar Ganesh Palani[1] · Anil Gopala[2] · Santosh Kumar Sarkar[3] · Venugopal Reddy Kandukuri[4]

Received: 14 September 2016 / Accepted: 12 January 2017

Abstract Establishment of analytical methods for detection and characterization of nanoparticles in the environment are gaining prominence across the globe. The present study was designed to quantify titanium (Ti) and to characterize titanium dioxide nanoparticles (TNP) from a municipal sewage treatment plant, by inductively coupled plasma mass spectrometry (ICP-MS). The concentrations of Ti & TNP were 1085 & 13.6 mg/kg in the influent sewage and 298 & 3.3 mg/kg in the aeration tank contents, respectively. The size of TNP ranged between 71–145 nm in the sludge fraction. Determining environmentally realistic concentrations of TNP could serve as a tracer material for characterization of those nanomaterials with similar size and aggregation properties. Furthermore, inference of Ti and TNP in municipal sewage in the study will also help in environmental risk assessment of nanomaterials.

Keywords Titanium dioxide (TiO_2) nanoparticles · Sewage · Sludge · Single-particle-ICP-MS

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TiO_2 nanoparticles (TNP) (size < 100 nm) are extensively used as vital ingredients in a wide range of personal care products such as sunscreen lotions and other products *viz.* toothpaste, implanted biomaterials, paints, printing ink, rubber, paper, cement, antimicrobial plastic packaging, and self-cleaning sanitary ceramics. They are also used as excipient in burn treatment creams, dental compositions and cosmetic lotions (Bindhu and Umadevi 2015). Currently, the market potential of TiO_2 production is approximately $2000 per ton (ICI 2015). The annual production of engineered TiO_2 nanoparticles already reached around 6 million tons (Jovanović and Guzmán 2014). Hence, release of TNP into the environment is inevitable during their production, transport, usage and final disposal (Soni et al. 2015; Bakshi et al. 2015; Sun et al. 2016). As a result, detection and characterization of TNP in environmental matrices have become essential to perform environmental risk assessments.

Studies based on exposure modeling revealed that predicted concentrations of TNP in wastewater effluents (0.7–16 µg/L) can be higher than that of predicted no-effect concentration (1 µg/L) (Gottschalk et al. 2009). Furthermore, toxicity potential of TNP was reported in the literature (Gottschalk et al. 2013; Shi et al. 2013; Li et al. 2014a, b). We also reported the ecotoxicity potential of TNP and associated confounding factors using earthworm as a sentinel (Siva Prasad and Sankar Ganesh 2014). Furthermore, occurrence of Ti in environmental matrices and its interaction with other metals including Zn and Cu has proven to induce toxicity in living systems (Holden et al. 2014; Cupi et al. 2015).

There exist knowledge gaps pertaining to the establishment of standard analytical methods to detect and characterize Ti and associated nanomaterials in the environment (Tourinho et al. 2012; Gottschalk et al. 2013). Few studies

Published online: 03 February 2017

attempted to elucidate occurrence of TiO_2 nanoparticles in wastewater and sludge. However, these studies employed techniques such as dynamic light scattering (Topuz et al. 2015) and atomic emission spectroscopy (Westerhoff et al. 2011) that could not *simultaneously* measure significant properties of nanoparticles such as size, concentration, and number (Pachapur et al. 2015).

Inductively coupled plasma mass spectrometry (ICP-MS) which facilitates precise quantification of specific element and characterization of metal based nanoparticles is gaining prominence. In recent times, operating ICP-MS in single particle mode (SP-ICP-MS) enabled rapid yet simultaneous analyses of elemental composition, number and size distribution of nanoparticles, such as TiO_2 (Kim et al. 2012; Laborda et al. 2014; Proulx et al. 2016), Ag and Au (Lee et al. 2014; Yang et al. 2016), ZnO (Hadioui et al. 2015) in the environmental samples.

Therefore, the present study was designed to quantify the concentrations of (i) Ti in the supernatant and sludge fractions of influent sewage, aeration tank contents and treated effluent of activated sludge process using ICP-MS and (ii) TNP in the sludge fraction by SP-ICP-MS.

Materials and Methods

Samples were collected from a central municipal sewage treatment plant that uses activated sludge process (Hyderabad, India). American Public Health Association Guideline No. 1060-A (APHA and AWWA 2005) was followed to collect samples of the influent sewage, aeration tank contents, and treated effluent of the treatment plant. These samples were then subjected to sedimentation (6 h) by gravity to separate the supernatant and sludge fractions of sewage. The samples were then subjected to digestion in closed Teflon vessels using a microwave digester (Milestone, ETHOS EZ). The supernatant (1 mL) and sludge (1 g) fractions of sewage were digested by adding 7 mL HNO_3 (65%), 1 mL H_2O_2 (30%). A temperature controlled microwave program ($T_1 = 200°C$ and $T_2 = 110°C$ for 15 min at 45 bar pressure and 1200 W power) was used to perform digestion of the samples. After digestion, samples were left to cool to room temperature and were transferred to metal free containers. Aliquots of these samples were used to determine the concentration of Ti using ICP-MS. The microwave digest of the sludge fraction from influent sewage and aeration tank contents were filtered through 0.22-micron cellulose acetate membrane (Whatman) to eliminate the interference of other particulates in the digest that can interfere during ICP-MS analysis. The filtrate was sonicated for 5 min in a water bath (SONICA) at 40 kHz frequency. These samples were analyzed using SP-ICP-MS to characterize TNP.

Concentration of Ti in digested wastewater and sludge samples was determined as per Fabricius et al. (2014) using ICP-Quadrupole-MS (ICP-MS; Agilent 7700 series, Agilent Technologies, Germany). The ICP-MS was equipped with a PEEK Mira Mist nebulizer and a PFA inert sample introduction kit with a sapphire injector (inner diameter 2.5 mm). Measurements were conducted at an RF power of 1550 W and a carrier gas flow of 1.17–1.18 L min. The samples were introduced directly into the ICP-MS system using standard peristaltic pump with Tygon tubing (internal diameter of 1.02 mm), and ASX-520 autosampler. The multi-element standards supplied by Agilent Technologies were used as calibration standards to measure Ti from TiO_2. The concentration of Ti was calculated considering the percent of Ti (≈60%) in the total mass of TiO_2. The isotope ^{47}Ti was chosen for calibrating and measuring Ti in the samples because it minimizes the polyatomic and isobaric interferences during the analysis. Sensitivity of ICP-MS (cps/µg/L) for Ti was calibrated using rutile nanoparticle (<100 nm, Sigma–Aldrich) as standard, thereby differentiating nanoparticle signals converted into mass concentration of the element measured.

Application of SP-ICP-MS in the time resolved analysis (TRA) mode is currently conceded as the standard method for characterization of nanomaterials (Degueldre and Favarger 2004; Sannac et al. 2013). In this study, TRA for 3 and10 ms was used to characterize TNP in the sludge fraction of influent sewage and aeration tank respectively. Measurements were conducted at an RF power of 1550 W and a carrier gas flow of 1.05 L min. Nebulizer pump was set to 0.1 rps and the total acquisition time was set to 60 s, with an integration time of 10 ms. Spray chamber temperature was set to 2°C. Nebulizer efficiency and sensitivity of the measurements were calibrated using rutile form of TiO_2 nanoparticles (<100 nm). Time-resolved analysis of SP-ICP-MS was carried out with the help of spreadsheet developed by the National Institute of Food Safety in the Netherlands (RIKILT Wageningen 2013). Density of TiO_2 (4.23 g/mL) was entered into the spreadsheet, to calculate the intensity i.e., number of particles per unit volume. From this data, a size distribution plot was obtained from which the median nanoparticle size of TiO_2 was calculated (Sannac et al. 2013).

Results and Discussion

Concentration of Ti in the supernatant fraction of influent sewage, aeration tank contents and treated effluent was found to be 3.47 ± 1.06, 2.15 ± 0.6 and 0.71 ± 0.2 mg/L respectively, (detection limit = 2.9 ppb, $y = 44.9x + 70.38$, $R = 0.99$) (Fig. 1). Concentration of Ti in the influent sewage, reported in this study was found to be higher than the

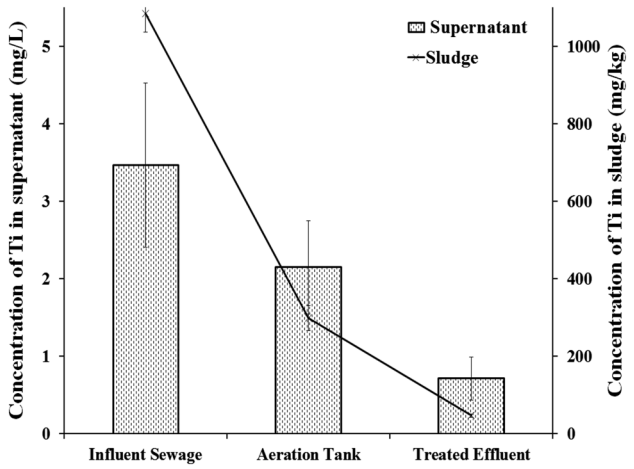

Fig. 1 Concentration of Ti in supernatant (mg/L) and sludge (mg/kg) fractions of influent sewage, aeration tank contents, and treated effluent of a municipal sewage treatment plant. The data represent concentration ± S.D

values reported in the literature, 3 mg/L (Kiser et al. 2009), 0.03 mg/L (Johnson et al. 2011), 1.8×10^{-3} mg/L (Khosravi et al. 2012). Gottschalk et al. (2009) and Westerhoff et al. (2011) predicted that the concentration of Ti in the influent sewage is in the range of 0.021–1.233 mg/L. Likewise the measured concentrations are higher in magnitude than that of predicted concentrations of Ti in the environment (Neale et al. 2013). Characteristics of municipal sewage, sample preparation method and the analytical technique used would have contributed to the variation in Ti concentration reported in this study, as compared to the above references. Currently, data pertaining to the concentration of Ti in the environment is meagre. In this regard, results of this study will contribute in developing "point of reference" to determine the distribution of Ti in the influent sewage, aeration tank contents and treated effluent of activated sludge process. Such data help in risk assessment of TiO_2 and in studying the ecological fate and behavior of TiO_2 in aquatic environment. Concentration of Ti in sludge fraction of influent sewage, aeration tank contents, and treated effluent were found to be 1085 ± 48, 298 ± 32 and 47 ± 4 mg/kg respectively (Fig. 1). Lowest concentration of Ti was found in the sludge sample obtained from treated effluent.

Results indicate that accumulation of Ti is higher in the sludge fraction as compared to the supernatant. In the presence of high molecular weight organic compounds in the wastewater, TiO_2 undergoes heteroaggregation (Topuz et al. 2015). Therefore, from our results we surmise that presence of organic molecules could have facilitated agglomeration of Ti. Hence it could have resulted in the observed highest standard deviation of Ti measured in the supernatant fraction of influent sewage. As it is evident that Ti can accumulate in sludge fraction of sewage, disposal of sludge at the end of sewage treatment can contribute to accumulation of Ti in soil, which is the ultimate sink of terrestrial environment. Modelling study done by Mueller and Nowack (2008) emphasized that predicted Ti concentrations in municipal sewage (0.7–16 µg/L) are higher than their no effect concentrations (1 ug/L). Gottschalk et al. (2009) also predicted that concentrations of Ti could range from 100 to 802 mg/kg in sewage sludge. Therefore, our results are in concordance with predicted concentrations of Ti, as reported in the above studies. Outcomes of the study contribute to demonstrate the distribution of Ti in various environmental compartments. Accumulation of Ti (10 mg/kg) in river sediments facilitates adsorption of various substances including phosphorous, arsenic etc., to the sediment (Luo et al. 2011). Adsorption capacity of Ti is directly proportional to its concentration. As the concentration of Ti reported in our study (47–1085 mg/kg) is much higher than that was reported by Luo et al., cited above, so the adsorption capacity of Ti will be also be higher. Hence outcomes of the study will pave a way to understand the possible interactions of Ti with other components in municipal sewage. The study presents the first report that highlights distribution of Ti in supernatant and sludge fractions of municipal sewage.

In this study, SP-ICP-MS was used to characterize TNP in sewage sludge. TRA for influent sewage and aeration tank sludge fractions is shown in Fig. 2 (A1 and B1). The raw data was curated to eliminate the background signal ($y = 8035x + 225{,}695$, $R = 0.98$). The remaining intensities were converted into their corresponding particle size to obtain distribution plot of TNP [Fig. 2 (A2 and B2)]. From this size distribution plot, median particle size, particle number and mass concentration of TNP were calculated and presented in Table 1. The median particle size of TNP in the sludge fraction of influent sewage sample was 145 nm whereas this was almost half (71 nm) in aeration tank. The distribution of nanoparticle number in the influent sewage sludge fraction (4.8×10^{10} particles/kg) is higher than that of aeration tank contents (1.1×10^{8} particles/kg). The concentration of TNP determined from sludge fraction of influent sewage (13.6 mg/kg) is significantly higher than that of the aeration tank samples (3.3 mg/kg). Decrease in the size of TNP in the sludge fraction of aeration tank contents indicates that mechanical agitation of reactor contents by the sparged air could have resulted in dis-agglomeration of TiO_2 from various organic and inorganic constituents of the sludge, forming smaller size/ aggregates of the particles.

Our results also indicate that the dwell time of 10 ms (for influent sewage) and 3 ms (for aeration tank contents) allows accurate characterization of TNP by SP-ICP-MS. Therefore, calibration of appropriate dwell time is the key factor in SP-ICP-MS analysis for precisely obtaining the

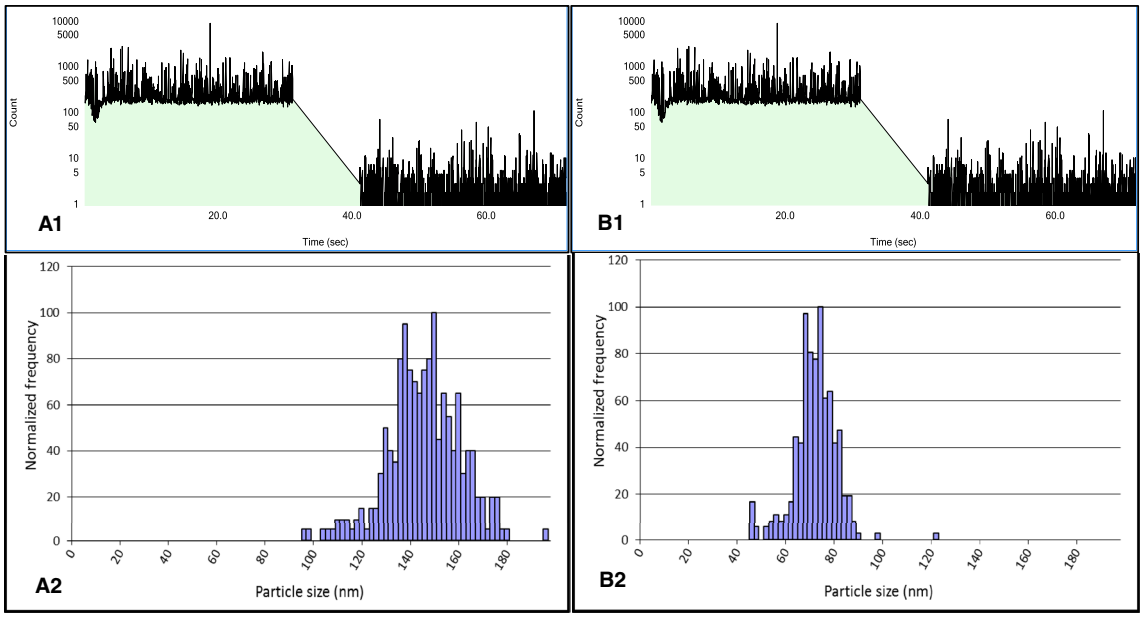

Fig. 2 Time resolved analysis of SP-ICP-MS signals of sludge fraction of influent sewage (A1) & aeration tank contents (B1) and corresponding particle size distribution (A2) & (B2)

Table 1 Characteristics of TNP detected in the sludge fraction of influent sewage and aeration tank contents

Parameter	Influent sewage	Aeration tank contents
Concentration (particle number)	4.8×10^{10} particles/kg	1.1×10^{8} particles/kg
Concentration (mass)	13.6 mg/kg	3.3 mg/kg
Median particle size	145 nm	71 nm

chemical composition and mass concentration of the analyte. Nebulizer efficiency (@ 0.1 rps) helped in minimizing the background noise caused by other constituents of the sample during measurements. ^{47}Ti isotope analysis used in the study will definitely help in establishing the ratio of various stable isotopes of Ti i.e., ^{46}Ti to ^{50}Ti in sludge samples thereby understanding their potential to accumulate in the soil environment. Though result of the current study is limited to ^{47}Ti, it will definitely complement reports on other isotopes of Ti in sewage sludge. Compiling such data will help in understanding speciation of Ti in a given environmental sample. TNP particle size distribution measured in the present study is within the estimated size limits of TNP reported by Lee et al. (2014) that can be detected using SP-ICP-MS as well as the measured size limits reported by Kim et al. (2012) using analytical scanning electron microscopy. Application of SP-ICP-MS in *simultaneous* determination of mass concentration, particle number and particle size of TNP from sewage sludge is the novelty of present study. Therefore, approach and outcomes of this study can serve as proxies in improving ICP-MS based analytical methods to characterize TNP, in terms of their quality and consistency. However, further investigations on reference nanomaterials that can be used for environmental monitoring of nanoparticles are warranted.

Size and functionalization of nanoparticles can also influence their distribution in municipal sewage (Guo et al. 2006; Brar et al. 2010). Wastewater treatment system is the sink for municipal sewage that contains remnants of products such as cosmetics and sunscreen lotions that contain functionalized nanoparticles that could have contributed to the presence of TNP in sewage. Moreover, TNP could pose environmental hazards even in low concentrations, owing to their unique physicochemical properties such as surface area, size and charge. Furthermore, as the usage of TNP is alarmingly increasing in various products, it is expected that in the near future, their presence in sewage also will increase accordingly. Being metal based nanoparticles, TNP do not get biodegraded, hence get discharged out of the wastewater treatment systems, either with the sludge or treated effluent and eventually reach the environment (water and soil). Over time, TNP accumulate in the environment and could elicit toxicity in organisms that get exposed to them. Elsewhere we have also carried out a systemic study to unveil the toxicity of TNP in earthworm. The studies have revealed that bioaccumulation of TNP in the worms

led to their mortality by inducing oxidative stress (Siva Prasad and Sankar Ganesh 2014). Therefore, further investigations on TNP in the environment are imperative.

To date, relevant risk assessment and safety guidelines for TNP are not well documented. In this context, appropriate analytical methods for characterization of TNP under realistic environmental concentrations are needed. Present research is the first of its kind conducted on municipal sewage sludge to report the concentrations of Ti using ICP-MS and TNP using SP-ICP-MS. Occurrence of TNP in environmental samples, could serve as a tracer material for detection and characterization of other nanomaterials with similar size and aggregation properties, thereby assisting in their risk assessment. The study also contributes towards finding the ratio of ^{47}Ti to other stable isotopes of Ti. The authors believe that outcomes of the current study will contribute primarily for quantifying the concentration of TNP using SP-ICP-MS and ultimately for assessing the environmental risk posed by them.

Acknowledgements SGP, VGRK and SPB as principal investigator, co-investigator and senior research fellow respectively, thank Department of Biotechnology, Government of India, for funding this research study vide Project No. BT/PR6592/GBD/27/446/2012. The views and opinions expressed in this research article are solely of the authors and should not be construed as opinions of the affiliating organizations.

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Article-21

Poll Res. 36 (2) : 313-319 (2017)

ISSN 0257–8050

EVALUATION OF ANTIOXIDANT ENZYME RESPONSES IN EARTHWORMS EXPOSED TO POLYSTYRENE NANO PARTICLES AND PAMAM DENDRIMERS

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(Received 20 January, 2017; accepted 1 March, 2017)

ABSTRACT

Rapid yet cost-effective toxicity testing methods to evaluate the toxic potential of engineered nanoparticles are of high priority in the era of emerging contaminants. In this pursuit, efforts are underway to explore the applicability of existing toxicity testing methods that are used for conventional chemicals. The present work focuses on toxicological evaluation of two polymeric nanoparticles i.e., polystyrene (PS) and polyamidoamine dendrimers inearthworm *Eisenia fetida* through the dermal contact method of OECD-207. Dynamic light scattering technique was used to determine size and charge of polymeric nanoparticles. Polystyrene had the size of 111± 11.5 nm and charge of-64.9 ± 5.8 mV. Size and charge of polyamidoamine dendrimers were 386 ± 15 nm &-27.5 ± 3.2 mV respectively. Though exposure of earthworm to these anionic polymeric nanoparticles showed no mortality, at sub-lethal concentrations, perturbations in antioxidant enzymes such as glutathione reductase and superoxide dismutase were observed. Thus outcomes of the study will help in screening the toxicity of polymeric nanoparticles in earthworm using dermal contact.

KEY WORDS : Polystyrene nanoparticles, PAMAM dendrimers, *Eisenia fetida*, Dermal contact, Antioxidant enzymes

INTRODUCTION

Toxicological evaluation of engineered nanoparticles is not a clear-cut scenario because the altered physical and chemical properties of these nanoparticles induce varied toxic end-points in living systems (Maynard and Aitken, 2007; Tian Xia *et al.,* 2008). The difference in the results could be attributed to the type and properties of the nanoparticles, method, and sentinel that was adopted to evaluate the toxicity. Therefore, inter and intra lab studies that were conducted to evaluate nanoparticles toxicity show different results. In the light of the above facts, *in vitro* and *in vivo* toxicity assessments of engineered nanoparticles are apparently crucial to elucidate the subtle responses at the organism level. Hence lab based preliminary investigations are required to establish the baseline datawhich could help inelucidating the mechanism of toxicity of the nanoparticlesconcerned (Fubini *et al.,* 2010). Recent advances in polymer engineering led to the manufacture of polymeric nanoparticles such as polystyrene and dendrimers with unique size and charge. Among the crucial factors that influence toxicity and eventual mode of action are the size and charge of nanoparticles (Bhattacharjee *et al.,* 2010). Hence, nanoparticles necessitate toxicological testing that aids in understanding how their surface charge and particle size might induce the toxic effects that can be elucidated in lab based studies.

Polystyrene (PS) nanoparticles are widely used in biomedical applications owing to their rapid synthesis and feasible functionalization (Canesi *et al.,* 2016). PS is also used in packaging and manufacturing of insulating materials and different

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types of toys (Andrady, 2011). Hence the use of PS nanoparticles as models in nanotoxicity testing is increasing. For instance, cytotoxicity potential of PS nanoparticles (Frohlich *et al.,* 2009; Xia *et al*., 2008) their toxic effects on macrophages (Xia *et al*., 2006) and their ability to cause skin lesions (Yanagisawa *et al.*, 2010) are well established. However, data on toxicological evaluation of PS nanoparticles in invertebrates such as earthworm, which is an universally accepted terrestrial model for toxicity testing is scarce.

Polyamidoamine (PAMAM) dendrimers are hyper-branched polymeric nanomaterials that have globular shape, functionalized with various surface groups. Due to their encapsulation ability, they can undergo internalization in cells and potentially induce toxicity in organisms (Fernandez *et al.,* 2015). Albeit, few studies demonstrating the cytotoxicity of dendrimers (Duncan and Izzo, 2005; Chen *et al.,* 2004) and their ability to perturb cell membranes thereby cell lysis (Rittner *et al.,* 2002) are available, toxicity studies to establish the dermal contact and subsequent effects in invertebrates such as earthworms aresparse.

Despite the advances in nanotoxicology testing methods, elucidation of toxicity through dermal contact is very important to understand the toxic effect of nanoparticles in terrestrial models likeearthworm.Responses of antioxidant enzymes during the dermal exposure ofearthworms are scarce in the literature. Furthermore, application of filter-paper contact method standardized by Organization for Economic Cooperation and Development (OECD), is cost effective for screening toxicity of various chemicals (Fitzpatrick *et al.,* 1996). However, application of this method for assessing nanoparticle toxicity is not well documented. The antioxidant enzymes CAT, SOD & GR and lipid peroxidation in terms of TBARS were chosen in this study because these biochemical responses can serve as biomarkers to assess the toxic potential of polymeric nanomaterials by inducing the oxidative stressin environmentally sentinel organisms (Siva Prasad and Sankar Ganesh 2014). Therefore, we made an attempt to (i) evaluate the toxicity of polystyrene and PAMAM dendrimers in *E. fetida* (Savigny 1826) through dermal exposure(OECD 207), (ii) measure response of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) & glutathione reductase (GR) and (iii) determine the release of thiobarbituric acid reactive substances (TBARS) to evaluate the potential of nanoparticles to perturb membrane function.

MATERIALS AND METHODS

Measuring size and charge of nanoparticles

Suspensions of polystyreneand polyamidoamine (PAMAM) dendrimers-zeroth generation (G0) both having the mean particle size of 100 nm (Sigma Aldrich) were used in this study. Working stocks of these polymeric nanoparticles were prepared in Milli-Q-water. Size and charge (zeta potential) of nanoparticle suspension was performed using dynamic light scattering method using zeta sizer (ZS90, Malvern, United Kingdom), which is equipped with 4-mWHe-Ne 633nm laser (for measuring size and charge)and an electric eld generator (for measuring charge).

Collection and acclimitization of earthworms

Eisenia fetida (Savigny, 1826) earthworms were collected from the vermiculture facility maintained at BITS Pilani, Hyderabad Campus. Adult worms with well-developed clitella having anaverage weight of 600 mg were used in the experiment. These worms were acclimatized in the lab for 7 days, before the experiments.

Exposure of earthworm to nanoparticles

To assess the toxicity of nanoparticles, filter paper contact method of the earthworm, Acute Toxicity Test No.207 was employed (OECD 1984). Earlier studies also demonstrated that it is an efficient method to assess toxicity of organic chemicals and metals (Van Leemput *et al.*, 1989) r-TiO_2-ENP in the earthworm (Siva Prasad and Sankar Ganesh 2014). Prior to the experiments, earthworms were kept on moist filter paper for three hours under starvation to clear their gut contents. Each earthworm was kept in one Petri dish, containing cellulose filter paper (Whatman Grade 1), having a surface area of 68 cm^2. The Petri dishwas placed in dark and the temperature was maintained at 20 ± 2^{o}C. To provide amoisture content of 35 ± 2%, the petri dish was intermittently sprinkled with distilled water whenever necessary. Aliquots of polystyrene (100, 200, 400, 800 and 1000 µg/cm^2) and PAMAM dendrimers (125, 250, 500, 1000, 2000 µg/cm^2) weretested to elucidate their toxicity in the earthworms. As on date, meagre data exist on laboratory testing of polystyrene nanoparticles and PAMAM dendrimers, the exposure concentrations

were chosen based on a literature study (Canesi *et al.*, 2016; Miyazaki *et al.*, 2013; Labieniec *et al.*, 2014; Watala *et al.*, 2016). For each concentration of the nanoparticles, 10 worms were exposed, and the experiment was repeated thrice for obtaining statistically significant data. Controls were run in parallel with Milli-Q-water *sans*nanoparticle suspension.

BIOCHEMICAL STUDIES

Tissue preparation

After 48 h exposure, earthworms exposed to nanoparticles were homogenized in 0.1 M phosphate buffer (pH 7.5) using Potter-Elvehjam homogenizer, which is equipped with a Teflon pestle. The homogenates were centrifuged at 5000g for 10 min in Beckman (TLX-361544) centrifuge, and the supernatant was further centrifuged at 5000g for 10 min. Subsequently, supernatant from the second round of centrifugation was used to estimate theactivity of antioxidant enzymes and lipid peroxidation. All enzyme preparations were carried out at 4°C. Protein content was estimated by the method of Lowry *et al.*, (1951).

Estimation of antioxidant enzymes and lipid peroxidation

All estimations were performed with aminimum of three replicates per enzyme assay and lipid peroxidation. Tissue homogenates were used as the source of protein to assess theactivity of Catalase (EC 1.11.1.6), Superoxide dismutase(EC 1.15.1.1) and Glutathione reductase (EC1.6.4.2) enzymes. Spectrophotometer (Beckman Coulter)was used for estimating the enzyme activities. Changes in the specific activities of enzymes and lipid peroxidation in earthworms that were exposed to nanoparticles, as compared to controls were expressed as mg of protein. Corresponding blanks were run for each assay containing all components except the enzymesource.

Catalase (CAT) (EC 1.11.1.6) was assayed by decomposition of hydrogen peroxide (H_2O_2) using the modified method of Claiborne (1985). The assay mixture contained 950 µl of distilled water, 2.0 mL of 0.059 M H_2O_2 in phosphate buffer and 50 µl of theenzyme. Change in absorbance was measured at 240 nm against theblank at 10 seconds intervals for 2 min.

Glutathione Reductase (GR) (EC. 1.6.4.2) was assayed by the method developed by Schaedle and Bassham (1977). Enzyme activity was determined by monitoring the Glutathione dependent oxidation of NADPH in a reaction containing 20 µl of 10 mM NADPH, 50 µl of 20 mM GSSG, 100 µl of10 mM EDTA, and 1330 µl of 0.1 M-phosphate buffer and 50 µl of sample. The contents were allowed to react for 10 min and the absorbance was read at 340 nm.

Superoxide Dismutase SOD (EC. 1.15.1.1) activity was determined by the method of Marklund and Marklund (1974) which is based onits ability to inhibit auto oxidation of pyrogallol. To 2.5 mL of the assay mixture containing 2.2ml of pyrogallol (1,2,3-trihydroxybenzene, Sigma Aldrich), 300 µl of thesample were added to initiate the reaction. Change in absorbance of thesample was read at 420 nm for 10 min in kinetic mode.

Lipid peroxidation was determined by the formation of thiobarbituric acid reactive substances (TBARS) as described by (Fatima*et al.*, 2000).0.5 mL of protein was incubated for a period of 30 min at 37°C with an incubation mixture of 350 µL (10 µM $FeSO_4$, 200 µM Ascorbate, 0.125 M KCl, 0.02 M Tris-HCl buffer, pH 7.4). Peroxidation was terminated by adding 250 µl of 20% trichloroacetate (TCA). Subsequently, 500 µl of 0.67% thiobarbituric acid (TBA) was added to the above mixture and heated in water bath at 100°C for 10 min. Centrifugation was performed to remove the precipitated proteins. The absorbance of the supernatant was read at 532 nm to measure TBARS.

Statistical analysis

Size and charge of nanoparticles were represented as mean values taken at poly-dispersity index<0.5. The final data of antioxidant enzymes and TBARS was expressed as mean ± S.D of three separate preparations, each assayed in triplicates. The statistical significance of all parameters was determined by unpaired Student's *t* test and $p<0.05$ was considered significant when compared to controls.

RESULTS AND DISCUSSION

Size and charge of the nanoparticles are one of those crucial factors that influence the toxicity of nanomaterials and their eventual mode of action. In this study, size and charge of PS nanoparticles and PAMAM dendrimer suspension in Milli-Q-water (1 mg/ml) were determined using dynamic light

scattering technique and the results are presented in Table 1. The PAMAM dendrimer showed agglomerates of size 386 nm whereas PS nanoparticles did not demonstrate such property because the size was 111 nm only. In contrast, the charge of PS nanoparticles was two folds higher than PAMAM dendrimers. From the results, it is evident that size and charge of the nanoparticles are directly proportional to each other. This could be attributed to increasing inter-particle repulsion, there byVander Walls attractive forces decrease, leading to anoverall decrease in the size of the particles (Lagan, 2016; Vishaka, 2016). Therefore, PS nanoparticles havea tendency to form amono dispersive suspension in water, hence can be used as standards in toxicity testing.

Table 1. Size and charge of nanoparticles

Parameter	PS nanoparticles	PAMAM dendrimers
Size (d.nm) ± S.D	111± 11.5	386 ± 15
Charge (mV) ± S.D	-64.9 ± 5.8	-27.5 ± 3.2

Exposure of earthworms to PS nanoparticles (100 to 1000 µg/cm^2) and PAMAM dendrimers (125 to 2000 µg/cm^2) via dermal contact showed no mortality. Despite difference in their sizes, both types of polymeric nanoparticles used in this study, resulted in no toxic effects in terms of mortality. Therefore, it is concluded that anionic polymeric nanoparticles such as PS nanoparticles and PAMAM dendrimers did not induce acute toxicity in the earthworm. This could be attributed to the fact that electrostatic interaction of anionic polymeric nanoparticles with the earthworm's skin is less when compared to the corresponding cationic polymeric nanoparticles such as cationic polyethylenimine and cationic dendrimers (Schulz *et al.,* 2012). Furthermore, investigations that dealt with thetoxicity of PAMAM dendrimers in different cell systems revealed that generation (G-number of branched monomers), dose, exposure duration, species and charge of the terminal groups influence their toxic potential (Jain *et al.,* 2010; Malik *et al.,* 2000; Mukherjee and Byrne, 2013). For a given type of dendrimer, lower generation (G0-G3) PAMAM dendrimers elicit aless toxic response than their higher generation (G4 and above) counterparts (Gonzalo *et al.,* 2015; Jevprasesphant *et al.,* 2003; Roberts *et al.,* 1996). Results of the present study substantiate that PAMAM dendrimers of zeroth generation could not elicit a significant toxic response in earthworm via dermal exposure (Lagan 2016).

To elicit subtle sub-lethal effects, the earthworms were exposed to the nanomaterials for evaluating their effect on the antioxidant enzymes. Exposure to polystyrene nanoparticles for 48 h, increased catalase activity and decreased GR activity in earthworms, as compared to controls (Figure 1A and B). However, there was no significant difference in SOD activity between treated and control worms, except for the PS nanoparticle concentration of 1000 µg/cm^2 (Figure 1C). Furthermore, TBARS that are produced during the exposure to polystyrene nanoparticles were found to be significantly less than that of control worms, except for the PS nanoparticle concentration of 200 µg/cm^2 (Figure 1D). It is also evident that PS nanoparticles could trigger the activity of CAT which could have helped in mitigating the stress being imposed by reactive oxygen species such as peroxides released during the exposure. Moreover, SOD activity could also have assisted in the process of balancing the redox cycle. Thus exposure of PS nanoparticles could not elicit lipid peroxidation. Similar results were reported the study done by Hoet *et al.,* (2004) in which nanoparticles that entered rat liver induced oxidative stress. When polystyrene nanoparticles at the concentrations of 20 and 100 mg/kg were administered intravenously into the rat liver either as a single dose in one day or in repeated doses for 14 days has resulted in depletion of oxidized glutathione (GSSG) and reduced glutathione (GSH). Furthermore, the activity of SOD got inhibited and the catalase activity got increased. Therefore, it was concluded that polystyrene nanoparticles with a mean particle size of 111 nm, did not cause mortality in earthworm at the concentration ranging from 100 to 1000 µg/cm^2. It indicates that earthworms could overcome the oxidative stress being imposed by polystyrene nanoparticles, mainly by the activity of catalase(Vishaka 2016).

On the other hand, exposure to PAMAM dendrimers increased CAT activity (except in the concentration of 500 µg/cm^2) and SOD activity (except in the concentration of 2000 µg/cm^2) as depicted in Figure 2A and C. However, there was no significant difference in GR activity between treated and control worms (Fig. 2C). Furthermore, TBARS that were produced during the exposure to dendrimers were found to be significantly lesser in the exposed worms than that of control worms

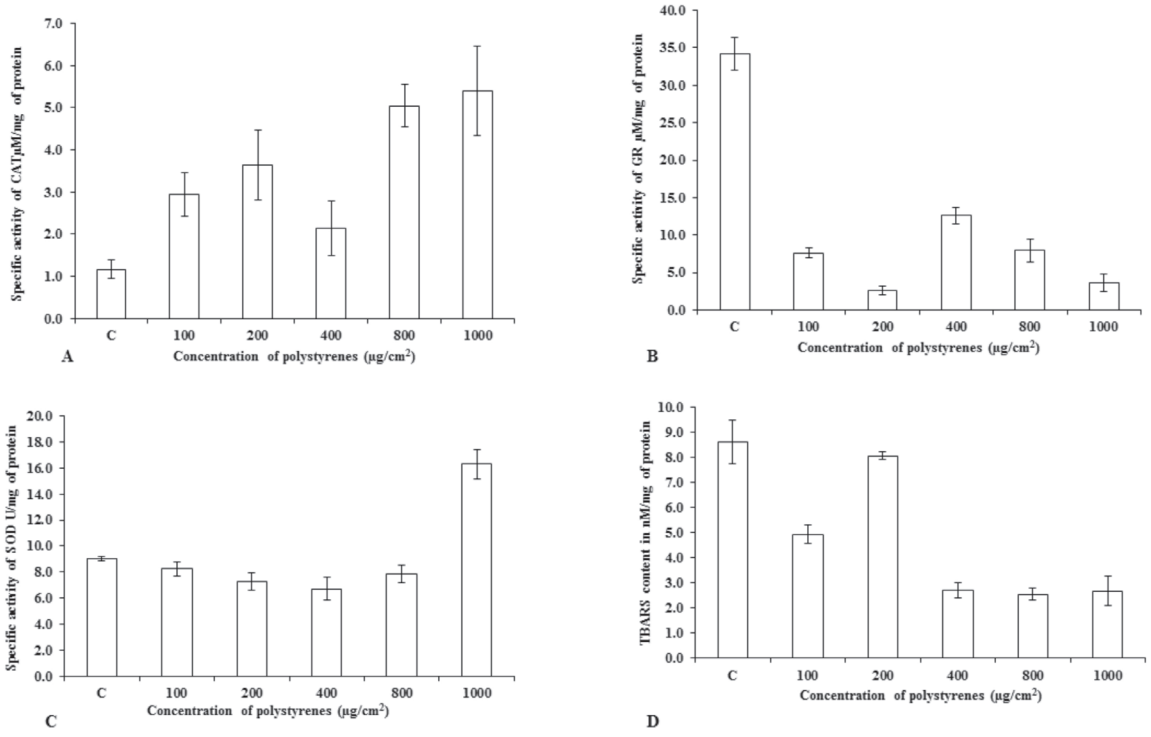

Fig. 1. Specific activities of antioxidant enzymes and lipid peroxidation (TBARS) in tissue homogenate of earthworm exposed to polystyrene nanoparticles. Final data represents mean ± S.D (n=3, p<0.05)

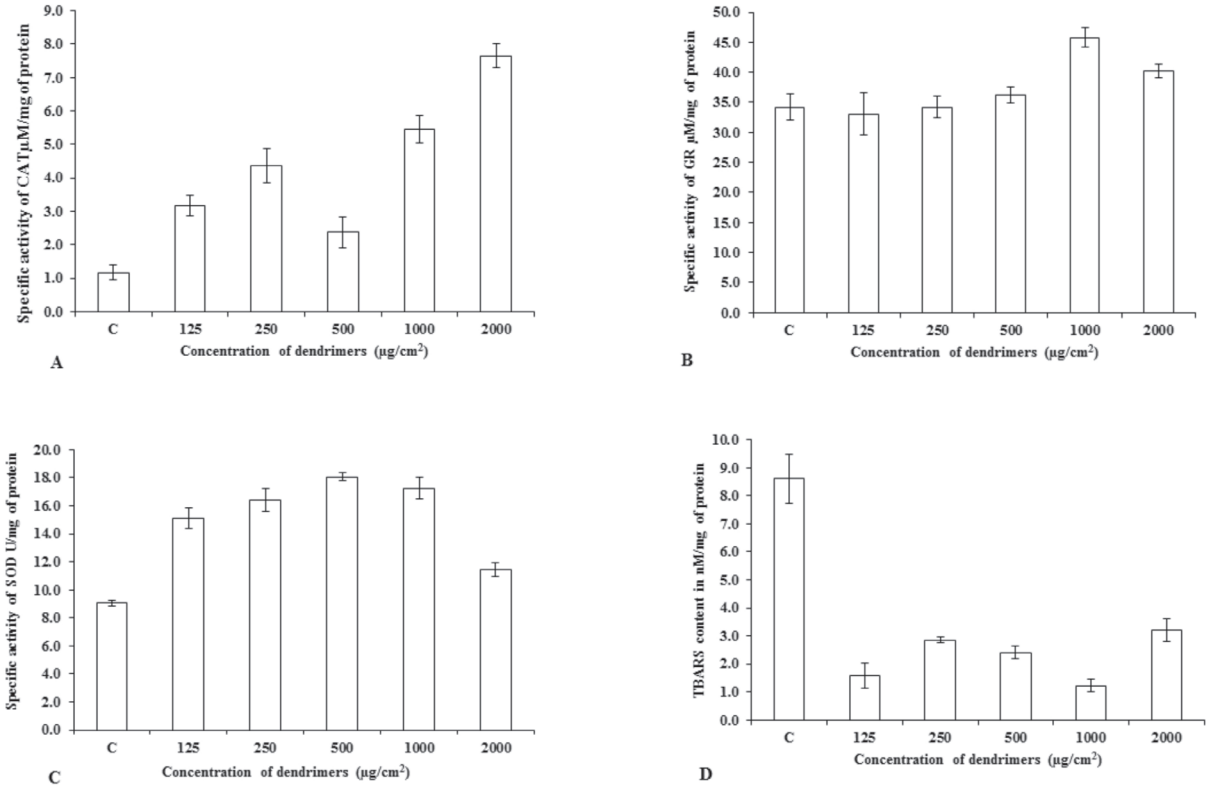

Fig. 2. Specific activities of antioxidant enzymes and lipid peroxidation (TBARS) in tissue homogenate of earthworm exposed to PAMAM dendrimers. Final data represents mean ± S.D (n=3, p<0.05)

(Figure 2D). From the above results it can be assumed that the production of reduced glutathione increased which had mitigated the effect of reactive oxygen species, hence PAMAM dendrimers of zeroth generation could not elicit oxidative stress in the earthworm. Charge of the amino groups present on the surface of dendrimers is probably not sufficient to cause significant changes in the overall oxidative stress in the earthworm. Therefore, PAMAM dendrimers at a concentration range from 125 to 2000 µg/cm^2 could not cause any mortality in earthworms.

CONCLUSION

In summary, results of the present work indicate that polymeric nanoparticles such as PS nanoparticles and PAMAM dendrimers, without any functionalization could not elicit toxicity in earthworm. However, sub-lethal concentrations of PS nanoparticles induced CAT and inhibited GR activities whereas PAMAM dendrimers induced activities of both CAT and SOD. Hence it is evident that CAT can act as the biomarker to indicate the antioxidant potential of earthworms exposed to PS nanoparticleṡ and PAMAM dendrimers.Being anionic nanoparticles, both the polymeric nanoparticles could not significantly induce lipid peroxidation in earthworm, when exposed via dermal contact. Since data on toxicity evaluation of polymeric nanoparticles is scarce, outcomes of this study will help in establishing baseline limits for conducting toxicity tests for PS nanoparticles and PAMAM dendrimers.

ACKNOWLEDGEMENT

PSGand BSP thank Department of Biotechnology, Government of India for funding support in the form of Project No. BT/PR6592/GBD/27/446/2012 and Senior Research Fellowship respectively. Authors acknowledge Dr. Vamsi Krishna Venuganti, Department of Pharmacy, BITS Pilani, Hyderabad Campus for reviewing the manuscript. The views and opinions expressed in this research article are of the authors and should not be construed as opinions of the affiliating organizations.

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Nanowaste and Environment: Current Understanding and Way Forward

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Abstract

Manufacturing and use of nanomaterials in various commercial products across the globe indicates possibility of nanomaterials entry into environment. Nanomaterials or synthetic byproducts of nanoscale dimensions that are found in environmental matrices are termed as nanowaste. Currently, investigations to delineate the occurrence, fate, transport and associated risk of nanomaterials in various environmental compartments are gaining attention in waste management research. Till date, most of the research outcomes raised more hypothesis than true answers to address the fate and transport of nanomaterials in waste water and in the environment. Furthermore, research in this domain lags far behind when compared to the developments in nanotechnology*per se*. Hence there is a need for concerted efforts to pursue research to understand fate and behavior of nanomaterials especially in sewage and sludge. In order to address the complex problem involving various disciplines, the 'System of Systems' approach should be adopted to implement the principles of conventional green chemistry to mitigate the effects of nanowaste. Thus repositioning of chemistry to address the nanowaste paradigm will definitely aid in promotion of nanotechnology.

Keywords: Nanotechnology, nanoparticles, nanowaste, environment, toxic

Introduction

Miniaturization of particles at nanoscale led to their widespread applications in industrial, biomedical and personal care products.[1,2] In the last three decades, emergence of engineered nanomaterials led to enhanced market potential of engineered nanoparticles (ENPs). The production of ENPs is found to double every three years. For instance, application of silver nanoparticles as antibacterial agents led to their projected market value from 0.79 billion in 2014 to $2.54 billion by 2022.[3,4] Integration of ENPs in various products will reach $3 trillion market by 2020.[5,6] Categories of products that used nanomaterials for their production in 2013 are presented in the Figure 1.

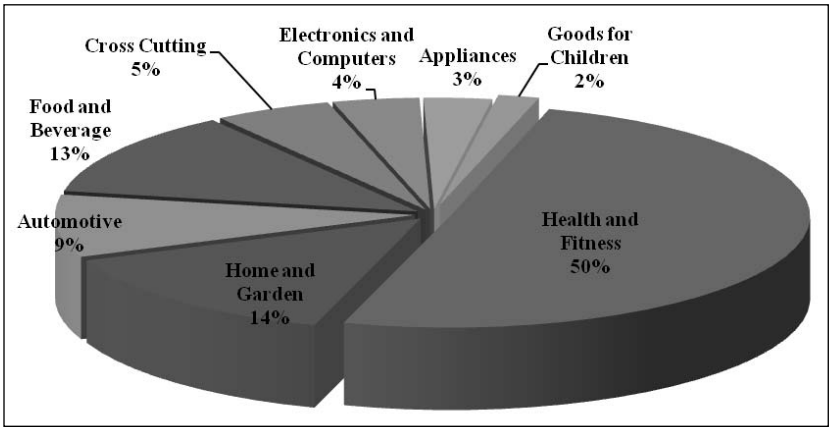

Figure 1. Categories of products that are incorporating nanomaterials during their manufacture by the year 2013.
Source: ***http://www.nanotechproject.org/cpi/site/assets/files/3551/chart_2.png***

Though nano based products are gaining societal interest, properties of ENP and their environmental impacts led to conflicts rather than answers for promoting safety of nanotechnology. Moreover, the behavior and effects of nanomaterials on the environment are largely unknown. Recent review by Krug[7] on nanomaterial safety highlighted the evolution of nanotoxicology and need for the waste management to understand impact of nanomaterials on human and environmental health. In this regard, new developments in nanotechnology contributed to the production of new forms of waste that can accumulate in the environment.[8]

What is nanowaste?

Waste stream(s) containing nanomaterials or synthetic by-products of nanoscale dimensions, generated either during production, storage and distribution, or waste stream resulting from the end of lifespan of formerly nanotechnology-enabled materials and products, or items contaminated by nanomaterials such as pipes, personal protection equipment are classified as nanowaste[8]. Nanowastes that enter aquatic environments include wastewaters discharged from manufacturing industries like pesticides,

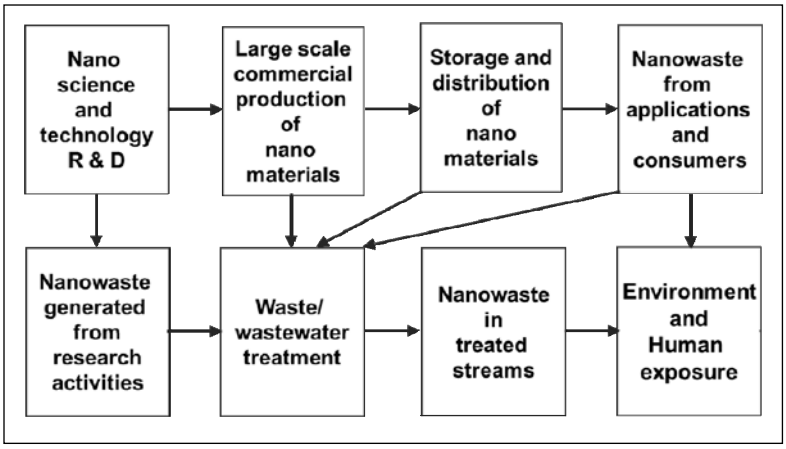

Figure 2. Nanowaste generation and exposure pathways[8]

fertilizers, pharmaceuticals, personal care products, etc., or processing sites, landfill seepage, or construction debris. Uncontrolled release of nanomaterials into the environment will grow dramatically in the near future necessitating safe handling of nanowaste. Possible generation and exposure pathways are presented in Figure 2.

Environmental exposure of nanomaterials is likely to cause potential adverse effects on flora and fauna even at very low concentrations. Therefore, it is essential to ascertain and validate their ultimate fate and distribution in the environment and their potential for causing toxic effects in various life organisms. Establishing the fundamentals of various processes responsible for transformation, transport, toxicity and ultimate fate of nanomaterials and the rates of these processes, should be one of the research priorities in the area of nanowaste management. These research findings will benefit stake holders in environmental health and safety research.[9]

Current understanding of nanowaste

Analytical methods that can detect nanomaterials from environmental matrices are still emerging. Therefore, global research in this sector mainly have focused on the release and fate of selected nanoparticles including TiO_2, ZnO, Ag, fullerenes in surface waters, wastewater effluents, soil, and sewage but without appropriate analytical method validation. Occurrence of TiO_2 in textile industry wastewater was reported.[10] Surface water concentrations of TiO_2 nanoparticles vary from 0.012–0.057 µg L^{-1} in Europe, 0.002–0.010µg L^{-1} in the U.S., and 0.016–0.085 µg L^{-1} in Switzerland.[11] A study on the fate of Zn, either normally present in the wastewater or added in the form of soluble salt or ZnO nanoparticles, indicated that Zn nanoparticles undergo considerable changes in speciation during the anaerobic phase of wastewater sludge treatment.[12] Release of silver nanoparticles from eight different commercially available textiles manufactured with silver, during the washing and rinsing cycle was concluded.[13] Effective separation of ionic gold species and gold nanoparticles from the wastewater and natural water samples was obtained by using cloud point extraction technique.[14]

Aggregation behavior of aqueous suspensions of C_{60} nanoparticles and their effect on transport, bioavailability, and removal during wastewater treatment was investigated.[15] Naturally occurring riverine macromolecules affect the fate of citrate and acrylate stabilized gold nanoparticles.[16] The influence of riverine molecules on the stability of the gold colloids was studied as a function of pH by UV–visible absorption spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM). Quantification and the removal efficiency of silver, titanium dioxide, and carbonaceous nanomaterials from simulated wastewater and into biosolids using lab-scale sequencing batch reactors (SBRs) to evaluate the effects of nanomaterials on the function of bacteria in wastewater treatment plants was reported.[17]

The U.S. National Research Council and National Nanotechnology Initiative has recently proposed a new framework for environmental health and safety of ENM.[18] This committee recommended that research should focus on understanding "critical elements of nanomaterial interactions", that are needed for assessing exposure, hazards, and hence risks posed by nanomaterials. These critical elements include physical, chemical, and biological interactions that ultimately influence nanomaterial persistence, bioavailability, reactivity, and toxicity in the environment.

However, such scientific data/ investigations on the fate and behavior of nanomaterials are scarce. There is a need for the development of infrastructure, human resources and research and simultaneous monitoring of needs and issues in this area. Beyond this limited research, there is little, if any, data which brings a huge uncertainty in risk assessment of nanomaterials in environmental matrices. The management of nanowaste is one of the needs for sustainable nanotechnology.[19] Therefore, it is essential to promote the research programs on risk assessment of nanotechnology which can underpin the necessary and anticipatory governance to ensure the benefits of nanotechnology. A proactive response in this context is in the best interests of public health, environmental health, the

advancement of science, and ultimately the sustainability of relevant industries.

Quo Vadis?

Extensive production and use of nanoparticles have led to calls for more information regarding the impacts that they may have on the environment and human health.[20–22] Physico–chemical and biological interactions of nanomaterials in both environmental and biological systems alter their fate, transport, and toxicity compared to their bulk counterparts.[9] However, the main issue pertaining to the lack of knowledge on sources and subsequent behavior and effects of nanomaterials is one of the major lacunae to assess the risk posed by nanowaste.

The risk assessment strategies are likely to be even more challenging for a densely inhabited country like India. Rapid urbanization, industrialization and technological advance in the past have also led to increased use of consumer products containing potentially toxic nanomaterials. Thus the emergence of nanomaterials and their release into the air,[23] water, and soil[11] would show exacerbation on environmental health.[19]

The number of personal care products and consumables containing nanomaterials continues to increase despite insufficient knowledge about their environmental risk.[18] Because of their widespread use in consumer products, it is expected that engineered nanoparticles will find their way into the environment in spite of their fate and behavior being largely unknown.[24] Present and envisaged future applications of nanomaterials are the most significant sources of large quantities of nanomaterials into the water bodies through waste streams.[25]

As opposed to the several environmental benefits from nanotechnology for improving the water quality, the environmental exposure and potential adverse risk from nanomaterials in water is currently ill-defined.[26] Mobility and transport of engineered nanoparticles into the soil matrix was also highlighted in the literature[27]. Possible sources that release engineered nanomaterials into the environment are aqueous discharges from industries and wastewater treatment plants. Depending on the manufacturing process and the type of product, there are likely to be both solid and liquid wastes that may contain nanomaterials in the aqueous discharge. But the potential for nanomaterials to end up in aqueous discharges is currently unknown.

Future discharge rates of nanomaterials into the environment are unknown and scarce data on scientific investigations on risk assessment of nanomaterials in nanowastes exists, compromising the ability to predict their impact on environmental compartments.[28,29] Ascertaining the risk of chemicals to cause adverse impacts on different environmental compartments depends on various intrinsic characteristics of a chemical such as: bioaccumulation, persistence, toxicity, and transport. These characteristics are independent of the chemical's quantity. Besides intrinsic characteristics of a chemical, the quantity of chemicals released into the environment are important in ascertaining their realistic hazard and risk.[30–33]

Mode of interactions and transformations of nanomaterials will readily occur in the environment and *in vivo*, which greatly affects their properties, behavior, and effects. Research on development and validation of standard analytical methods to detect and quantify nanomaterials at the appropriate concentrations in environment is scarce; this is true for aquatic ecosystems too. Consequently, the scientific data on the risk assessment of nanomaterials in the aquatic environment is in its infancy. However most of the research work done on toxicity, fate, and transport till date have focused mainly on pristine form of nanomaterials, which will behave differently than the transformed ones.[9] For example, silver nanoparticle might be readily sulfurized in wastewater treatment plants to Ag_2S nanoparticle. Aggregation of TiO_2 individual nanoparticles can inhibit the growth of microbes in surface water.[34]

Modeling of chemical constituents in environmental compartments is used to assess the fate and transport of organic chemicals for 30 years.[35] Establishing relationship between physicochemical properties of a chemical and its behavior in the environment encompassed with environmental processes enable predicting their environmental concentrations. In this context, development of modeling procedures for depicting environmental fate and behavior of nanoparticles is still in its infancy. However, few approaches to address the challenges in exposure modeling of engineered nanomaterials were documented in the literature. Predicted environmental concentrations of Ag, TiO_2 and CNTs (carbon nanotubes) in air, soil, and water with a substance flow analysis of engineered nanoparticles from different products are documented.[36–38]

Though some studies have been conducted during the last few years on the effects of nanomaterials on organisms and their environments, a comprehensive scientific data has not been established because of their large variety and differing characteristics. Fundamental data to understand bioaccumulation of nanomaterials at various trophic levels is scarce. Because little is known about the biodegradation of nanomaterials, which may

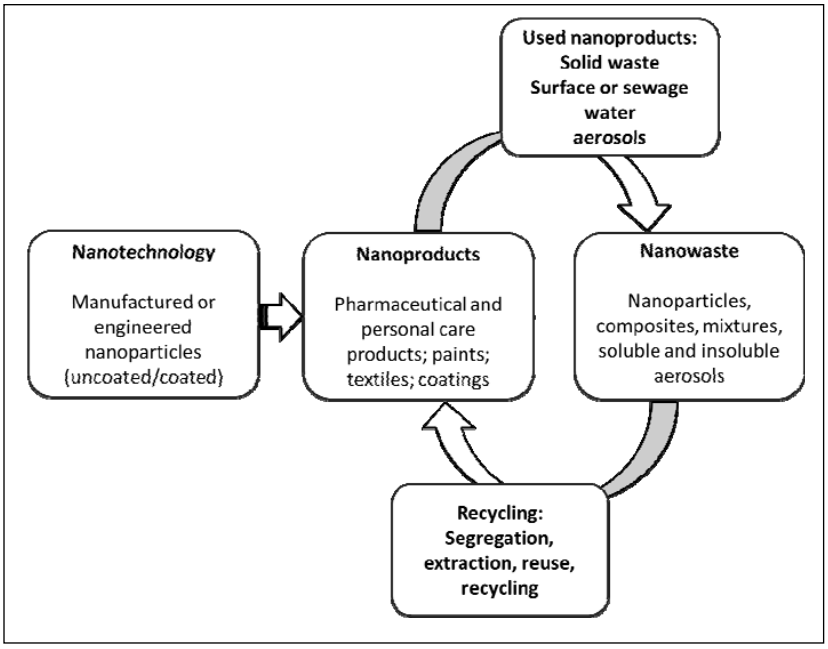

Figure 3. Recycling approach of green nanoscience to address the challenges in evolution of nanowaste from nanotechnology[39]

result in the changes in their physical structure or surface characteristics, and the majority of nanomaterials are not expected to biodegrade, bioaccumulation is an essential characteristic of nanomaterials to study. However, it is still not clear on how, at which concentrations, and in what forms nanomaterials will show their impact wastewater treatment and thereby on environment.

A way forward to green nanoscience

Ultimately, nanoparticles that are finding their entry into the environment are considered as different type of waste when compared to their bulk form.[39] Nanowaste during its life cycle (Figure 3) can pollute various compartments of environment. Hence conventional systems of waste management strategies are to be relooked or restructured to predict the fate and behavior of nanowaste. In this pursuit, regulating the levels of nanowaste has gained prominence. As on date, no nanowaste has been classified as hazardous waste. However, The Woodrow Wilson International Center's Project on Emerging Nanotechnologies (PEN), British Standards Institution (BSI) and United States Environmental Protection Agency (USEPA) are working in this regard. These organizations along with working party on manufactured nanoparticles (WPMN) are paraphrasing aspects relevant to the nomenclature & classification of engineered nanoparticles their products and life-cycle assessment of nanotechnologies.

Green chemistry concepts including the separation and removal of heavy metals from used materials like batteries help in minimizing their environmental contamination.[40] Similarly, extrapolation and application of such techniques for recovery of nanoparticles viz. zinc and iron could be applied to composite nanowaste after segregation of nanoproducts during their life cycle (Figure 3).

Besides physico-chemical methods in green chemistry, biological systems including plants, invertebrates and fungi are used as hyperaccumulators of heavy metals.[41] Hence identification of appropriate biological systems which have the potential to accumulate heavy metals, radionuclides and other xenobiotics will definitely assist in remediation and biotransformation of nanowaste in soil and water environment.Furthermore, following aspects are to be addressed in advancing nanowaste management:

- Universally accepted nomenclature for engineered nanomaterials (ENM) has to be paraphrased.
- Influence of dimensions/ properties *viz.* length, diameter, surface area, charge, capping agent on fate and behavior of ENM in the environment are to be investigated.
- Design and validation of analytical methods to measure the concentrations of ENM in environment and biological systems has to be given prime focus.
- Toxicological evaluation of ENM to frame nanowaste legislation should be carried out.
- Innovative waste management strategies to handle new forms ENP are required.
- Databases that provide information on ENM, toxicology of ENM and their life cycle assessment are need of the hour.
- Funding agencies and scientific community should prioritize the need for nanowaste monitoring and escalate the programs to train the manpower, dissemination of knowledge in this regard.

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Acknowledgement

Authors thank Department of Biotechnology, Government of India, for funding this research study vide Project No. BT/PR6592/GBD/27/446/2012. The views and opinions expressed in this research article are solely of the authors and should not be construed as opinions of the affiliating organizations.

The Toxicologist

Supplement to
Toxicological Sciences

57th Annual Meeting and ToxExpo™

San Antonio, Texas
March 11–15, 2018

OXFORD
UNIVERSITY PRESS

ISSN 1096-6080
Volume 162, Issue 1
March 2018

www.academic.oup/toxsci

The Official Journal of
the Society of Toxicology

SOT | Society of Toxicology

www.toxicology.org

2688 Dose Response of Multiwalled Carbon Nanotube (MWCNT)-Induced Lung Tumors

S. Reynolds. *NIOSH, Morgantown, WV.* Sponsor: L. Sargent

Mitsui-7 MWCNTs (MWCNTs) are strong lung tumor promoters in B6C3F1 mice. B6C3F1 mouse lung tumors have many molecular and morphological similarities to human pulmonary tumors. In previous work, we demonstrated that exposure to inhaled MWCNTs following exposure to a DNA damaging agent caused potent promotion of lung tumors. To investigate a possible threshold for MWCNT-induced carcinogenesis, we exposed B6C3F1 mice to a single dose of either methylcholanthrene (MC, 10 µg/g BW, i.p.) or vehicle (corn oil). One week after i.p. injections, mice were exposed by inhalation to MWCNTs (5 mg/m3, 5 hours/day, 5 days/week) or filtered air (controls) for a total of 2, 5 or 10 days. At 17 months post-exposure, mice were euthanized and examined for lung tumor formation. Thirty six percent of the filtered air controls, 33% of the MWCNT- exposed, and 47% of the MC-exposed, had a mean of 0.33, 0.33 and 0.4 tumors per mouse, respectively. By contrast, 94% of mice receiving MC followed by 10 days MWCNT had an average of 2.9 tumors per mouse while 81% of mice exposed to MWCNTs for 5 days had an average of 1.9 tumors per mouse, and 73% of mice exposed to MWCNTs for 2 days had an average of 1.2 tumors per mouse. Additionally, mice exposed to MWCNTs or MC followed by MWCNTs had larger tumor volumes than their corresponding control groups. Preliminary data indicate a dose response in the percent of animals with tumors as well as the number of tumors per animal following exposure to MC and MWCNTs. In this study, mouse MWCNT lung burden approximates feasible human occupational exposures. Therefore, the results of this ongoing study indicate that caution should be used to limit human exposures to MWCNTs.

2689 Label-Free Quantitation of Proteomic Responses Triggered by Rutile Titanium Dioxide Nanoparticles

S. Bitragunta, and S. Palani. *Birla Institute of Technology and Science Pilani, Hyderabad Campus, Hyderabad, India.*

Proteomics can serve as a promising tool in establishing gene, protein or metabolite changes which indicate perturbations that are caused by a foreign body (compound or chemical) on the organisms. However, considerable information on protein profiling using proteomic approaches in earthworms in response to TiO_2 nanoparticles is scanty when compared to vertebrate sentinels. In light of this scenario, invertebrate based proteomic approach is followed in the present study to identify proteins that portray toxicological effects of rutile titanium dioxide nanoparticles (r-TiO_2-ENP). Earthworms were exposed to r-TiO_2-ENP via skin contact as per OECD-207 guidelines. Label free quantitation technique was employed to demonstrate specific signatures of the proteins in *Eisenia fetida* exposed to LC_{50} (0.15 mg/cm^2) and LC_{10} (0.05 mg/cm^2) of r-TiO_2-ENP via skin contact. Relative quantitation of the non-conflicting peptides was carried out to identify the proteins. Sequence coverage of the peptides and proteins was assessed through MASCOT analysis that matched 100% with databases against *Eisenia fetida*. Fold change in the proteins is presented in terms of average normalized abundancies. From the results, it was observed that 22 proteins that were identified through label free quantitation, met the requirements of 1 fold change (either decrease or increase) greater than 1 ($p<0.05$). It was observed that 16 proteins were upregulated, whereas 6 proteins were down regulated when compared to their respective controls. This study underlines the role of various proteins that affect stress-responsive system and behavior upon exposure to r-TiO_2-ENP in earthworms. This is true with regard to upregulation of (i) heat shock protein 70 (ii) beta-adrenergic receptor kinase 1-4 (iii) valosin containing protein-2 (iv) pyruvate carboxylase (v) HSP60 (vi) lombricine kinase (vii) cytochrome c oxidase subunit II and (viii) protein kinase C1 which indicate the significance of mitochondria when metabolism gets disturbed. Besides these proteins, this study also identified down regulation of metallothionein, valosin-containing protein, precursor protein EEP-2 and cyclophilin-A that assist in paraphrasing toxicity assessment of r-TiO_2-ENP. Outcomes of the study also assist in better understanding of the r-TiO_2-ENP toxicity and concomitant survival mechanisms in earthworms.

2690 Biochemical and Histopathological Evaluation of Graphene Oxide in Sprague-Dawley Rats

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Graphene Oxide (GO) due to its unique physico-chemical properties is a promising candidate for biomedical applications. However, the available reports on potential toxicity of GO are limited. The aim of the study was to determine the hepatotoxic and oxidative stress potential of GO in male Sprague-Dawleys rats. Five male rats per group were orally administered GO, once a day for 5 days with doses of 10, 20 and 40 mg/Kg GO respectively. Deionized water was used as a control. Twenty four hours after the last treatment blood and liver were collected following standard procedures. Exposure to GO was shown to enhance the induction of reactive oxygen species (ROS), activities of certain liver enzymes (alanine (ALT), aspartate (AST) aminotransferases, alkaline phosphatases (ALP), lipid hydro peroxide (LHP) concentration and damage to liver tissue compared to control. Statistically significant ($p<0.05$) increases in the above mentioned results were evident in the highest two doses 20 mg/Kg and 40 mg/Kg GO respectively. Aspartate aminotransferases (AST) activity showed no effect on GO exposure. Our results indicate that hepatotoxicity induced by GO might be mediated through the mechanism of oxidative stress. Although it is most likely that this impairment in hepatotoxicity biomarkers is associated with GO toxicity, further experiments are needed to elucidate the biochemical mechanisms involved.

2691 Adjuvant Effects of Redox-Modified Cerium Oxide Nanomaterials to Promote Airway Sensitization in BALB/c Mice

J. G. Wagner[1], S. Dekkers[2], E. Eldridge[1], R. J. Vanderbriel[2], J. R. Harkema[1], and F. Cassee[2]. [1]*Michigan State University, East Lansing, MI; and* [2]*RIVM, Utrecht, Netherlands.*

Inhalation of ambient ultrafine particles and engineered nanomaterials are associated with adverse airway responses, including allergic asthma. Generation of reactive oxygen species by inhaled nanoparticles (NPs) may activate immunological adjuvant pathways and enhance local sensitization to environmental and occupational allergens. To test the hypothesis that NP redox status is associated with adjuvant activity, we modified the redox activity of cerium dioxide (CeO2) NPS by incorporating increasing quantities of zirconium (Zr) into the crystalline structure of the CeO_2 NPs. Female BALB/c mice were intranasally sensitized with ovalbumin (OVA) or OVA + 200 µg CeO_2 (doped with 0%, 27% or 78% Zr) on days 1, 3, 6, and 8, and then challenged with OVA alone on days 22 and 23. Twenty-four hours later serum was collected to assess OVA-specific IgE and IgG1, bronchoalveolar lavage fluid (BALF) collected for quantitation of inflammatory cells, and lungs processed for detection and morphometric evaluation of eosinophils and intraepithelial mucosubstances (IM). OVA-sensitized and -challenged mice had minimal increases in serum IgE or IgG1 compared to non-sensitized mice (PBS control). However, marked increases in IgE and IgG1 were observed after co-sensitization with CeO_2 (doped with 0%, 27% and 78% Zr) compared to OVA alone. OVA-sensitized and -challenged mice had increased BALF macrophages and eosinophils compared to controls. Sensitization with OVA + CeO_2 (0% Zr) enhanced BALF total cells (2.3-fold increase), macrophages (2.8-fold), eosinophils (15-fold), and lymphocytes (3.8-fold) compared to OVA alone. Doping of CeO_2 with 78%, but not 27% Zr further enhanced BALF total cells, macrophages, and eosinophils by 50-100% compared to CeO_2 without Zr doping. Allergic inflammatory, epithelial, and mucous cell responses were minimal in lung tissues of OVA-sensitized and challenged mice, but marked eosinophilic alveolitis and bronchiolitis, and a modest increase in IM were induced by co-sensitization with OVA and all zirconium-doped CeO_2 NPs. Mice sensitized with CeO_2 (78%Zr) had more parenchymal eosinophils than CeO_2 (0%Zr), but mucous and inflammatory cell responses were similar for all CeO_2 NPs. Our results suggest that CeO_2 can act as potent airway adjuvant for allergic sensitization, and that the redox activity of engineered NPs can affect the character and severity of allergic airway responses.

Recent Advances in Toxicology of Gold Nanoparticles

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Contents

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C. M. Hussain (ed.), *Handbook of Environmental Materials Management*,
https://doi.org/10.1007/978-3-319-58538-3_59-1

Abstract

Application of engineered nanoparticles (ENPs) in biomedical and industrial applications is gaining prominence across the globe. The unique properties of ENPs, such as size, shape, charge, etc., facilitate their specific cellular and subcellular interactions. Among the emerging ENPs, the exceptional characteristics of gold nanoparticles (Au-NPs) including biocompatibility, facile synthesis, and optical properties make them ideal candidate particles for biocatalysis, imaging, and drug delivery. However, widespread use of Au-NPs may result in adverse impacts on environment and health. Thus, toxicity assessment starting from their manufacture to end of the life cycle is an important requisite for risk assessment of Au-NPs. Moreover, understanding the cellular interactions of Au-NPs assists in assessing biochemical effects and establishing methods of toxicity assessment. Therefore, this chapter is aimed at providing an account on cellular interactions of Au-NPs with an emphasis on the role of protein corona in nanoparticle uptake by cells. The chapter highlights the importance of physicochemical properties in evaluating the toxicological profile of Au-NPs. Furthermore, the chapter focuses on effects of Au-NPs on different types of cells such as renal and dermal cells. Thus, main findings of the study will help in divulging cytotoxicity and associated biochemical mechanisms of Au-NPs.

Keywords

Gold nanoparticles · Physicochemical properties · Cytotoxicity · Protein corona

Introduction

Miniaturization of materials at nanoscale resulted in extensive growth of nanotechnology over the past two decades, thereby leading to widespread application of nanoparticles (NPs) in almost all sectors including cosmetics, therapeutics, and electronics. The size of the nanoparticles gives it a large surface area thereby rendering them more reactive than their bulk counterparts. Among the different NPs available in the market, gold nanoparticles (Au-NPs) are widely used because of their unique properties. The history of application of Au-NPs dates back to fourth century AD, followed by the use of these Au-NPs in medicinal preparation of *Swarna Bhasma* in seventh century AD (Paul and Sharma 2011) (Fig. 1). Use of Au-NPs in cancer therapy is increasing due to its ability to absorb near-infrared rays. It produces hyperthermic effect on tumor cells due to its enhanced permeability and retention effect (EPR) thereby accumulating more in tumor tissue than in normal tissue (Kumar et al. 2013; Wei 2008). Increased surface area of Au-NPs renders them as the best vehicle for delivery of large biomolecules into cells (Ghosh et al. 2008). Due to their unique optical property, they are also used in biosensors for detecting heavy metals in polluted water (Li et al. 2010; Darbha et al. 2008). They are also used in pollution control for carbon monoxide removal. Colloidal Au-NPs gives different optical properties depending on size; e.g., 25 nm is red in color, 50 nm is green in color, and 100 nm is orange in color. Hence the mode of interaction of

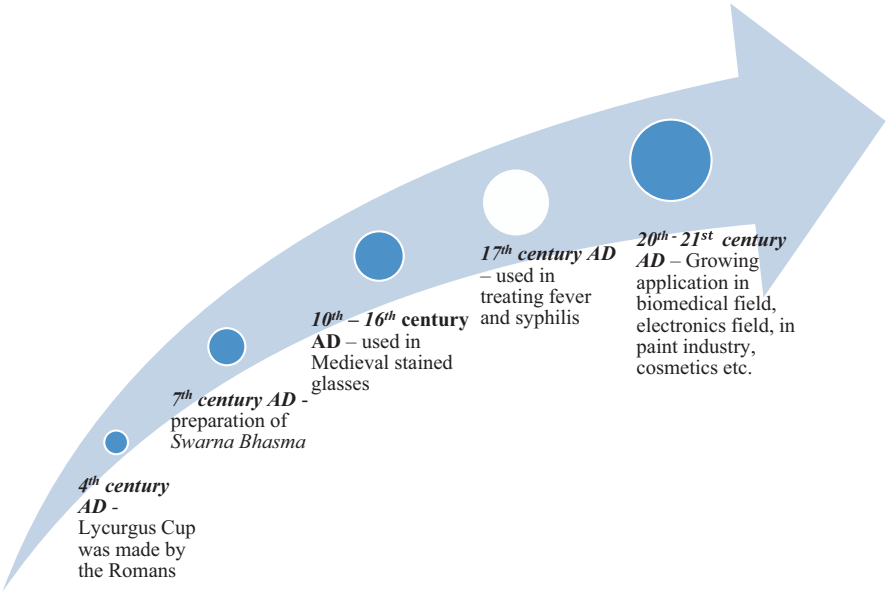

Fig. 1 The advent of the use of Au-NPs starting from fourth century AD to twenty-first century AD (Reference: Melo 2008; Paul and Sharma 2011; Wilson 2008)

these NPs with various components in the environment will also differ with size. To date, there are different consumer products that are incorporating Au-NPs (Wilson center database – http://www.nanotechproject.org/cpi/). As per the database, Au-NPs were not incorporated in consumer products in 2006; however by 2011 the use of Au-NPs in various commercial products gained momentum. As per the last updated version of the repository in the year 2013, around 19 consumer products that are employing Au-NPs are recorded. However there is no data regarding current status of the use of Au-NPs in consumer products, but the repository includes only those products that claim to use nanotechnology either by the manufacturer or by any third-party source. Thus, there would be many other products in the market with no description about the use of NPs. With increasing production of different-sized Au-NPs and their application, the concern about their impact on the environment and human health is gaining attention across the globe.

Though Au-NPs is the least disputed nanoparticle in terms of adverse impact on environment and health, the concern increases due to its increased reactivity at the nanoscale and thus the ability to interact with different components in the environment (Dwivedi and Ma 2013). Therefore, it is important to study how these NPs interact and enter the cells. In this regard, this chapter highlights the possible pathways/modes of Au-NPs interaction with cells and the way forward. Interesting questions such as how does the physiochemical property of the Au-NPs affect the mode of entry? and what are the consequences after Au-NPs enter the cell? are the starting point for the present work to shed light on recent advances in toxicology of Au-NPs.

Life Cycle of Nanoparticles

See Fig. 2.

Mode of Entry of Nanoparticles and their Physicochemical Properties

Understanding the mode of entry of nanoparticles is important to assess their toxicity (Chithrani and Chan 2007). They can enter the cell either by receptor-mediated endocytosis like clathrin-mediated endocytosis or caveolae-mediated endocytosis or phagocytosis (Shang et al. 2014). However, endocytosis is the major mode of entry into phagocytes and endothelial cells or in the presence of opsonins; otherwise, the NPs with size <100 nm enters the cell through pericellular space (Neuss-stein et al. 2007). NPs size, shape, surface charge, and surface chemistry determine their mode of entry into cells (Shang et al. 2014). To elucidate intercellular response of NPs, it is very important to understand their physiochemical properties (Walkey and Chan 2012) (Fig. 3).

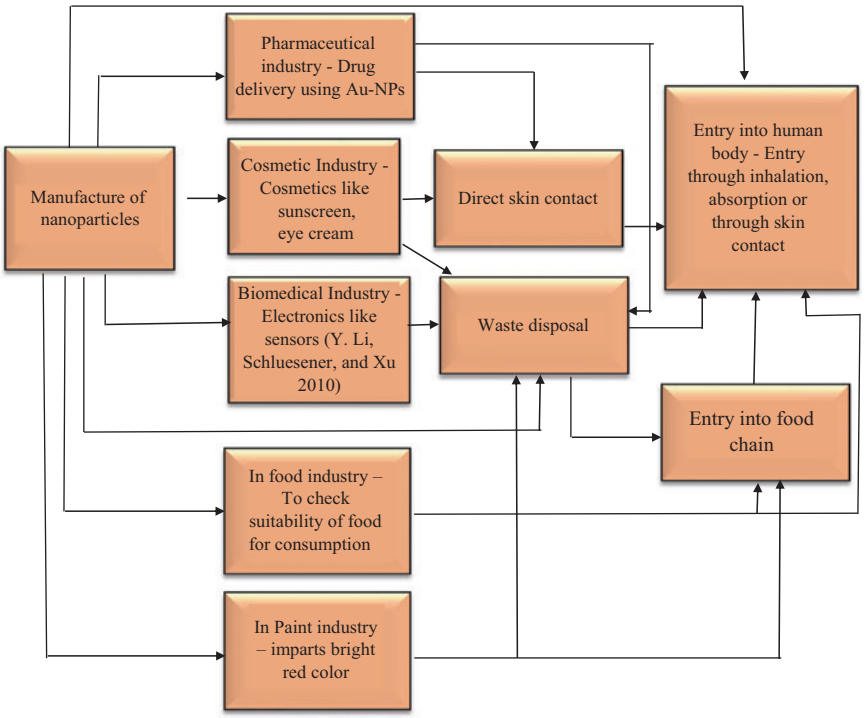

Fig. 2 Life cycle of nanoparticles from manufacture to their disposal

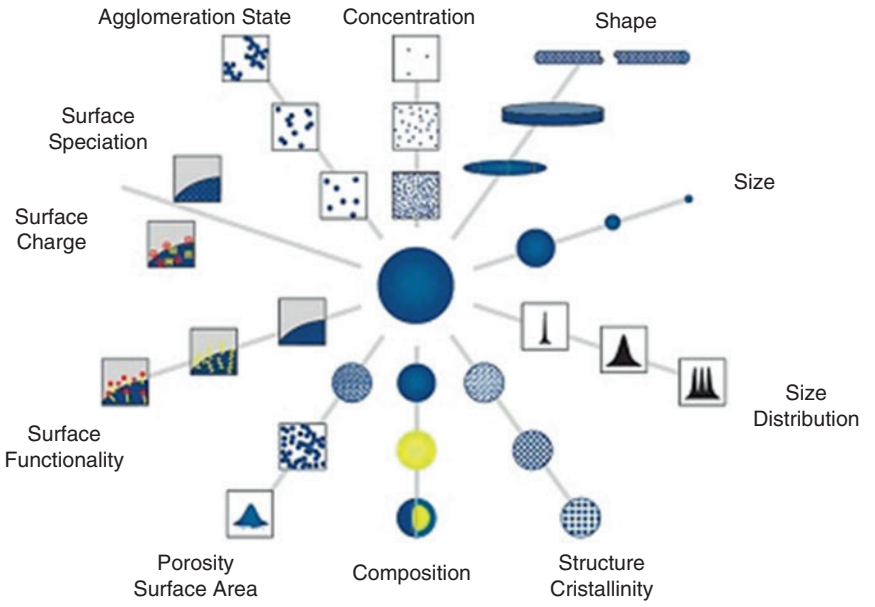

Fig. 3 Various physiochemical properties responsible for determining mode of entry of nanoparticles into the cells (Hassellöv and Kaegi 2009)

Size of Au-NPs

Size of NPs is very important to understand their agglomeration in a given environment or physiological conditions. Size plays a major role in determining their mode of entry into the cellular system and its interaction with biomolecular (e.g., proteins, DNA) and cellular (e.g., mitochondria and endoplasmic reticulum) components. Moreover, stabilizers being used to coat the NPs also play an important role during their cellular interactions. For instance, uptake of citric acid-stabilized Au-NPs (14, 30, 50 and 100 nm) by mammalian cells (HeLa cells) revealed that 50 nm Au-NPs was taken up more readily than 14 nm or 75 nm NPs resulting in a nonlinear relationship between size and uptake rate (Chithrani et al. 2006). This could be attributed to the fact that NPs with a size of 50 nm would facilitate the free energy availability upon binding with the receptor while entering cell, whereas 14 nm NPs would not. Clustering of 14 nm NPs would be required before the NPs can enter the cell, resulting in more time for their accumulation inside the cell. When comparing 50 nm and 74 nm NPs, the wrapping time required for large particles (74 nm) are more which will result in less uptake rate (Gao et al. 2005). Thus it can be concluded that the 50 nm Au-NPs can enter the cell more easily than particles with smaller or larger sizes; thus it is very important to study the toxic effect of Au-NPs with regard to size and stabilizing agents under a given condition.

Cells can take up Au-NPs (14–100 nm) by vesicle-mediated endocytosis (Chithrani et al. 2006). Receptor-mediated endocytosis through clathrin-coated

vesicle formation can be inhibited by pretreating HeLa cells with sucrose and potassium-depleted medium. Under such conditions, the concentration Au-NPs coated with transferrin (14 nm and 50 nm) in the cells decreased, indicating that Au-NPs coated with transferrin followed clathrin-mediated endocytosis. When the size of citrate/Au-NPs was <13 nm, the mode of entry was found to be phagocytosis (non-receptor-mediated endocytosis) (Mironava et al. 2010). However, this was found to be contradictory to the results obtained by Cheng et al. (2015) who observed that the uptake of 5 nm Au-NPs was through caveolin-mediated endocytosis by RAW 264.7 and Hep G2 cell lines. It was observed that 50 nm Au-NPs caveolin-mediated pathway was dominant in the case of Hep G2 cells, whereas scavenger receptor-mediated endocytosis was dominant in RAW 264.7 cells. However, in the presence of serum-containing media, clathrin-mediated endocytosis dominates as serum prevents the recognition of Au-NPs by scavenger receptors (Cheng et al. 2015). Jiang et al. (2015) also showed that the uptake rate of Au-NPs by HeLa cells, in the presence of serum-containing media, was less when compared to serum-free media. It was also suggested that the mode of entry for 2 nm Au-NPs coated with -COOH was taken up by dynamin-mediated endocytosis as opposed to the phagocytic uptake of Au-NPs <13 nm (Jiang et al. 2015). Analysis of the results indicates that the mode of uptake of nanoparticles is dependent not only on the size of NPs but also to the cell type and media composition. But it is evident that one key factor is size of NPs that is influenced by the media composition, and hence characterizing the size of NPs in exposure media is imperative. It will assist in underlining the mode of interaction of NPs with the cell in a given physiological environment.

Till date, studies about the fate of nanoparticle during cellular interactions showed that cells do not undergo exocytosis. As a result, Au-NPs can accumulate in the cell. Principal way for reduction reactions of Au-NPs in cells is cell division (Mironava et al. 2010). Though Au-NPs are considered as nontoxic to cells (Asharani et al. 2011; Villiers et al. 2010; Jiang et al. 2015), Au-NPs with sizes of 3, 8, and 30 nm were found to be cytotoxic, whereas 5, 6, 10, 17, and 45 nm particles showed no cytotoxicity (Kumar et al. 2013). Incubation of 30, 50, and 90 nm Au-NPs with human fibroblasts for 24, 48, and 72 h revealed that 30 nm particles have more potential to induce cytotoxicity than that of 50 nm and 90 nm particles (Mateo et al. 2015). Thus cytotoxicity of Au-NPs is majorly dependent on size. However, Au-NPs that have not exhibited immediate cytotoxicity may accumulate under chronic exposure conditions, thereby eliciting toxicity. Hence it is very important to study the chronic effects of nanoparticles in biological systems.

Charge on Au-NPs

The charge or zeta potential of Au-NPs is another crucial parameter that influences the interaction of NPs with the cell membrane and its entry into the cell. The charge of NPs is altered by various capping agents (e.g., citric acid). Capping of Au-NPs with critic acid imparts a negative charge to the Au-NPs. Hence NPs-binding efficiency of NPs with the cell surface decreases (Chithrani et al. 2006). Such reactions are very

important in receptor-mediated endocytosis of Au-NPs because they cause binding or adsorption of serum proteins present in the exposure media (DMEM) onto the nanoparticles (Chithrani and Chan 2007). Therefore, the charge of NPs has the potential to dictate the microenvironment towards the formation of protein corona. For instance, hydrodynamic size of Au-NPs increased on incubation with human plasma (Dobrovolskaia et al. 2009). However, the role of protein corona and its composition (albumin, apolipoprotein, immunoglobulin complement, and fibrinogen) on NPs is a further matter of research. Transferrin-coated Au-NPs is taken up by mammalian cells through transferrin-specific receptor (Chithrani and Chan 2007). However, uptake of transferrin-coated Au-NPs is less when compared to citric acid-stabilized Au-NPs because the latter interact with serum proteins and form a protein corona. Furthermore, transferrin-coated Au-NPs undergoes endocytosis through transferrin-specific receptors. A similar mechanism is envisaged in the case of NPs that is introduced into the bloodstream. Thus, the surface charge and net charge of Au-NPs in a given environmental condition play a major role in understanding their cellular entry. However capping of the particle would also be a synergistic factor that affects composition of the protein corona during the cellular interaction of NPs.

Entry of a positive [coated with thioalkyl tetra (ethylene glycol) and trimethyl ammonium (TTMA)], negative [coated with carboxylate (COOH) coated], and neutral Au-NPs (coated with zwitterion resulting in two types of particle – outermost positive charge and outermost negative charge) into HeLa cells is facilitated by zwitterions with outermost positive charge entering the cell more efficiently than the outermost negatively charged particle (Jiang et al. 2015). This can be attributed to the negative charge of the cell membrane thereby resulting in a repulsive force for the particle with outermost negative charge. It was also understood that the rate of uptake of positively charged particle increases with an increase in size, but a contrary trend is observed in the case of the negatively and neutrally charged particles (Jiang et al. 2015). An increase in the size of negatively charged particles leads to the increase in charge density thereby promoting repulsion of the particles with the cell membrane.

Shape of Au-NPs

Shape of the nanoparticle also plays a major role in cellular uptake of Au-NPs. In HeLa cells, the rate of uptake of citrate-stabilized rod-shaped Au-NPs is more than the spherical counterpart. The rod-shaped Au-NPs can interact with more than one receptor on the cell when compared to the spherical Au-NPs. The presence of cetyltrimethylammonium bromide (CTAB) (used during synthesis) on rod-shaped Au-NPs did not allow the replacement by citric acid. Hence impairment of protein corona formation affects the receptor binding (Chithrani et al. 2006). Au nanorods of size 2.1, 2.5, 3.0, 3.3, and 3.5 nm were found to be toxic due to the presence of CTAB, whereas the spherical Au-NPs (5–70 nm) were nontoxic to HaCaT keratinocytes (Wang et al. 2008). Stabilizing agents like CTAB with (polystyrene sulfonate) (PSS) which impart a negative charge to Au-NPs reduce the toxicity of Au-NPs (Wang et al. 2008). Cytotoxic effect of flower-shaped Au-NPs is greater than

that of spherical shape because of more internalization of flower-shaped Au-NPs in the cells. Therefore, the rough surface of flower-shaped Au-NPs enabled the higher probability of binding to the surface receptor of the cell hence cytotoxicity (Sultana et al. 2015). Thus, conclusions drawn from in vitro studies have to be carefully interpreted in terms of experimental conditions, cell lines, and exposure media which can affect the shape, size, and charge of Au-NPs to report the cytotoxicity.

Protein Corona and its Role in Internalization

Serum is used as one of the major components of the cell culture media. It contains many kinds of proteins. Hence these proteins such as IgG, albumin, fibrinogen, and apolipoprotein. These proteins can interact with NPs in biological fluids. Thus the adsorption of proteins onto the surface of NPs leads to the formation of protein corona. There are two types of corona – hard corona and soft corona. Proteins that adsorb with high affinity are called hard corona, and those with low affinity are called soft corona. Initially, the most abundant protein gets absorbed onto surface of the cell. Upon certain interval, it is replaced with protein with low affinity (Hunter et al. 1981). It is very important to note that the presence of protein corona affects the uptake of the nanoparticle by endocytosis (Cheng et al. 2015). In this context, serum decreases the uptake rate of Au-NPs. Inhibitory effect of serum on Au-NPs is very significant in the case of phagocytic cells than non-phagocytic cells. The protein corona inhibits the initial step of the endocytosis process, i.e., adhesion to the cell membrane, thereby preventing the uptake of NPs. Such inhibition process is dependent on the size of Au-NPs in the presence of serum media (Cheng et al. 2015). Depending on the type of cell culture medium and size of the NPs, uptake rate of proteins by a cell shows a linear relationship. Therefore, protein corona is another significant factor that affects the uptake of Au-NPs by cells.

Capping and Functionalization of Au-NPs

In order to increase the stability of NPs under dynamic environmental conditions, they are usually capped with agents like dihydrolipoic acid (DHLA), citric acid, polyvinylpyrrolidone **(**PVP), starch, gum arabic, etc. (Kumar and Ganesan 2011). Capping prevents adsorption of proteins and toxic effect of nanoparticles (Tournebize et al. 2012). PVP-capped Au-NPs (size range of 15–35 nm) were nontoxic to zebrafish embryo (Asharani et al. 2011). In this case, capping of Au-NPs would have resulted in less agglomeration (Kumar and Ganesan 2011). However, an exposure of colloidal form of Au-NPs at varied sizes (3, 10, 50, 100 nm) resulted in less than 3% mortality rate of the zebrafish embryo indicating that it was slightly toxic (Bar-Ilan et al. 2009). It was also shown that PEGylated nanoparticle induces less cytotoxicity when compared with the non-PEGylated ones which might be due to decreased internalization efficiency of the PEGylated particles by HUVEC cells. Because PEG coating shifts the surface charge close to zero, it is difficult to enter the cell (Sultana et al. 2015).

Functionalized Au-NPs were found to produce different biological responses depending on the functional group attached. Functionalization of Au-NPs (1.5 nm) with different ligands *N*,*N*,*N*-trimethylammoniumethanethiol (TMAT) [imparts positive charge], 2-mercaptoethanesulfonic acid (MES) [imparts negative charge], or 2-(2-(2-mercaptoethoxy) ethoxy) ethanol (MEEE) [imparts neutral charge] resulted in differential toxic effect. TMAT-Au-NPs (250 μg/ml) were found to be fatal to zebrafish embryo (120 h postfertilization); MES-Au-NPs were found to produce sublethal toxicity, whereas MEEE-Au-NPs did not induce any toxic response. The difference in biological effect is due to upregulation and downregulation of certain gene transcript related to inflammation and G-protein-coupled receptor signaling (Wnt signaling and NOTCH signaling), respectively, and not dependent on the uptake rate (Truong et al. 2012).These signaling pathways are important in cell proliferation in a developing embryo. NPs are able to pass the human placenta and hence would affect the embryo development. However, only small NPs of size 14 nm and 18 nm can pass through the placenta, whereas large particles cannot. It can also be concluded that the entry of particle through the placenta is largely size dependent and smaller particles have more probability for entry through the placenta (Semmler-Behnke et al. 2014). Polystyrene beads with a diameter of up to 500 nm were taken up through the mouse fetal placenta, and NPs of size 20 nm (200 μg/ml) and 40 nm (500 μg/ml) would induce trophoblast cell apoptosis (Huang et al. 2015). However, these results are not straight to understand Au-NPs toxicity because of the difference in terms of surface properties of polystyrene beads with Au-NPs. However, insights from the study can serve as cues for designing further studies with regard to the size-dependent passage of Au-NPs via placenta and associated mechanisms.

Cytotoxicity of Au-NPs

Au-NPs is considered as not cytotoxic (Asharani et al. 2011; Villiers et al. 2010; Jiang et al. 2015) but can result in the impairment of cellular functions at sublethal doses. In a long run, it is expected that sublethal responses will lead to adverse effects on living tissues/cells (Pernodet et al. 2006). Though many papers have reported that NPs to be nontoxic, the toxicity of Au-NPs is dependent on the cell type (Mironava et al. 2010). It has also been reported that Au-NPs increases the susceptibility of cell to death (Leite et al. 2015).

Effect of Au-NPs on Skin Cells

As the use of Au-NPs is becoming prevalent in cosmetics and personal care products, the mode of entry of these NPs into the human system is through dermal tissue. Hence it is very important to study their effect on skin cells. The stratum corneum of the epidermis and the tight junctions provide an excellent barrier to entry of foreign particles. However due to the unique property of NPs, and depending on the dosage of NPs, they are found to accumulate in the liver and

spleen (Smijs and Bouwstra 2010). Tissue distribution after skin adsorption mostly happens through blood circulation (Doudi and Setorki 2014). Under physiological conditions using Franz cell, Au-NPs (12.6 ± 0.9 nm) at a concentration of 100 mg/l can permeate through the human abdominal (full-thickness) skin. After 24 h of incubation, it was observed that higher the number of Au-NPs, higher the permeation. This would mean that higher the availability of Au-NPs, the higher the rate of permeation of these NPs through the skin into the biological fluid by systemic diffusion (Larese Filon et al. 2011). Citrate/Au-NPs (13.1 ± 1.4 nm) changes the morphology of human dermal fibroblasts after 6 days of incubation. Increase of concentration (0.1–0.6 mg/l) leads to a decrease in cell adhesion thereby decrease in cell number. It indicates that Au-NPs altered the actin structure which impaired cell division (Pernodet et al. 2006). Change in cytoskeletal elements/structures would have resulted due to the change in ECM composition, high fibronectin, and low collagen. Au-NPs (13 nm and 45 nm) caused apoptosis in dermal fibroblast cells (CF-31) indicating their harmful effect (Mironava et al. 2010). Au-NPs (30, 50, and 90 nm) at 1–25 μg/ml were found to be slightly cytotoxic when incubated at 24, 48, and 72 h. However, the cytotoxic effect of 30 nm was more than that of 50 nm and 90 nm particles. There was also a decrease of cellular metabolic activity in this context. Au-NPs (50 nm) generated reactive oxygen species (ROS) immediately after 1 h of exposure/incubation and reached maximum after 3 h when compared to 30 nm and 90 nm particle at IC50 (17.9, 18.0, and 19.3 mg/ml for 30, 50, and 90 nm, respectively) (Mateo et al. 2015). Thus, Au-NPs are toxic to skin fibroblasts as they can interact with the extracellular matrix proteins of the dermis thereby affecting the cell adhesion and cell proliferation and ultimately leading to their cytotoxic effect. It is interesting to note that particles with small diameters are found to have more adverse effects than larger particles.

Effect of Au-NPs on Hepatocytes

Au-NPs get accumulated in the liver after intravenous administration (Sonavane et al. 2008; Cho et al. 2009; Balasubramanian et al. 2010). Hence it is very important to study the effect of Au-NPs on hepatocytes. PEGylated Au-NPs did not induce any cytotoxic effect on HepG2 on short-time incubation, but, on prolonged incubation, there was a decrease in cell viability and also an increase in ROS indicating their potential to cause oxidative stress. It is fascinating to note that there was an increase in antioxidant enzymes in a short period of time (2 h) which might have kept the ROS levels down, but upon further incubation, there was no increase in the antioxidant enzyme which disturbed the ROS homeostasis leading to increased oxidative stress (Thakor et al. 2011). Au-NPs stabilized with polyamidoamine (7.2 ± 2.7 nm) and Au-NPs stabilized with citrate (7.3 ± 1.2 nm) showed a genotoxic effect in addition to cytotoxic effect and oxidative stress (Maria et al. 2012). Au-NPs did not cause any cytotoxic effect in Au-NPs on HepG2 cell line after incubating for 36 h (Patra et al. 2007). Hence a functional group of capping agent is another remarkable feature in elucidating the Au-NPs.

Effect of Au-NPs on Renal Cells

Elimination of NPs from the body happens mostly through renal cells. Hence study of the interaction of Au-NPs with renal cells is critical to understand their biological phenomena of expulsion. Renal sediments that were incubated with Au-NPs for 15 min penetrated the renal cells (Sereemaspun 2008). Therefore, Au-NPs have the potential to enter the cells. Gold nanorod with average size of 10–40 nm impedes cell growth of PK-15 (porcine kidney) cell line at a concentration of 360 ng/ml and 720 ng/ml, whereas, in Vero (African green monkey kidney) cell line, cell growth was inhibited at concentration $\geq$ 180 ng/ml. Cell viability also decreased at a concentration of 180 ng/ml and 720 ng/ml in Vero and PK-15 cell lines, respectively. Thus, the effect of Au-NPs depend on the sensitivity of each cell line and not on their intercellular accumulation. Apart from inhibition of cell growth and a decrease in cell viability, Au-NPs also induced apoptosis in Vero cell line (Chueh et al. 2014). Au-NPs (15 nm) did not produce any cytotoxic effect; however, the decrease in size ($<$5 nm) of Au-NPs that are being used in laser irradiation can cause cytotoxicity (Abdelhamid et al. 2012). This sheds light on the risk of using Au-NPs in laser-induced hyperthermia for cancer treatment.

Effect of Au-NPs on Intestinal Cells

Inhaled nanoparticles can enter the bloodstream through the respiratory and gastro-intestinal layer. Hence it is important to study its effects on intestinal cells before it can enter the bloodstream (Kumar et al. 2013). Au-NPs (prepared using electrolysis method) with slight negative charge (average size of 5–15 nm) applied to human intestinal epithelial cells (INT-407) at a concentration of 13 μg/ml did elicit cytotoxic effect. However, chronic exposure of Au-NPs showed suppression of the colony forming efficiency (Jo et al. 2015). Adsorption, accumulation, and toxicity of Au-NPs (100 nm, 50 nm, 15 nm) under in vitro conditions, in intestinal epithelial cells (CaCO2), showed that smaller particle size enables Au-NPs to penetrate and accumulate (in terms of particle number) in the cells. It was seen that some of the nanoparticles that entered the nucleus caused the genotoxicity. 50 nm NPs were accumulated at a higher concentration than NPs of 15 nm and 100 nm. In this context, 50 nm would have provided enough binding energy between the ligand and the receptor and enough free energy for particle envelopment. When the particle is too small, then it does not provide enough free energy for envelopment. Furthermore, the particle has enough free receptors to provide the ligand-receptor binding energy (Yao et al. 2015).

Effect of Au-NPs on Endothelial Cells

Intravenous mode of drug administration is a common practice. When Au-NPs are used as drug delivery agents, studying their effect on vascular endothelial cells is

crucial for tracing their transport to the target tissue in the body. Under dynamic conditions (flow rate – 5 μl/min), agglomeration and accumulation of Au-NPs will be less and further leads to decreased cytotoxic effect at high concentration. No significant difference in effect was observed under low NPs concentrations. At a high concentration (10^{12} NPs/ml), the cell viability was found to be ~ 70% under static flow and 90% under dynamic flow. This experimental condition can be considered as more realistic mean of assessing the effect of NPs under in vitro conditions by taking into account the complexity of the circulatory system (Fede et al. 2014). Au-NPs exposure did not result in cell cytotoxicity but induced oxidative stress (Klingberg et al. 2015). Earlier studies on assessing the cytotoxicity of Au-NPs on human endothelial cell lines (HDMEC, human dermal microvascular endothelial cells; HCMEC, human cerebral microvascular endothelial cells) have shown marked decrease in cell viability on Au-NPs treatment. They have also shown that the cytotoxic effect increases with increase in concentration and surface modification using sodium citrate (Freese et al. 2012b). However it is intriguing to note that the marked decrease in viability is observed only at a concentration above 500 μM. Similar kind of results were reported elsewhere which showed no cytotoxicity of Au-NPs in HDMEC cells (Freese et al. 2012a; Bartczak et al. 2012). Thus we can conclude that concentration of Au-NPs is critical for understanding their effect on endothelial cells irrespective of the capping agent.

Effect of Au-NPs on Skeletal Muscle Cells

PEG-Au-NPs (4.5 nm) did not cause any cytotoxic effect on skeletal muscle cells at a concentration up to 50 μg/ml. However, there was an increase in ATP level leading to reduced phosphorylation of the protein in Akt signaling pathway (Leite et al. 2015).

Effect of Au-NPs on Fibroblast Cells

Studying the influence of Au-NPs on fibroblast cells will assist in delineating their role in the repair of damaged tissue. Such studies revealed that citrate-stabilized Au-NPs (5 nm) at a concentration of 2, 10, 20, 39.2, and 58.8 μg/ml in fibroblast cell line (Balb/3 T3) decrease colony forming efficiency. But did not affect the cell viability. Transmission electron microscopy showed internalization of NPs by non-receptor-mediated endocytosis. Au-NPs can disrupt F-actin fibers resulting in the change of cell shape and thereby affecting the cell survival. However, this cannot be an affirmative result as immortalized cell lines are different from normal cell lines. The rate of apoptosis is concentration dependent and increases with increase in Au-NPs concentration when 13 nm and 45 nm Au-NPs were tested on primary human dermal fibroblasts (Coradeghini et al. 2013). Au-NPs (45 nm) disrupt actin fibers by interfering with the signaling pathway required for actin fiber assembly (Mironava et al. 2010). Despite the disruption of actin filament, it was also shown that Au-NPs of size 20 nm (pretreated with FBS to mimic real environment) induce

oxidative stress in MRC-5 lung fibroblast cell line leading to upregulation of autophagy genes (ATG 7 and MAP-LC3-II) (autophagy is a type 2 programmed cell death and cell survival mechanism during stress) that are needed to combat the oxidative stress (Li et al. 2010). However, there was no increase in nonviable cells at a concentration of 0.1, 0.5, and 1 nM, incubated for 21, 48, and 72 h, but the number of cells decreased when incubated with 1 nM Au-NPs for 72 h. It indicates that there is a decrease in cell proliferation. It was further confirmed by the downregulation of certain cell cycle genes (Li et al. 2008). The lethal dose of Au-NPs differ with their shape and surface chemistry i.e., gold nanosphere (61.46 nm) was found to be lethal at a concentration of 40 μg/ml, whereas the lethal concentration of gold nanostar (33.69 nm) was found to be 400 μg/ml (Favi et al. 2015).

Genotoxicity

Binding of functionalized Au-NPs to DNA changes the conformation of the DNA that is incompatible for ethidium bromide (EtBr) binding. This change in conformation might result in inhibition of transcription by occluding RNA polymerase binding (Goodman et al. 2006). Au_{55} clusters of size approximately 1 nm can enter the nucleus and bind to the DNA irreversibly (Tsoli et al. 2005). This proves that Au-NPs can cause genotoxicity. Au-NPs (20 nm) induced oxidative stress in MRC-5 cell line at a concentration of 0.5 nM and 1 nM in terms of 8-hydroxydeoxyguanosine (8-OHdG) as a marker. There was an increase in expression of DNA repair gene (BRCA2) to combat with the DNA damage induced by Au nanorods (Chueh et al. 2014). However further incubation with Au-NPs for 72 h resulted in downregulation of DNA repair gene (BRCA2) leading to impairment of DNA repair mechanism (Li et al. 2008)). Though Au-NPs has been used in the biomedical application, there is still a lack of complete knowledge on its toxic effect. Besides the above mentioned effects, Au-NPs conjugated with 2-mercapto-1-methylimidazole having an average diameter of 5 nm was shown to affect the dimethylation level of histone protein H3 which was evident from the western blot. There was a decrease in methylation level at lysine 4 residue but an increase in lysine 9 residue of H3 causing epigenetic modification after 1 h of exposure. This change in methylation level would change the expression of genes (Polverino et al. 2014). Hence further emphasis and extensive research are required to elucidate the effect of Au-NPs on histone modification, and related the changes during gene expression.

Conclusion

The mode of entry of nanoparticles is largely dependent on their physiochemical properties such as size, charge, and functionalization of a capping agent. Au-NPs have the potential to induce toxicity at sublethal concentration. However, integrated approaches to ascertain the toxicity of Au-NPs are the need of the hour to advance

the current application of Au-NPs. Besides *in vitro* studies, there is a need for the development of *in vivo* models that assist in understanding cytotoxicity of Au-NPs. There is a growing need to standardize protocols for testing the toxic effect of nanoparticles. In the way forward, emphasis should also be given to the environmental impacts caused by a specific type of Au-NPs and its combination with other NPs to divulge the possible implications. Such studies will help in comprehending the safety promotion of nanotechnology.

Cross-References

► Nanoscale Materials Toxicity on Living Organisms and Environment

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The Toxicologist

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UNIVERSITY PRESS

ISSN 1096-6080
Volume 150, Issue 1
March 2016

www.toxsci.oxfordjournals.org

The Official Journal of the Society of Toxicology

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1501 Integrated 'Omics' of Coelomic Fluid: An Attempt to Understand Phenomics of TiO2 Nanoparticle Toxicity

S. P. Bitragunta and S. G. Palani. *Department of Biological Sciences, Birla Institute of Technology and Science Pilani, Hyderabad Campus, Hyderabad, India.*

Background: Toxicological evaluation of TiO2 nanoparticles (TNP) has gained prominence across the globe to promote their environmental and health safety. Despite progress in assessing ecotoxicity of (TNP), mechanistic aspects of the same are unclear. Moreover, standard methods of toxicity testing in sentinel organisms, dose-response metrics in ecotoxicology studies are inadequate to substantiate TNP exposure in the environment. In this context, high-throughput methods which can facilitate ecotoxicological evaluation of nanoparticle are inevitable in environmental monitoring studies. Methods: Physicochemical characterization of TNP and integrated 'omics' approach that incorporate proteomics and metabolomics were used to divulge toxic effects of TNP on earthworm, *Eisenia foetida*. Earthworms were exposed to TNP of concentration from 0.05 to 0.25 mg/ml as per OECD-207 guidelines. Characterization and quantification of TNP were carried out by dynamic light scattering and ICPMS respectively. LCMS-based proteomics and metabolomics approach was used to screen biomarkers related to amino acid metabolism and oxidative stress in coelomic fluid of earthworms exposed to TNP. Results: It was found that pH of the dispersion medium influenced the agglomeration behavior of TNP. Multivariate statistical analysis of 'omics' data revealed that TNP could alter the levels of amino acids including L-alanine, L-aspartate, L-methionine, S-adenosylmethionine and L-lysine thereby affecting oxidative stress in the earthworm. TNP exposure led to the differential expression of heat shock proteins and metallothioneins in coelomic fluid. Conclusions: Results substantiated the importance of coelomic fluid as a complimentary biological medium that can help in delineating TNP toxicity. TNP could trigger sub-lethal effects in earthworm by altering the levels of amino acids and proteins thereby metabolic pathways. Integrating 'omics' assisted in identifying the plethora of metabolites that can significantly fluctuate during earthworm exposure to TNP. Outcomes of the study will definitely assist in paraphrasing subtle responses that are vital in understanding the phenomics of earthworm exposed to nanoparticles.

1502 Cholesterol-Induced Phenotypic Alterations in Cellular Response to Nanoparticles

J. H. Shannahan, V. C. Minarchick and J. M. Brown. *Department of Pharmaceutical Sciences, University of Colorado, Aurora, CO.*

Nanoparticles (NPs) are being synthesized for biomedical applications, which require their direct introduction into the circulatory system. A significant and growing portion of the population exists with high circulating levels of low-density lipoprotein (LDL). This physiological environment high in LDL is known to induce phenotypic changes in macrophages and endothelial cells allowing for disease progression. These alterations include increased surface expression of scavenger receptors (SRs) that facilitate both the uptake of LDL and NPs. Therefore we hypothesize that the presence of LDL will result in macrophage and endothelial cell phenotypic alterations resulting in altered cellular-NP interactions and toxicity. Macrophages (RAW264.7) and rat aortic endothelial cells (RAEC) were grown in either media without LDL present or with LDL at 25mg/dl. Alterations in cell surface expression of SR-BI, SR-AI, and MARCO were assessed by flow cytometry. The presence of LDL resulted in increased expression of all SRs evaluated, of which SR-BI was the most induced. Cells grown in these conditions were exposed to increasing concentrations (0, 6.25, 12.5, 25, or 50µg/ml) of 20nm silver NPs (AgNPs), 100nm AgNPs, or 20nm Fe3O4 NPs and no differences in cytotoxicity were determined through 3h. Following a 2h exposure to NPs at 50µg/ml, cells grown in LDL-rich media were found to internalize fewer NPs compared to cells grown in the absence of LDL. Lastly, cell activation was analyzed following a 1h exposure to NPs at 50µg/ml. Cells grown in LDL-rich media, that overexpressed SRs, induced a greater inflammatory response as measured by IL-6 mRNA expression following NP exposure. General inhibition of SRs by dextran sulfate resulted in decreased NP uptake in both environments as well as a reduction in the inflammatory response of cells in the LDL-rich environment. These findings demonstrate the possible impact of disease-related cellular phenotypic alterations on NP toxicity thus influencing their utility for biomedical applications. This work was supported by NIEHS K99/R00 ES024392.

1503 Topical Skin Treatment with Nanoparticles Modulates the Contact Hypersensitivity Response in a Murine Model

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Nanoparticle engineering is a growing field due to the vast array of applications. For example, titanium dioxide and zinc oxide nanoparticles are included in a number of sunscreens for their ultraviolet light filtering properties, and silica based nanoparticles and carbon nanotubes are being researched as potential drug delivery vehicles through skin. There is a need to test the dermal toxicity of these nanoparticles, and since skin is exposed to a number of potential allergens we find it equally important to examine the effect of nanoparticle application on immune system activation after allergen exposure. Here we examine the Type IV immune response in the contact hypersensitivity (CHS) model after sensitization and challenge with dinitrofluorobenzene (DNFB). We have found that unmodified 20 nm silica nanospheres reduce the swelling response in the contact hypersensitivity model. The swelling is only reduced when the silica nanospheres applied up to one hour before or one hour after the chemical hapten challenge, and the response appears to be related to the size and surface charge of the nanoparticles. Importantly, we have also identified a reduced swelling response when silica nanoparticles are co-administered along with 2-deoxyurushiol, another skin irritant that resembles the chemical structure of the allergens in poison-ivy. In search of a potential mechanism, we have found both reduced inflammatory cytokine (IL-6, IL-1β, KC, MIP-2) levels and reduced mast cell degranulation when 20 nm silica nanospheres are administered along with DNFB in the challenge phase. Interestingly, we have found opposite trends in skin swelling and inflammation when carboxylated multi-walled carbon nanotubes (30 nm diameter, 5-20 µm length) are administered along with DNFB in the challenge phase. Future work will include continued research into mechanisms of action and exploration of nanoparticles as a potential preventative treatment for contact dermatitis, but will also focus on carbon nanotubes since they exacerbate CHS responses, and represent an interesting toxicological concern due to their increased use in biomedical science.

1504 A Computational Framework for Interspecies Pharmacokinetics, Exposure and Toxicity Assessment of Gold Nanoparticles

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Interspecies and *in vitro* to *in vivo* extrapolation of pharmacokinetic and toxicology data is crucial for successful translation from laboratory studies to humans and for proper risk assessment of nanomaterials. This manuscript reports a comprehensive computational framework built on physiologically based pharmacokinetic (PBPK) modeling that successfully simulated tissue distribution of gold nanoparticles (AuNP) across a wide dose range and several species, including mice, rats, pigs, and humans. This framework was evaluated with multiple independent datasets, demonstrating excellent predictive capability. Cross-species extrapolation evaluation was done from mice, rats, and pigs, respectively, to humans. The simulation results suggest that rats and pigs seem to be more appropriate models than mice in species extrapolation of AuNP pharmacokinetics and that the dose and age should be considered in this extrapolation. These results partially explain the current low translation rate of nanotechnology-based drug delivery systems from mice to humans. Also, it provides a scientific basis for researchers to select the most appropriate animal model and dosing paradigm for conducting future nanomaterial studies to increase the research relevance to humans. This framework showed that by incorporating *in vitro* and/or *in vivo* cellular uptake and toxicity data into PBPK models, risk assessment and interspecies extrapolations can be improved. This simulation approach may be applied to other types of nanomaterials and provide guidance to the design of future translational, pharmacokinetic, and toxicological studies. (Supported by the Kansas Bioscience Authority)

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5E. GLOBAL PERSPECTIVES IN TOXICOLOGICAL RISK ASSESSMENT OF NANOPARTICLES IN ENVIRONMENT

5E.1

ASSESSING SUB-LETHAL EFFECTS OF GOLD NANOPARTICLES ON OXIDATIVE STRESS AND NEUROTOXICITY INDUCTION IN EARTHWORM, EISENIA FETIDA

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In recent times, miniaturization of materials at the nanoscale is gaining prominence across the globe. As a result, production and application of nanoparticles in various commercial and industrial processes are paramount. Gold nanoparticles (Au-NPs) are among the most widely used nanoparticles in various applications including the health and cosmetics sectors. However studies on ecotoxicity assessment of pristine or altered forms of Au-NPs are inadequate. Besides, nanotoxicology studies that integrate physicochemical characterization and ecotoxicity assessment of Au-NP are not well documented. Therefore, the present study is aimed at characterization and ecotoxicity assessment of Au-NPs. Size and charge of Au-NPs in biologically relevant media were determined using dynamic light scattering technique. Subsequently, toxicity potential of Au-NPs (50 nm) in the earthworm, Eisenia fetida was ascertained at concentrations ranging from 2.5 to 40 µg/ml using OECD-207 guidelines. Results indicated that Au-NPs did not agglomerate in biological buffers at pH ranging from 3 to 8. Earthworms that were exposed to Au-NPs through direct contact and soil at concentrations ≤ 40 µg/ml showed no lethal effects. However, exposure of earthworm to Au-NPs ≤ 40 µg/ml led to the inhibition of antioxidant enzymes viz. superoxide dismutase, catalase and glutathione reductase. Elevation of lipid peroxidation was also observed in these earthworms. Furthermore, Au-NPs inhibited acetylcholinesterase activity in earthworms. Hence, it is concluded that even sub-lethal concentrations of Au-NPs have the potential to induce oxidative stress and inhibition of cholinesterase function in earthworms. Thus, outcomes of this study will aid in assessing the behavior and ecotoxicity of Au-NPs, thereby paraphrasing benchmarking methods to evaluate ecotoxicity of Au-NPs.

Keywords: 1) Gold nanoparticle, 2) physico-chemical characterization, 3) ecotoxicity, 4) earthworm, 5) antioxidant enzyme, 6) acetylcholinesterase

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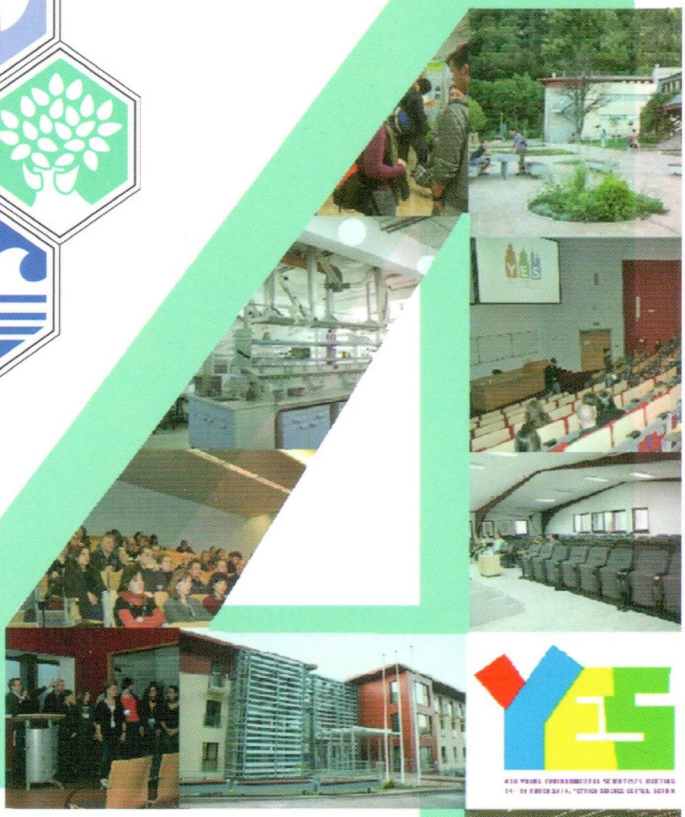

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Effect of TiO_2 nanoparticles on trace element homeostasis: An inquiry into metallomics in earthworm, *Eisenia foetida*

S. Bitragunta, Birla Institute of Technology & Science Pilani, Hyderabad Campus / Biological Sciences; S. Palani, Birla Institute of Technology & Science, Hyderabad Campus / Biological Sciences

Widespread use of titanium dioxide (TiO_2) nanoparticles in personal care products and biomedical applications inevitably increase their release into environment. In this regard, toxicological assessment of TiO_2 nanoparticles to determine their fate, transport, and effects on environment gained prominence in nanoecotoxicology. However, recent studies were largely focused on biological responses such as oxidative stress, inflammation and necrosis in sentinel species exposed to nanoparticles. Despite current progress in correlating properties of nanomaterials with their toxicity, little is known about bioaccumulation of TiO_2 nanoparticles and subsequent effects on trace element homeostasis in ecological indicator species. Therefore, current study was intended to delineate the influence of bioaccumulation of TiO_2 nanoparticles on concentration of electrolytes ($Na+$, $Mg+$, $K+$ and $Ca+2$) and trace elements (Se, Mn, Cu, Zn and Fe) in earthworm, *Eisenia foetida*. Earthworms were exposed to TiO_2 nanoparticles by paper contact method of OECD-207 guidelines. Inductively coupled plasma mass spectrometer (ICPMS) was used to determine bio-accumulation potential of TiO_2 nanoparticles, concentration of electrolytes and trace elements in earthworm tissues. Subsequently, neurotoxic effects and oxidative stress induced by TiO_2 nanoparticles were correlated with altered electrolyte and trace element levels in earthworm. Results indicated that concoction of primary particles caused bioaccumulation of TiO_2 nanoparticles in earthworm. Route of administration and bioavailability influenced bioaccumulation of TiO_2 nanoparticles. Consequently, TiO_2nanoparticles affected homeostasis of trace elements thereby ameliorating neurotoxicity and oxidative stress in earthworm. Thus, it was concluded that exposure route and internal dose of TiO_2 nanoparticles are vital to understand their metallomics in earthworm. Furthermore, it was found that size and charge affected accumulation of TiO_2 nanoparticles in earthworm. In conclusion, bioaccumulation of TiO_2 of nanoparticles alters metallome interactions in *Eisenia foetida*. Moreover, divulging such interactions will definitely aid in delineating toxicity of TiO_2 nanoparticles. Therefore, we believe that outcomes of current study assist in deciphering metallomics of nanoparticles in bioindicators. As a result, these indicator organisms can serve as proxies in environmental monitoring and safety assessment of metal nanoparticles.

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OXFORD UNIVERSITY PRESS

ISSN 1096-6080
Volume 138, Issue 1
March 2014

www.toxsci.oxfordjournals.org

PS 833 Unique Mode of Toxicity of Cu Nanoparticles Compared to Their Micron and Ionic Analogs in *E. coli* and *L. brevis*

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Nanotechnology has grown rapidly over the past decade, promising benefits in diverse areas of society. However, the rate of toxicological analysis of nanoparticles (NPs) has not kept pace with the rate of development, leading to concerns over the potential biological toxicity and environmental contamination of NPs. Here, we report toxicity ranking as well as mechanisms of toxicity for a series of Cu particles, including nano Cu, nano CuO, nano Cu(OH)2, micro Cu and micro CuO as well as ionic Cu (CuCl2 and CuSO4) in bacteria (*Escherichia coli* and *Lactobacillus brevis*). Fluorescent assays such as PI/SYTO, XTT, DiBAC, and H2DCFDA were used to measure viability, respiration rate, membrane potential, and ROS production, respectively. IC50 values were calculated from growth inhibition curves, revealing that Cu and CuO NPs are more toxic than their micro-sized counterparts, with toxicities approaching that of ionic Cu. Strikingly, the NPs showed distinct differences in their mode of toxicity when compared to the Cu ions, highlighting the unique toxicological properties of materials at the nanoscale. Moreover, cells treated with nano Cu showed higher levels of bioavailable Cu++ than cells treated with ionic Cu. 3D tomography from electron microscopy of cells exposed to the NPs revealed the presence of intact NPs inside the cells.

PS 834 Physicochemical Characterization and Ecotoxicological Evaluation of TiO_2 Nanoparticles in Earthworm *Eisenia foetida*

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Wide spread application and use of nanoparticles such as TiO_2 confers their release into various compartments of environment thereby raising concerns about their health impacts. It is essential to assess the ecotoxicity and implications of TiO_2 on the flora, fauna and ecosystem as a whole. Although there are studies reporting toxicity of TiO_2, but specific findings on its effect and underlining toxicity mechanisms in terrestrial organisms such as earthworms remain poorly understood and documented. Hence in this study, ecotoxicological evaluation of the rutile form (10-100nm) of TiO_2 nanoparticles on earthworm, *Eisenia foetida* was conducted as per the modified filter paper test of OECD-207 guidelines. Physicochemical characterization of TiO_2 nanoparticles was carried out using zeta sizer and scanning electron microscope. Earthworms were exposed to a series of concentrations (0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2) of TiO_2 nanoparticles. Mortality of earthworms was determined after 48h exposure to TiO_2. Activity of the enzymes, catalase, super oxide dismutase and glutathione reductase was assessed in whole body homogenate of the worms surviving after exposure. Lipid peroxidation was measured to define the levels of oxidative stress during toxicity assessment. Perturbations caused by TiO_2 nanoparticles resulted in the oozing of coelomic fluid and pale coloration of earthworm body. Mortality rate, activity of antioxidant enzymes and lipid peroxidation were influenced by size and charge of TiO_2 nano particles rather than concentration. Results of the study showed that agglomeration of TiO_2 nanoparticles is responsible for the variation in their size and charge. The finding is first of its kind to establish ecotoxicity of rutile form of TiO_2 nanoparticles on earthworms. Similar studies will eventually help in developing strategies to predict ecotoxicity of TiO_2 in the soil environment.

PS 835 Gene Expression Changes in Secondary Organs of Rats Intratracheally Exposed to Silver Nanoparticles

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Studies in rats have demonstrated the ability of silver nanoparticles (AgNP) to translocate to secondary target organs (e.g. liver or kidney) after administration by respiratory route (Takenaka et al, Environ Health Perspect, 2001). In this study, the expression profile of genes involved in oxidative stress (Gpx1, SOD, Gss, FMO2, Gsr, Txnrd1), metal toxicity (mt1), apoptosis/cell cycle (Casp3, p53), and protein folding (Hsp70) was investigated in liver and testis of rats at different time intervals after i.t. instillation of AgNP. AgNO3 was used as a positive control. Groups of adult male Sprague-Dawley rats received 50 μg/rat of AgNP (20 nm), 7 μg/rat of AgNO3 or 100 μL aqueous solution/rat (control). At days 7 and 28 post-administration, the transcriptional profile of selected genes was examined in tissues by cDNA microarray analysis coupled with bioinformatics and functional gene annotation studies. Semiquantitative followed by Real Time PCR was performed to quantify gene expression changes.
At day 7, changes in gene expression that selectively involved antioxidant enzymes were observed in both liver and testes. In particular, Gpx1, FMO2 and SOD genes were upregulated. No changes were seen for the other genes. At day 28 the AgNP-treated animals exhibited a tissue gene expression profile similar to control. None of the investigated genes was shown to be affected by treatment with AgNO3 at both time points considered. The results suggest the potential of AgNPs to cause, at low doses, subtle molecular changes in secondary target organs, in contrast with Ag ions, possibly reflecting the strong tendency of Ag ions to form inert complexes with cellular or blood components or be neutralized by defense mechanisms.
(Grants: Italian Ministries of Health, Research & Education; & CARIPLO foundation-Rif. 2011-2096).

PS 836 The Influence of UV Light on the Genotoxicity of Engineered Nanoparticles

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TiO2 nanoparticles (NPs) are one of the most commonly used engineered nanomaterials due to their use in a large variety of applications as photocatalytic agent. The inherent properties of TiO2 might however, lead to photo-induced toxicity in human and animal tissue. Under UV irradiation, TiO2 NPs can produce ROS which is damaging to DNA and other biomolecules.
In this study we used a chemical test for quantifying ROS generation in combination with the umu-test which is a short-term genotoxicity assay providing high throughput screening (96-well plates). Various nanoparticles (SiO2, CeO2, ZnO, and TiO2) were screened using concentrations of 0.0125-0.1 mg/mL with a 2 hour incubation/exposure time and no metabolic activation. No genotoxicity was induced for any of the NPs in the absence of UV light in the concentration range tested.
The next set of experiments involved the use of full spectrum UV light. Methylene blue dye decomposition was used for assessing the photocatalytic activity of TiO2 for an exposure period of 5-80 minutes. Maximal degradation of methylene blue occurred after 60 minutes. In order to investigate the effect of UV on Salmonella typhimurium bacteria, growth rate was investigated at 10, 20, 30, 40, 50, 70 min of exposure. Less than 15% decrease was observed for all the exposed bacteria compared to control. Therefore, an exposure period of 60 minutes was chosen to observe the ability of OECD reference hydrophilic (NM-104) and hydrophobic (NM-105) TiO2 NPs to induce genotoxic effects in the presence of UV light compared to absence of UV light. Concentrations of 0.25, 0.5, 1 mg/mL (45, 90, 180 μg/well) increased the β-galactosidase activity indicating a possible genotoxic effect in the presence of UV light.

PS 837 Monocyte Activation by Particulate Matter and Reactive Oxygen Species Formation

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Acute as well as chronic exposure to ambient air particulate matter (PM) has been implicated in playing a part in adverse health outcomes. Formation of reactive oxygen species (ROS) is one pathway by which PM exposure may disrupt normal physiological balance leading to disease pathogenesis. This study used human monocytic (THP-1) cells to assess potential activation and subsequent production of reactive oxygen species. THP-1 cells were seeded in 96-well plates and then exposed for 24 hrs to particulate matter collected from a site in Los Angeles. The particles were smaller than 100 nm in aerodynamic diameter and were collected in an urban site of downtown Los Angeles. Two different doses 2 or 20 μg/m3 were used. Levels of ROS were measured by a fluorescent method. After exposure, there was a dose-dependent increase in the amount of ROS formation. Mitochondrial activity, measured by the MTS assay, was increased at both doses. This increase was not due to changes in cell number. Indeed at the highest dose, we noted a decrease in cell number which may reflect decreased cell proliferation or viability. The data shows that THP-1 cells may be activated by PM leading to ROS formation. Further co-culture studies are needed to determine how activation of monocytes may affect normal human cells. This is the current focus of our laboratory.

Confounding factors and future challenges to delineate ecotoxicity of TiO_2 nanoparticles

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Abstract

Physicochemical and biological properties of TiO_2 nanomaterials differ from their bulk counterparts at atomic scale. Exploiting specific properties of TiO_2 nanoparticles in sunscreens & cosmetics, paints & coatings, water treatment, antimicrobial agents, health care products such as bandages and manufacturing of energy storage devices is paramount. As a result, TiO_2 nanoparticles find their entry into the wastewater and sludge thereby in soil. Occurrence of TiO_2 particles of 4-30 nm size is well documented in the literature. Therefore, it is imperative to validate current conventional methods of toxicity testing to adjudge their suitability to ascertain the toxicity of nanoparticles. In this context, current study was designed to validate paper contact method of OECD-207 guidelines to determine toxic effect of rutile form TiO_2 nanoparticles (size <100nm) on earthworm, *Eisenia foetida*. Altered size of TiO_2 nanoparticles in various dispersion media was determined by dynamic light scattering. Toxicity of TiO_2 nanoparticles on earthworm was determined by paper contact method. Oxidative stress of earthworms exposed to different concentrations (0.05, 0.1, 0.15, 0.2 and 0.25 mg/cm^2) of TiO_2 nanoparticles was described in terms of activity of catalase, superoxide dismutase and lipid peroxidation. Bio-accumulation behavior of TiO_2 nanoparticles in earthworms was depicted by ICPMS analysis. Results indicate that concentration dependent mixture of primary particles influence the agglomeration of TiO_2 nanoparticles in distilled water. Concoction of various primary particle sizes of nanoparticles leads to inadvertent dose responses of earthworm exposed to TiO_2 nanoparticles. As a result, various confounding factors contributing to the altered behavior of TiO_2 nanoparticles thereby their importance in designing and evaluation of methods of toxicity testing were discussed. Furthermore, challenges pertaining to the detection and quantification of nanoparticles from environmental matrices were elaborated. In this regard, outcomes of current study certainly assist in paraphrasing risk assessment strategies for nanowaste management.

Key words: TiO_2 nanoparticles; earthworm; OECD-207; agglomeration; bioaccumulation; nanowaste

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17 Nanomaterials and Nanotoxicology: Two Sides of the Same Coin

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Preamble:
Nanotechnology is a major innovative, scientific and economic growth area. Its ability to engineer matter at nanoscale enables production of materials with unique physical, chemical, and biological properties. These properties of nanomaterials may differ from the properties of bulk materials at atomic or molecular levels. With the advent of their unique properties, nanomaterials are used in numerous industries such as electronic components, medical devices, food, pharmaceuticals, and cosmetics.

Nanomaterials in defense and aerospace applications:
Application of nanomaterials in defense and aerospace sectors is inevitable. Carbon nanotubes and silica based nanomaterials are gaining prominence in these sectors. Nanomaterials are used in nanofabrics which are used for effective camouflaging. They are also used in designing liquid body armor to protect soldiers from bullet, sword and needle attacks. Principal areas of nanoscience research in defense applications deal with explosives, bio-medicine, sensors, electronics, energy storage, coatings and filters. Nanomaterials that are used in aerospace include graphene, multimetallic oxides like Barium Titanate ($BaTiO_3$), Barium Strontium Titanate ($BaSrTiO_3$) and perovskites. These are exceptionally small size (2–5 nanometers in diameter) and stable nanocrystals that can be dispersed and spin-cast to form high-quality, homogeneous, crack-free thin films on prefabricated infrared sensor circuitry. Durable and lightweight nanomaterials withstanding physical stresses during launch and operation of satellites is yet another application.

Interaction of nanomaterials with biological systems:
Production and utilization of nanomaterials for various applications including defense and aerospace will steeply increase in the upcoming years. Hence exposure of various living systems to nanomaterials is unavoidable. It is well established that nanomaterials can easily cross the cell membrane than their bulk forms. Therefore, usage of nanomaterials raises concerns about their risk of toxicity. In vitro and in vivo investigations of nanomaterials raise concerns about their adverse effects on respiratory & immune systems, inducing oxidative stress & cancer. For example, single and multiwall carbon nanotubes cause lung disease. Exposure of rodents to Titanium dioxide nanoparticles causes oxidative stress and DNA damage. However, research and scientific data on kinetics and toxicity of nanomaterials is limited. But complete understanding of the interaction between nanomaterials and biological systems, including nanomaterial uptake, distribution and biological responses, is vital to guide the design of safer and more effective nanomaterials than those that are currently available.

Toxicological evaluation of TiO_2 nanoparticles:

Therefore, to delineate toxicity of engineered nanomaterials, we started working on metal oxides the class that is widely used. We now present gist of the results on toxicological studies on TiO_2 nanoparticles which is one of the key components of various products including that of defense and aerospace. Physico-chemical characterization of Titanium (IV) oxide, (rutile, <100nm, 99.5%, Sigma Aldrich) was done by dynamic light scattering and

scanning electron microscopy. When TiO_2 nanoparticles are suspended in distilled water, they started aggregating. Consequently the predominant (27.5%) particle size was 122.4 d.nm as shown in Figure 1A. This size was far higher than its original form as purchased. Visual confirmation of particle aggregation was done using scanning electron microscopy, as presented in Figure 1B. To prove aggregation influenced bioaccumulation of TiO_2 nanoparticles, ecotoxicity studies were done in earthworm, Eisenia foetida as per OECD-207 guidelines. Worms were exposed to 5 different concentrations (0.05, 0.10, 0.15, 0.20 & 0.25mg/cm^2).

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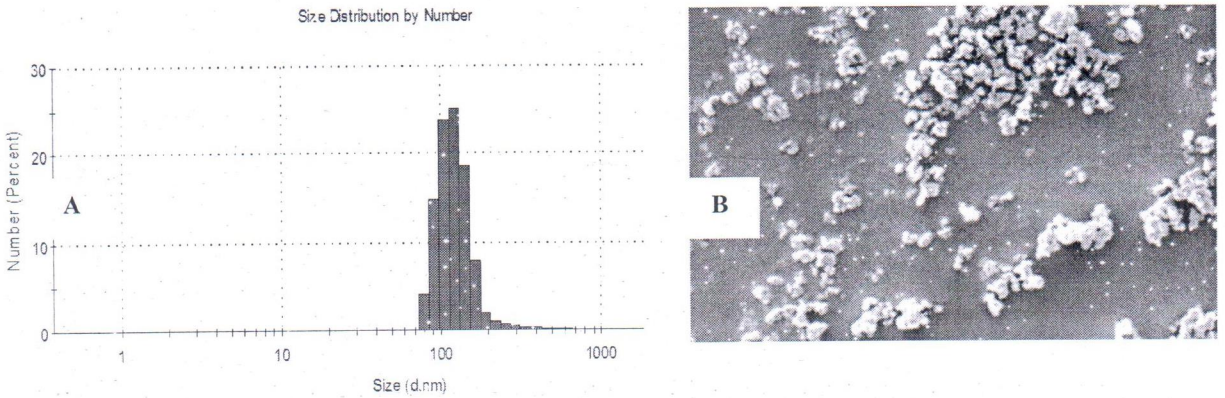
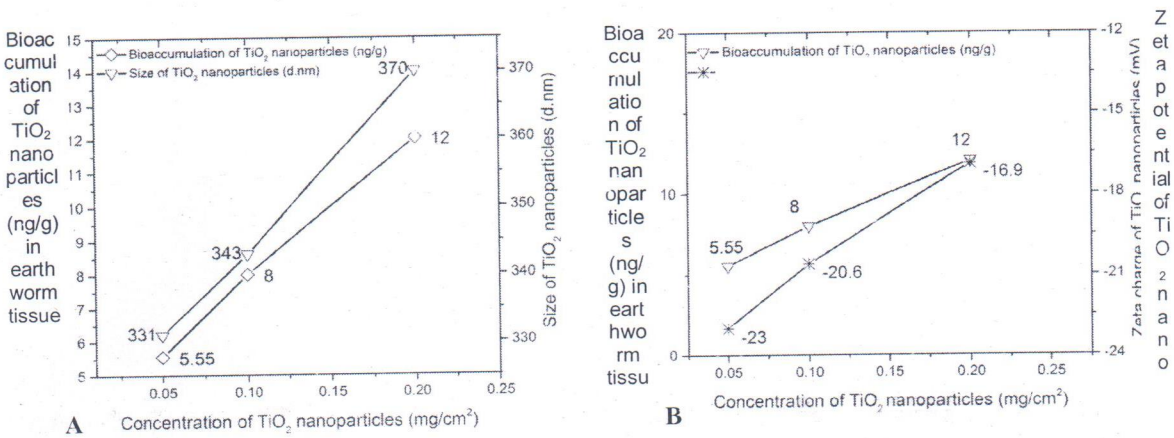


Figure 1: Aggregation of TiO_2 nanoparticles in distilled water suspension measured by dynamic light scattering (A), and scanning electron microscopy (B)

It was observed that as the concentration of TiO_2 nanoparticles increased, their size had also increased because of particle aggregation. It was also observed that bioaccumulation has increased with increase in particle size (Fig. 2A). This trend was for zeta potential also i.e with increased concentration of nanoparticles both zeta potential and bioaccumulation increased.

Figure 2: Influence of size (A) and zeta potential (B) on bioaccumulation of TiO_2 nanoparticles in earthworm tissue



Conclusions:

Size and charge mediated aggregation of TiO_2 nanoparticles influence their bioaccumulation in earthworm tissue. From our earlier studies, it was evident that if TiO_2 nanoparticles accumulate in earthworms, they induce toxicity in the form of oxidative stress. Consequently to overcome oxidative stress, the animals produced elevated levels of antioxidant enzymes (catalase, glutathione reductase and superoxide dismutase). The biochemical response to oxidative stress caused by TiO_2 nanoparticles remains conserved in all organisms. So, results of this study confirm that TiO_2 nanoparticles can induce toxicity in human beings also. Hence we recommend that through extensive toxicological research, the maximum allowable concentration of nanoparticles be determined and propounded in the form of regulations, which are to be strictly followed. As the usage of nanomaterials is increasing multifold, such regulations are to be made at the earliest, so that we could reap the benefits of nanotechnology without compromising on environmental health.

ABOUT THE BOOK

In 2014 a conference was held in BITS Pilani Hyderabad Campus as an attempt at bringing together policymakers, researchers and practitioners not only to reframe questions but also to think of novel solutions for sustainability in the areas of agriculture, human habitats and customary law in contrast to the modern legal framework. Titled *Technology Policy Community: Small Experiments in Sustainability* the conference engaged with eminent academicians, civil society activists and policy makers on a three major themes: Agriculture and Environment; Habitats and Environment Policy; and Law and Environment Policy.

The findings of the conference could be grouped into two categories. The academic research of the technical sessions and the intellectual capital of the panel discussions. Our attempt here is to bring these findings out in two volumes. The first *Small Experiments in Sustainability* contains research papers specifically dealing with the technical and policy aspects of sustainability. The second volume titled *Conversations on Sustainability* takes a more macro perspective on issues of sustainable agriculture, industry, indigenous communities, law and administration providing insight and direction to scholars, practitioners and the lay person alike.

ABOUT THE BOOK

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Shamuel Tharu is currently visiting faculty at the Department of Humanities and Social Science, BITS Pilani Hyderabad Campus. He has a worked as a journalist, taught international relations and political science in several universities across the country and currently is engaged in research on human rights and anti-insurgency protocols. This is his first work in sustainability studies.

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ISBN: 978-81-90293-11-2

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₹. 870/-

Small Experiments In Sustainability

SRINIVAS SAJJA
SHAMUEL THARU

Small Experiments In Sustainability

Srinivas Sajja, Shamuel Tharu

CHAPTER 8

Should we say NO to NaNO?

Preliminary Study to Corroborate Occurrence of Nanoparticles in Treated Wastewater Samples

B. Siva Prasad, Ashwini Sri Hari, P. Sankar Ganesh

Abstract

Extensive production and use of nanotechnology based products; thereby their release into environment via sewage is currently occurring. Concentration of nanomaterials in wastewater and wastewater treatment plants will inevitably increase in the future. Wastewater is one of the important sources of nanomaterial discharge into the environment. Various forms of the source include treated effluent, biosolids, and plant-generated aerosols. As nanomaterials have been used in various personal care products and environmental technologies, they may find their entry into various compartments of environment. Detection of such nanomaterials in wastewater entering environment is one of the needs of hour to advance the current wastewater treatment technologies to treat nanowaste. Therefore, the current study was designed to find the nanoparticle size distribution in wastewater collected from 340MLD, Sewage Treatment Plant located at Amberpet, Hyderabad. Collection and hotplate digestion of wastewater samples from a conventional activated sludge wastewater treatment facility was performed as per the guidelines of *American Public Health Association (*APr Digested samples were subjected for filteration through 0.22 micron

filter. Samples were subjected for water bath sonication to facilitate the homogenous dispersion of particles. DLS provides statistical representative data about the hydrodynamic size of nanomaterials. In situ, real-time monitoring of particle size distribution by DLS was carried out to delineate useful information regarding the aggregation process and, at the same time, gives quantitative measurement on the size of the particles in samples. Raw sewage entering the plant showed the particle z-average size 398 nm, while aeration tank sample showed particle z-average size 1067 nm. Samples showed the polydispersion due to their aggregation or sedimentation in the sample. Results suggest that significant fractions of nanoparticles are present in the wastewater. Therefore, outcome of the study can aid in further scientific analysis of detection of nanoparticles in wastewater thereby contributing to understand various challenges associated with the promotion of nanotechnology.

KEY WORDS: *Nanotechnology, wastewater, nanomaterials, aggregation, dynamic light scattering*

1. Introduction

Extensive production and use of nanotechnology based products; thereby their release into environment via sewage is currently occurring (Benn and Westerhoff, 2008). Concentrations of nanomaterials in wastewater and wastewater treatment plants will inevitably increase in the future. Wastewater is one of the important sources of nanomaterial discharge into the environment. Various forms of the source include treated effluent, biosolids, and plant-generated aerosols. Therefore, information on the occurrence of nanoparticles in wastewater will be an important contribution to understand their behavior and fate in aquatic ecosystem.

Titanium dioxide (TiO_2), Silver (Ag), and Fullerenes (C_{60}) are currently the most produced and hence found in a wide range of commercial products. Recent studies on exposure modeling of nanomaterials indicated that predicted concentrations of nano-TiO_2 in wastewater effluents (0.7–16 μg/L) were higher than the predicted no-effect concentration level (1 μg/L) (Gottschalk, Sonderer, Scholz, Nowack, 2009). As nanomaterials have been used in various personal

care products and environmental technologies, they may find their entry into various compartments of environment. Detection of such nanomaterials in wastewater can assist in understanding their fate in the environment while passing through a wastewater treatment plant. Therefore, the current preliminary study was designed to find the particle size distribution in wastewater collected from 340MLD, Sewage Treatment Plant located at Amberpet, Hyderabad.

2. Materials and methods

2.1 Collection and hotplate digestion of wastewater sample

Wastewater samples were collected from a conventional activated sludge wastewater treatment facility at Amberpet, Hyderabad. *American Public Health Association (*APHA) (APHA, AWWA, WEF, 1992) guideline No. 1060-A was followed to collect wastewater sample. The samples were subjected for hot plate digestion as per modified guideline 3030-G reported by Westerhoff et al. (2011) (Paul Westerhoff, Guixue Song, Kiril Hristovski, Mehlika A. Kiser, 2011; Alex Weir, Paul Westerhoff, Lars Fabricius, Kiril Hristovski, and Natalie von Goetz, 2012). In the hot plate digestion method, sample was added to a 150 mL polytetrafluoroethylene (PTFE or Teflon) beaker along with 10 mL of hydrogen peroxide and 2 mL of nitric acid. The beakers were heated at 120°C for 4 hours to digest the organics. The beakers were removed from the hot plate and allowed to cool. When 0.1 to 0.5 mL of solution remained, the beakers were removed from the hot plate and allowed to cool. Then the beakers were rinsed three times with a solution of 2% nitric acid in nanopure milli q water into a 25 mL volumetric flask. The samples were filtered through 0.22 micron filter p

2.2 Characterization of wastewater sample by dynamic light scattering

Several light scattering methods are among the ensemble approaches, and they can provide representative size distribution characterization of particles at relatively low concentrations (ca. 10^7-10^8 mL^{-1}) in a suspension. Dynamic light scattering (DLS), also called photon correlation spectroscopy, measure the laser light scattering by particles

to calculate their hydrodynamic radius (r) distribution of particles in a suspension. DLS provides statistical representative data about the hydrodynamic size of nanomaterials. In situ, real-time monitoring of particle size distribution by DLS provides useful information regarding the aggregation process and, at the same time, gives quantitative measurement on the size of the particle clusters formed. Therefore, DLS was performed to ascertain the particle size distribution in the wastewater samples. All DLS measurements were performed with a Malvern Instrument Zetasizer Nano Series (Malvern Instruments, Westborough, MA, USA) equipped with a He-Ne laser ($\lambda = 633$ nm, max 5 mW) and operated at a scattering angle of 173°. In all measurements, 1 mL of sample was placed in a 10 mm × 10 mm quartz cuvette and size distribution was measured in the given sample. Samples were diluted in 1:8 dilutions in milli q water to attain the optimum polydispersity index during size distribution measurement in the water samples.

3. Results and discussion

Raw sewage entering the plant showed the particle z-average size 398 nm (range of the particle size was found to occur between 69 to 398 d.nm), while aeration tank sample showed particle z-average size 1067 nm (range of the particle size was found to occur between 77 to 892 d.nm) as represented in Figure 1.

Samples showed the polydispersion due to their aggregation or sedimentation in the sample (Donselaar, Philipse, 1999; Phenrat, Saleh, Sirk, Tilton, Lowry, 2007). Results suggest that significant fractions of nanoparticles are present in the wastewater. However, the interpretation of DLS data involves the interplay of other parameters, such as the size, concentration, shape, polydispersity, and surface properties of the particles involved. Hence, careful analysis of occurrence of nanoparticles in the sample should be confirmed by employing other confirmatory techniques such as nanoparticle tracking analysis, electron microscopy (SEM and TEM). It is essential to ensure the class or type of nanoparticle in samples by ICPMS techniques to conclude the species of materials present in the given sample (Nowack, Bucheli,
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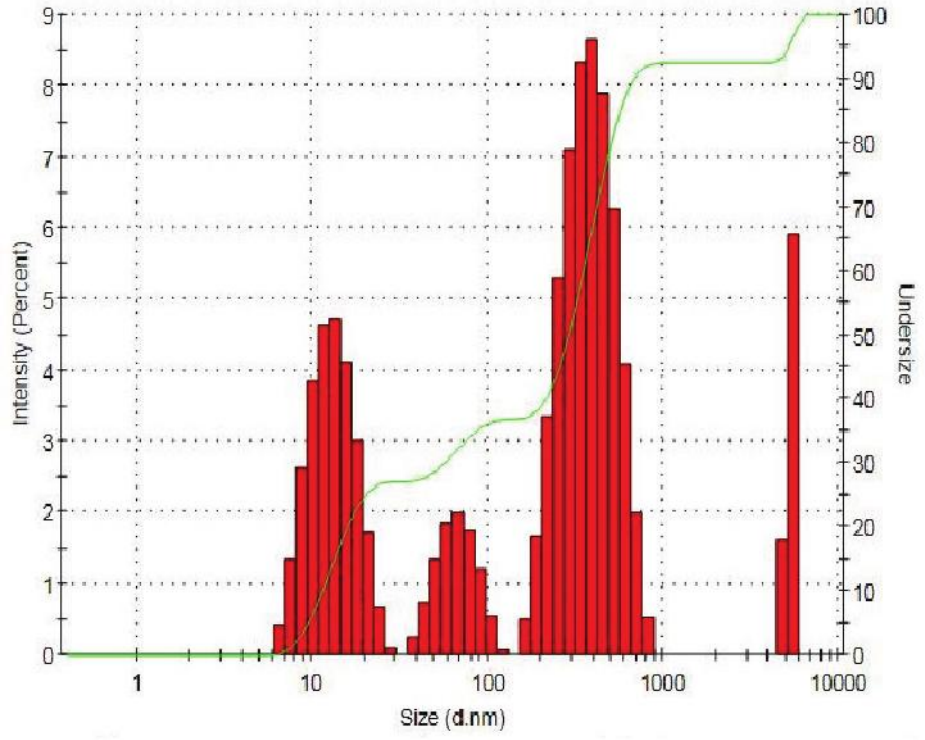
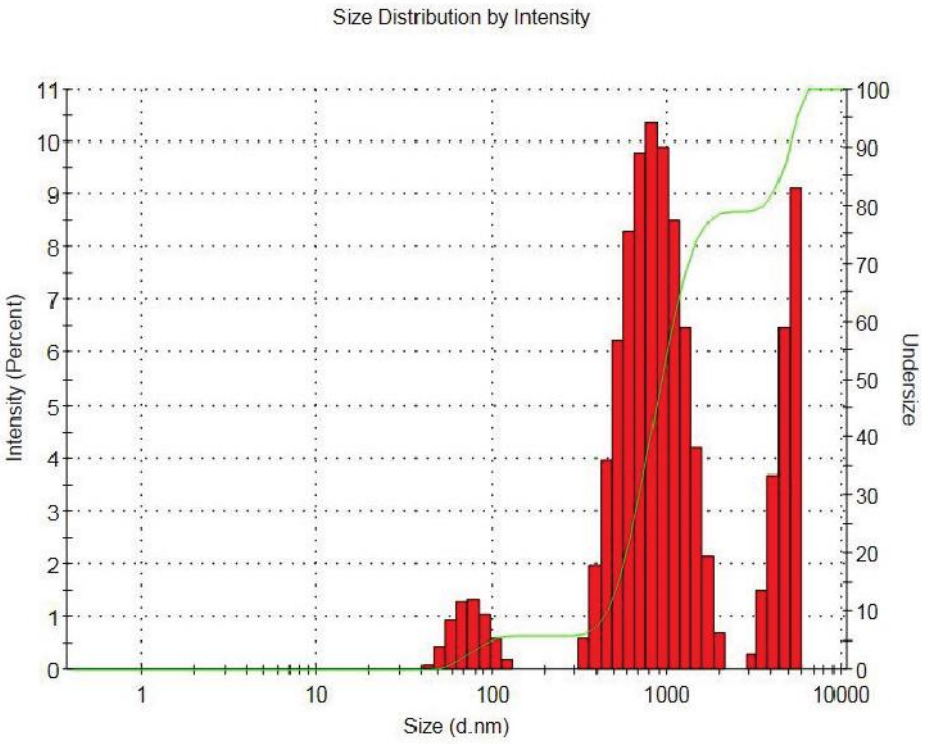


(A)



(b)

Figure 1: Size distribution of particles in wastewater sample of raw sewage (A) and aeration tank sample (B) of activated sludge determined by DLS. The Z-average of particle size distribution was calculated at particle distribution index (Pdl) 0.37 and 0.54 for A and B samples respectively.

4. Conclusions

This study can infer the basis of association of nanoparticles in wastewater samples. However, the relevant nanomaterials and their concentrations in wastewater samples can be established only by employing other analytical techniques such as ICPMS and electron microscopy. Thus, it is concluded that use of complementary techniques i.e. combination of mass spectrometry, light scattering and electron microscopy can provide substantial data to adjudge the characterization and quantification of nanomaterials in aquatic environment.

5. Acknowledgment

PSG and BSP thank Department of Biotechnology, Government of India for funding support in the form of Project No. BT/PR6592/GBD/27/446/2012 and Junior Research Fellowship, respectively. ASH thanks BITS Pilani, Hyderabad Campus for Teaching Assistantship.

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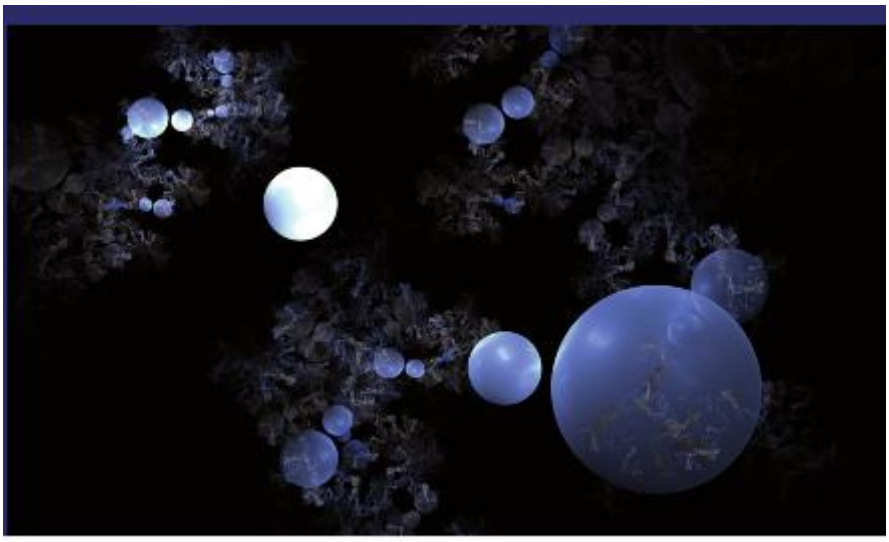

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