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Ph.D in Biology

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UNIVERSITY OF GAZİANTEP
GRADUATE SCHOOL OF
NATURAL & APPLIED SCIENCES

EFFECTS OF POLYSTYRENE NANOPARTICLES ON SOIL MICROBIOTA

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AWET TEKESTE TSEGAI

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Effects of Polystyrene Nanoparticles on Soil Microbiota

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Awet Tekeste Tsegai

ABSTRACT

EFFECTS OF POLYSTYRENE NANOPARTICLES ON SOIL MICROBIOTA

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The increasing production of nanoplastic and the fragmentation of microplastics into smaller particles pause a plausible yet unclear hazard in the natural environment, such as soil. In this research the effects of polystyrene nanoparticles (PS NPs) on the activity and biomass of soil microbes were investigated. Soil samples were spiked with environmentally relevant concentrations of 0 (control), 10, 100 and 1000 µg PS NPs kg^{-1} dry soil, respectively. Ecotoxicological effects were assessed at 1, 14, and 28 days of the incubation periods. The results showed a significant decrease in MBC in the PSNP-100 and PSNP-1000 treatments throughout the incubation period. Moreover, dehydrogenase activity and activities of enzymes involved in N-(leucine-aminopeptidase), P-(alkaline-phosphatase) and C-(β-glucosidase and cellobiohydrolase) cycles in the soil were significantly reduced at day 28 suggesting a broad and detrimental impact of PS NPs on soil microbiota and enzymes. Basal respiration and metabolic quotient increased with increasing PS NP application throughout the incubation period, this, coupled with decrease in MBC clearly indicate detrimental effects of PS NPs on soil microbes that possibly by diverted energy use from growth and reproduction to damage repair. The findings demonstrated for the first time the potential antimicrobial activity of PS NPs in soil, and this may serve as an important resource in environmental risk assessment of PS NPs in the soil environment.

Keywords: Polystyrene, nanoparticles, ecotoxicology, soil, microbial biomass, enzyme activities.

ÖZET

POLYSTYRİN NANOPARTİKÜLÜNÜN TOPRAK MİKROBİYOTASINA ETKİSİ

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Nanoplastik üretiminin artması ve mikro plastiklerin daha küçük parçacıklara parçalanması, toprak gibi doğal ortamlarda makul ve net olmayan bir tehlike oluşturmaktadır. Bu çalışmada, polistiren nanopartiküllerinin (PS NP) toprak mikroorganizmalarının aktivitesine ve biyokütlesine etkileri araştırılmıştır. Toprak örnekleri, çevresel olarak ilgili konsantrasyonlarda sırasıyla, 0 (kontrol), 10, 100 ve 1000 µg PS NP kg-1 kuru toprak ile karıştırılmıştır. Ekotoksikolojik etkiler, inkübasyon sürecinin 1, 14 ve 28 günlerinde değerlendirilmiştir. Sonuç olarak, inkübasyon süresince PSNP-100 ve PSNP-1000 uygulamalarında mikrobiyal biyokütlede önemli bir düşüş gözlenmiştir. Ayrıca, dehidrojenaz ve N- (lösin-aminopeptidaz), P- (alkalin-fosfataz) ve C- (β-glukosidaz ve sellobiohidrolaz) döngülerinde yer alan enzimlerin aktiviteleri 28. günde önemli ölçüde azalmıştır. Bu sonuçlar, PS NP'lerin toprak mikrobiyota ve enzimleri üzerinde geniş ve zararlı bir etkisi olduğunu düşündürmektedir. İnkübasyon dönemi boyunca artan PS NP uygulaması ile bazal solunum ve metabolik oran artmıştır. MBC'deki düşüş ile birleştiğinde, bu bulgu PS NP'lerin, enerjiyi büyüme ve üremeden hasar onarmaya yönlendirerek substrat kullanım verimliliğini düşüren toprak mikroorganizmaları üzerindeki zararlı etkilerini açıkça göstermektedir. Bu çalışma ile PS NP'lerin topraktaki potansiyel antimikrobiyal aktivitesi ilk kez gösterilmiştir ve çalışmanın sonuçları toprakta PS NP'lerin çevresel risk değerlendirmesinde önemli bir kaynak olarak kullanılabilir.

Anahtar Sözcükler: Polistiren, nanopartiküller, ekotoksikoloji, toprak, mikrobiyal biyokütle, enzim aktiviteleri

I would like to dedicate this work to my parents.

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TABLE OF CONTENT

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ILLUSTRATIONS

Page

LIST OF ABBREVIATIONS

AMC	Amino methyl coumarin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CEC	Cation exchange capacity
DHA	Dehydrogenase activity
DLS	Dynamic light scattering
dM	Dry mass
DNA	Deoxyribose nuclic acid
FISH	Fluorescent in situ hybridization
IAA	Indole-3-acetic acid
ICP-MS	Inductively coupled-plasma mass spectrometry
IRGA	Infrared gas analyzer
MBC	Microbial biomass carbon
MUB	Methylumbelliferone
MWCNT	Multi walled carbon nanotubes
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NGS	Next generation sequencing
NM	Nanomaterial

NP	Nanoparticle
PLFA	Phospholipid fatty acid
PS	Polystyrene
PS NP	Polystyrene nanoparticle
qCO_2	Metabolic quotient
REM	Reflection electron microscope
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SWCNT	Single walled carbon nanotube
TEM	Transmission electron microscopy
TN	Total nitrogen
TOC	Total organic carbon
TPF	Triphenyl formazan
T-RFLP	Terminal-restriction fragment length polymorphism.
TTC	Triphenyl tetrazolium chloride
WHC	Water holding capacity
wM	Wet mass

CHAPTER 1

INTRODUCTION

1.1 Background

Since the 1980s, there has been increased interest in the exploitation of materials at their smaller size both at molecular and atomic level. This has given rise to a vital technology of the 21st century, nanotechnology (Klaine et al., 2012). Nanotechnology deals with particles at dimensions of less than 100 nm and is capable of manipulating particles on a molecular and atomic level. Due to the increasing applications of nanoparticles the development of nanotechnology based materials and products are experiencing unprecedented expansion in the 21st century (Maynard, 2006). This expansion is strongly attributed to the nanoparticles novel physiochemical properties. NPs high surface area and small size as compared to their corresponding bulk-scale materials not only attribute to their novel properties but also to their toxic effects (Lewinski et al., 2008; Esmaeillou et al., 2013; Tang et al., 2015). As the application and production of nanomaterial increases, the exposure and subsequent risk is increasing. Concerns are thus, raised by the increased use and potential environmental and ecological risks of NPs.

Nanotoxicology studies the potentially harmful effects of nanoparticles. The source of those nanoparticles could be from natural (volcanic eruption) manufacturing (grinding) and combustion (diesel) (Donaldson et al, 2004). The long run objective for nanotoxicology is to categorize nanoparticles by understanding their physical and chemical properties, distributions, route of entry and the extent of their toxicity. The biotoxicity of NPs has not yet been well understood, it is only after the Food and Drug Administration in 2011 set guideline for evaluating the use of nanomaterials that the attention on the study of the potential toxicity of nanomaterials increased.

1.2 Motivation and objectives of the research

Polystyrene is one of the top five globally produced plastics. Global production of plastics has greatly increased in the 21st century (PlasticsEurope, 2015). Pollution by plastic particles in the environment is considered as an emerging worldwide threat. Most of plastic wastes are disposed in landfills with growing accumulation of plastic waste in the environment (North, 2013). Polystyrene is one of the most extensively used types of plastic. Its use has increased over time mainly due to consumerism because of its convenience and comparatively low price. Therefore, their potential effects on the environment such as the soil needs to be considered.

Polystyrene nanoparticles may enter the soil through various pathways, for example by weathering through UV radiation, mechanical abrasion, biological degradation, and disintegration. Many reports suggest a subsequent degradation of plastics including polystyrene into nanoparticles (Andrady, 2011, Lambert et al., 2013; Mattsson et al., 2015). That is, once in the environment such as landfills, plastics can undergo fragmentation and degradation mechanically and/or biologically in to smaller size that could eventually form nanoparticles (Mor, 2008, Singh, 2015; Lambert and Wagner, 2016). As a result, nanoplastics including PS NPs are expected to increase with time in the environment. Soil is the major sink for plastic pollution, therefore, the ecotoxicological effects of nanoplastics in the soil environment needs attention (Corsi et al., 2014).

In soil, PS NPs might potentially affect soil organisms and thus, soil functioning. Though, bulk sized polystyrenes are considered non-toxic, their increased usage coupled with their potential degradation in the environment could potentially impact the soil microorganisms and the services they provide. That is, nano-size polystyrene particles affect living organisms distinctly compared to their bulk materials (Ward et al., 2009). Moreover, studies have shown that physicochemical properties of NPs could be altered in environmental media (Liu et al., 2014; Lowry et al., 2012). Soil matrix is a complex medium unlike the air and aquatic environment it constitutes particles such as minerals, heavy metals and dissolved organic matters. PS NPs can potentially interact with soil particles and could lead to a change in its physicochemical properties which in turn leading to significant toxicity. Therefore, assessment of the potential toxic effects of PS NPs on soil microorganisms deserve attention.

To date, no data of PS NPs amounts in soil are available. The available studies suggest several effects of PS NPs mainly on aquatic environment but as far as the current study there were no previous studies on the effects of PS NPs on soil microbes and their activities. Unlike many other ecotoxicological studies that use synthetic media or one population, this study was conducted at a community level and therefore presents a better environmental risk assessment. Moreover, the present literatures on the ecotoxicological effect of NPs are largely based on high concentrations than would normally be found in the environment (Simonin & Richaume, 2015). In this study, environmentally relevant concentration of PS NPs were used. To assess the environmental significance of PS NPs in soil, we investigated the effects of PS NPs on soil microorganisms and enzyme activities involved in the C-, N- and P-cycles as sensitive bio-indicators in soil.

In brief, this study investigated the ecotoxicological effects of PS NPs on the soil microbiota and their functional diversity namely enzyme activities, basal respiration rate as well as microbial biomass carbon. Soil samples were spiked with environmentally relevant concentrations of 10, 100 and 1000 µg PS NPs kg^{-1} dry soil. Potential ecotoxicological effects of PS NPs in the sampled soil were assessed as a change in soil microbial properties. That is, soil microbial biomass, respiration rate and enzyme activities were assessed at 1, 14, and 28 days of incubation. Microbial biomass carbon was determined by fumigation extraction method, enzyme activities were determined by Triphenyltetrazolium chloride and Fluorimetric microplate enzyme assay methods for intracellular and extracellular activities respectively, soil basal respiration rate (CO_2-C) was measured with infrared gas analyzer (IRGA) and metabolic quotient (qCO_2) was calculated as a ratio of basal respiration to metabolic quotient (CO_2-C / MBC).

This research, demonstrated for the first time, the potential antimicrobial activity of PS NPs in soil. This may serve as an important resource in environmental risk assessment of PS NPs in the soil environment.

1.3 Thesis outline

The thesis is arranged as follows:

Chapter 1 provides the background information, motivation and objective of the thesis. It also states the unique contributions of this dissertation, and briefly presents the experimental design.

Chapter 2 presents the literature review on nanoparticles, physicochemical properties and related environmental effect with emphasis on soil environment. It also describes soil properties in relation to nanoparticles.

Chapter 3 presents available literature specifically on PS NPs and their potential source to the environment. It also describes the PS NPs physicochemical properties and their potential interaction in the environment as well as their toxicological effects

Chapter 4 outlines the experimental details of the research. It presents methods used to determine the soil microbial properties and activities and their underlining principles. It outlines the procedures of fumigation extraction method in microbial biomass carbon determination, Triphenyltetrazolium chloride method and Fluorimetric microplate enzyme assay in determination of enzyme activities and Infrared gas analyzer in soil basal respiration rate determination.

Chapter 5 presents experimental results of soil microbial properties and their activities. It outlines the analyses and comparisons among the different PS NPs dose treatments and the control. It also presents the calculated result of metabolic quotient.

Chapter 6 discuses the experimental results. It details the possible cause for the change in microbial properties and activities. Moreover, it outlined antimicrobial effects of PS NPs that could be related to their nano-size and their interaction with soil matrix.

Chapter 7 Finally concludes the research findings and provides a summary of key findings as well as suggestions and recommendations for future research work.

CHAPTER 2

NANOPARTICLES AND SOIL ENVIRONMENT

2.1 Nanoparticles definition and applications

The prefix "nano" of nanoparticles is a Greek word originated from “nanos” which means, “dwarf”. Nomenclature in nano-science and nanotechnology was debatable, however, often the term "nanomaterials" (NM) includes particles having a size of at least 100 nm or less while “nanoparticles” refers to particles with two dimension of sizes 1 nm to 100 nm (Klaine et al., 2008). According to the EU nanomaterials are defined as “ a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.” (EC, 2011). This definition is an inclusive definition that includes all nanoparticle classes including carbon based materials such as polystyrene nanoparticles, single walled carbon nanotubes (SWCNTs) as well as metals and their oxides such as Au-NPs, TiO_2 particles, semi-conductor nanocrystals such as quantum dots, and zero-valent metals (Bhatt and Tripathi, 2011). The above mentioned EU definition, however, requires further characterization such as the chemical composition, the particle charge or surface area that could determine the potential hazard of NPs that are not regarded by this definition that was solely based on size. This calls for a new classification or grouping of NPs in the research community (Lynch et al., 2014). Furthermore, available analytical methods to detect and quantify NPs within environmental samples or products and tissues that may undergo internalization and modification are often established based on the analysis of chemical substances with known properties. However, NPs can behave completely different due to their novel properties and thus the development of new analytical measurement tools is required (Borm et al., 2006; Marquis et al., 2009).

Due to the increasing applications of nanoparticles, nano based materials and products are experiencing unprecedented expansion in the 21st century. The nanotechnology

based materials and products are expected to rise from 2,000 tons in 2004 to 58,000 tons in 2020 (Maynard, 2006). This expansion is strongly attributed to the nanoparticles novel physiochemical properties. Currently there are numerous commercially available products that are using nanotechnology such as ingredients of cosmetics, food, packaging material, electronics and medicine (Lowry et al., 2010). Depending on their application, nanomaterials are manufactured from different sources.

Though there are many potential environmental hazards from nanomaterials, nanotechnology has been used in recovering contaminated environments through developing new ways to such environmental contamination problems. One of such novel uses of nanomaterials includes the use of nanomaterials in detecting, preventing and removing environmental contaminants (Kamat and Meisel, 2003; Lee et al, 2005). For example, the adsorption of contaminants to NPs is of a potential use in the environmental remediation of polluted water bodies and soil (Zhang 2003; Quinn et al., 2005). Moreover, nanotechnology can be used to create more environmentally friendly products and cleaner industrial processes as well (Kamat et al., 2002).

Engineered nanomaterials in contrast to naturally found NPs which occur polydispersed and have more complex structures, are more monodispersed particulates (Casals et al., 2008). That is because engineered nanomaterials are usually synthesized under more control environmental conditions of pH, temperature and concentration so that they can have the required shape and size (Klaine et al., 2008) and other physicochemical properties that will make them appropriate in specific applications for example drug delivery and tissue restoration (Mahmoudi et al., 2009).

According to Roco (2005), four generations of nano-technology advancement were envisioned from 2000 to 2020 in an advancing order. Starting with the first generation, it includes simple components such as nanotubes, nanolayers and nanocoatings. The second generation which was predicted for after 2005, includes nanostructures that can alter properties (for example, shape, morphology, magnetic and biological) at some point in process. With the potential to be used in medical applications and electronic devices. In medical application in the field of cancer diagnosis and therapies NPs were successfully used in developing targeted drug delivery mechanism (Davis, 2008; Baker, 2009). The third generation which was predicted after 2010 includes self assemble nano-systems such as organs and devices. Lastly, the fourth generation between 2015 and

2020 includes a nano-system with a molecule having definite structure as well as function in the system.

Today, two decades after nanomaterials were first deliberately used in consumer products, the extraordinary physiochemical properties of NPs have resulted in numerous applications. A wide variety of these materials have been incorporated into manufactured products. For instance, carbon nanotubes with high electrical conductivity, stronger than steel and harder than diamond find their application in the construction of electronic devices, aircrafts and cars (Kumar and Ando, 2010).

There are numerous NPs with specific application such as $CoFe_2O_4$-NP, Fe_3O_4-NPs, TiO_2-NPs, and cryptomelane type manganese dioxide NPs in catalytic activity. Bismuth containing oxide and carbon $Bi_2O_2(CO_3)$-NPs, poly (fluorenylene phenylene), celecoxib-NPs are used in medical applications. Li-complexes, copper–selenium-complexes, Co-B NPs and silicates rubber polypyrrole complexes are used in electronic equipments. Zn–Mn–Fe-NPs and Agl cysteine NPs in sensors. Cellulose manganese oxide are used in the removal of Pb from liquid composite (Peralta-Videa et al., 2011).

2.2 Soil overview

Soil is an ordered, diverse and irregular structure. It is a complex and dynamic mixture of different charged particles, dissolved inorganic and organic species, living organisms. Soils are spatially and temporally heterogeneous, where chemical equilibrium might not be achieved (Lindsay, 1979, Theng and Yuan, 2008). The thin surface layer of soil is a vital component of the earth's biosphere for food and fiber production. The majority of land based organisms depend on it for survival (Gliessman, 1984). Soil also contributes to the maintenance of environmental quality at local, regional, and global levels.

Almost 80 to 90% of the micro-organisms inhabit solid surfaces of the soil. Lithosphere, which is the most upper of the earth's surface contains soils that are rich in nutrients. It contains diverse organic matters both in living and dead forms and inorganic matters including minerals such as calcium, phosphorus, and potassium. The organic matters comprise humus, which is vegetation decayed by microbes. The amount of organic materials is reflected in the color of the soil. Color of the soil can be used to characterize soil property. For example, brown soil contains more organic content and has acidic pH

ranging between 5 and 7. These properties are characterized with high fertility that can support a range of plants.

Soil texture is determined by different percentage of soil minerals namely sand, silt and clay that are classified based on their size from the smallest to the biggest as sand, silt and clay. Different soils exhibit different degrees of acidic or alkaline properties. Acidity in a soil may indicate the absence of alkaline causing ions such as calcium and potassium and this can be as a result of leaching. Soil organic components include polysaccharides, enzymes, natural organic matter, and organo-metal complexes (Hayes and Bolt, 1991; Hassellov and von der Kammer, 2008).

The abiotic portion of soil matrix has an approximate volumetric composition of 50% solids, 25% liquids, and 25% gases (Lindsay, 1979). The solid and colloidal phases include minerals such as Fe, Al, and Mn oxides and hydroxides while the solid surfaces are often coated in natural organic matter (Hiemstra et al., 2010). Soil solution is defined as the liquid fraction, which passes through a 0.45 μm filter. Agricultural soil solutions have a pH in the range of 4.5 to 8.0, and contain a variety of charged species. Inorganic species include protons, cations (e.g., H^+, Ca^{2+}, and Mg^{2+}), anions (e.g., NO_3^- and PO_4^-), metal oxides and hydroxides.

Soil microbial population has high diversity and it is estimated around six thousand variety of unique bacteria per gram of soil. They play an essential role in nutrient cycle by converting dead and waste materials into useful plant nutrients. The microbial biomass of bacteria and fungus was estimated (1 to 2 tones) and (2 to 5 tone) per hectare of soil respectively (Torsvik et al., 1996; Killham, 1994). These high diversities make soil a complex microhabitat (Nannipieri and Badalucco, 2003). One of the main ecological challenges is predicting the exact species distribution in the soil. This leads to an absence of accurate information on the species that essentially contributed to experimental results. However, the growing new methods including use of laser scanning, genetic methods, electronic microscope were able to identify soil microorganism genera as well as organic- and inorganic nanoparticle (Forster, 1994; Assmus et al., 1995; Bakken, 1997).

2.2.1 Soil as a microbial habitat

Soil microhabitat is very dynamic in that it differs chemically, physically and biologically both spatially and temporally. Compared to vitro, which has well defined nutrients, soil usually constitutes less nutritional and energy resources for microbes that live in a unique microhabitat (Stotzky, 1997). The size of a habitat is proportional to the organism size that could range from micrometers such as bacteria to millimeters sized mulitcellular organisms (Coleman and Crossley, 1996). Though soil occupies a very vast area, microbes occupy only less than five percent of the space (Ingham et al., 1985). Only few habitats have the optimum environmental condition for biological life. The distribution of those microbes in the soil is spatially and temporally different. In 'hot spots', where there is high organic matter or manure, have higher microbial activities (Sexstone et al., 1985; Parkin, 1987; Petersen et al., 1996; Lynch, 1990; Pinton et al., 2001).

Quite a lot of environmental factors affect soil microbes' biology, activities and populations. Those factors include nutritional sources, organic matter, other microbes. Physicochemical factors include acidity or basicity, moisture, heat. These environmental factors can be highly dynamic and consequently change the soil microhabitats.

2.2.2 Soil and microbes interaction

All organisms including microbes, plants and animals that interact with soil, mix the soil and add fresh organic materials to the soil. The quality of the soil is reliant on its physicochemical, biological, and bio-chemical properties (Tate, 2000). Biochemical and biological properties particularly microbiological properties are very sensitive to the change in soil properties. The microbial activities in the soil such as enzyme activities greatly affect soil fertility, ecology and health.

The mechanism of soil and microbial interactions were well investigated. Some microbes for example, bacteria release polysaccharide in to the soil that interacted with soil clay (Chen 1998; Huang and Bollag, 1998). Soil can take up molecules by adsorption in to clay or humic such as protein, DNAs, RNAs and enzymes. Those molecules could be protected from degradation and denaturing by heat, alkalinity or acidity in the soil. For example, nucleic acids could be protected against degradation by

nucleases and subsequently they will be able to maintain their activity in recycling organic matter, bacterial genetic transformation(Nannipieri et al., 1990; Lorenz and Wackernagel, 1987; Khanna and Stotzky, 1992; Paget et al., 1992; Pietramellara et al., 1997).

Soil mineral components can involve in multiple catalase reactions. For example, clay minerals, Mn(III) and Fe(III) oxides involve in electron transfer reactions and abiotic reactions such as deamination, polymerization, and polycondensation which are an essential part of soil activities. Comparing between microbial related reactions and abiotic reactions, the biotic reactions are perceived to increase under natural conditions, however, abiotic onse are perceived to increase under a harsh conditions where the microbial activity is slow. It was therefore assumed that during hostile environmental conditions, abiotic activities take over microbial activity in soil (Huang 1990 and Ruggiero et al., 1996). However, there are no accurate methods to confirm the assumptions (Huang, 1990; Ruggiero et al., 1996)

2.2.3 Soil and nanoparticles interaction

Soil physicochemical properties have been found to influence NPs properties. For instance, aggregation rate of TiO_2 NPs was found to be negatively correlated to dissolved organic matter and clay contents, and positively correlated to the ionic strength, zeta potential and pH. Soil pH is one of the soil characteristics that can affect NPs properties. That is, property of the soil system such as pH and ionic strength strongly influence the charge of soil solid components. The charge of humic molecules and clay particles in turn influences their interaction with NPs.

The ionic strength of soil solution can affect the stability of particles electrical layers. For example, the effect of ionic strength was shown when aggregation of titanium dioxide in TiO_2 NP increase 50 folds in diameter as ionic strength increased from 1 to 100 mM (Tourinho et al., 2012). That is an increase in the ionic strength decrease the electrical double layer thickness, which will lead to an increase in agglomeration. Moreover, the effects of ionic strength were observed on aggregation of AgNPs that were both uncoated and coated (citrate, and sodium borohydridecoated-coated). Soils with low ionic strength and high dissolved organic matter have low sorption of NPs,

suggesting that these factors may affect the bioavailability of NPs in soils. However, the effect might not be the same when considering soil matrix instead of water suspension.

In soils, dissolved organic matter for example humic substances can be sorbed to NP surfaces, affecting NPs properties. The effect of organic substance on nanoparticles in turn is a function of soil characters such as soil pH. At environmental pH, humic substances are negatively charged therefore this will make the particle negatively charged. This can contribute to particle stableness and prevent aggregation as well as precipitation. Moreover, it may also reduce their bioavailability and uptake by decreasing their affinity to the cell membranes and thus possibly their ecotoxicity. On the other hand, Ghosh et al., (2008) showed humic acid to cause aggregation of Al_2O_3 NPs at low pH. Further, it was shown that ZnO NPs were attached with organic matter of soil matrix at acidic pH showing the possible effects of organic matter on nanoparticles distribution (Kool et al., 2011).

Stability and interaction of NPs in soils contribute an essential part in NPs transport, their fate, as well as toxicity. Moreover, in addition to the NPs concentration, their bioavailability, mode of exposure to organisms are essential in determining NPs ecological effects (Tourinho et al., 2012).

2.2.4 Soil physicochemical indicators

Soil quality is used as an indicator in assessing factors affecting soil functionality. Those indicators are useful in sustainable use of soil for agricultural purpose and in maintaining environmental and human health (Dexter, 2004; Larson and Pierce, 1994).

Soil physical indicators include soil texture, structure, consistence, density, pore space and color. Good physical quality of soil can be exhibited as having good water infiltration and run off, good air circulation, routable and workable. While soil chemical indicators include availability of nutrients and minerals and their cycling, salinity, pH, cation exchange capacity, pollutants and so on. These indicators can be used to predict the presence of organisms, the availability of nutrients and water, and mobility of contaminants (Anderson, 2003; Dexter, 2004)

2.2.5 Soil biological indicators

Microbial properties of soils are good indicators of soil health. These properties reflect a relationship between, ecosystem sustainability, microbes' diversity, and soil and plant quality (Hill et al., 2000). Soil biological indicators measure organic matter accumulation as well as mineralization. Biological indicators attempt to measure or monitor soil biological activities, structural developments and storage of nutrients (Gregorich et al., 1994). Biological indicators include microbial biomass, nitrogen mineralization, soil respiration, faunal population, decomposition rate and ratios of microbial biomass to total carbon and respiration rate to microbial biomass ratio.

Soil microbial activity plays an important role in the nutrient cycle that releases available nutrients for plants. It also contributes to mineralization and mobilizing of pollutants. Factors such as the availability of nutrients, water and oxygen, as well as temperature and availability of protons affect activity of microbes in the soil. Respiration measures microbial activities as a rate of CO_2 release that is related to decomposition of organic matter. In relation to respiration, a commonly used index is metabolic quotient (qCO_2), the ratio between respiration rate and microbial biomass. It describes the rate of mineralization of organic substrate to microbial biomass (Bastida et al., 2008).

Soil microbes are responsible for the biochemical processes that take place in the soil. Soil enzymes play important role in the overall biochemical functions (Ebersberger et al., 2003; Kandeler et al., 2006). In plant nutrition, the role of enzymes is quite essential in dissolving the nutrients in ionic forms, which are the key for the survival of life. Soil has many enzymes including Hydrolases, Lyases, Oxidoreductases, Isomerases, and Ligases. Soil enzymes play an essential role in biochemical processes of energy and material transformation (Gu et al., 2009). Enzyme activity assays are common measurement of microbial activity and can be used to determine effects of changes in the environmental condition for example due to pollution or land-managements (Burns, 1978).

Enzymes are the important indicators for the soil biogeochemical processes. They are active not only within living cells (intracellular) but also independently active as extracellular enzymes. Extracellular enzymes can be actively secreted either by plants

and microorganisms or from dead or decomposed cells. Extracellular enzymes in the soil have an important role in soil property, ecology, and health. They play an important role in agriculture. They catalyze several important reactions in soils including nutrient cycling and the decomposition and formation of organic matters (Sinsabaugh et al., 1991; Dick et al., 1994). The enzyme levels in soils depend on the composition and amount of organic matter as well as microbial processes and activities. The available substrate, which is the source of energy for the microbes and soil enzymes catalysis, is the main factor determining microbial activities. (Kiss et al., 1978).

For many years, the contribution of extracellular enzymes secreted by microbes and their role on the decomposition of organic matter have been ignored (Ceron et at. 2005). The activity of extracellular enzymes can be used as indicators for the quality of the soil, nutrient cycle and organic matter decomposition (Gelsomino et al., 2006). In addition to providing early information about the change in soils, the advantage of using enzymes as indicators is their proximity and relatedness to soils' organic matter, physical characteristics, and microbial biomass and activity (Dick 1996; Nielsen et al., 2001; Eldor, 2007). On the down side, the sources of soil enzymes could vary greatly. The soil enzymes could be from bacteria, fungi and plants, they could be from dead or living cells or they could be also intracellular or extracellular origin. Moreover, there could be variation due to association in soil matrix with clay or humic molecules. Therefore, it requires a great optimization to obtain the best values. Thus, laboratory conditions such as pH, treatment length or period, temperature, ionic strength and substrate concentrations need particular attention.

Dehydrogenases are one of the most important enzymes, and are a good indicators of soil microbial activity (Quilchano and Maranon, 2002; Gu et al., 2009; Salazar et al., 2011). Not only are they found intracellular but they are also found in all living microbial cells. Dehydrogenases activities are very important indications of soil fertility and their activities can be used to determine disruptions by pollutants such as pesticides or changes due to management practices (Wilke, 1991). Moreover, dehydrogenase activities are generally considered to be proportionally related to the soil microbial biomass.

Soil dehydrogenases represent majority of the oxidoreductase enzymes. They contribute in the production of energy by oxidizing organic compounds. That is, hydrogen atoms

are transferred from organic donor to inorganic acceptors, nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) (Zhang et al., 2010). Because hydrogen atoms take part in reductive biosynthesis, dehydrogenase activities in the soil depend on the activity of different dehydrogenases (Subhani et al., 2011). Even though, most dehydrogenases are produced by anaerobic microorganisms, dehydrogenases can still use the two oxygen atoms (O_2) as electron acceptors (Brzezińska et al., 2001). Therefore, dehydrogenases activity, could serve to measure soil microbial oxidative activities adequately.

2.3 Ecotoxicology of nanoparticles in soil

Soil has a great diversity of microbes including fungi, bacteria, and archaea. Microbial biodiversity includes variation at different levels. Those variations could be within species as in genetically or within community as in functional group, species abundance and richness (Torsvik and Ovreas, 2002). The available literatures suggest several effects of nanoparticles on the soil microbial community. Therefore, those effects of nanoparticles on microbial diversity can help explain the mechanism at which the soil ecosystem functioning is affected.

Studies have shown NPs affect soil microbial diversity by altering soil microbial community composition, though the archaeal and fugal communities are not well studied (Simonin and Richaume 2015; Prasad et al., 2016). For example, Ag NPs altered bacterial community structure, after short-time exposure at both low and high concentrations (Kumar et al., 2011; Colman et al., 2013).

Considering the critical ecosystem services delivered by beneficial microorganism including nitrifying and nitrogen fixing bacteria, rhizobacteria, and fungi, their sensitivity and the impact of NPs needs a greater attention. (Giller et al., 2009; Judy and McNear, 2015). Nitrifying bacteria are important soil microorganisms that through nitrification convert ammonia to nitrate (Roh et al., 2009). That is ammoniaoxidizing bacteria will first convert ammonia to nitrite afterwards nitrite is converted by nitriteoxidizing bacteria into nitrate. Multiple studies have demonstrated the sensitivity of nitrifying bacteria to AgNPs. It was shown that AgNPs decreased NH_3 oxidation, inhibited growth and abundance of nitrifying bacteria (*Nitrosomonas europaea*). Those effects were related to the change in expression of genes involved in the process of

energy production and nitrification (Choi and Hu, 2008 ; Yang et al., 2014; Radniecki et al., 2011).

Nitrogen fixation is one of the important roles played by the nitrifying bacteria in the soil. Fixed nitrogen is used in the biosynthesis of essential molecules including nucleic acids and proteins (Dimkpa, 2014). NPs can impact nitrogen fixation in plants. For example, when CeO_2 NPs and WO_3 NPs entered into roots and root nodules of leguminous plants (soybean) they showed detrimental effects on the growth of nitrogen fixing bacteria and subsequently on the plants (Priester et al., 2012; Allard et al., 2013). Fan et al., 2014 showed the effects TiO_2 NPs on cyanobacterium growth, and N_2-fixation and storage (Cherchi and Gu, 2010). It was shown that TiO_2 NPs negatively affected *Rhizobium leguminosarum* and legume (peas) symbiotic relationship. That is TiO_2 NPs caused distortion on the cell surface of bacterial cells. This disrupted root nodule development and subsequent nitrogen fixation, which in turn caused a change in structural composition nodules cell wall that resulted in reduced proliferation of lateral root. Moreover, Bandyopadhyay et al., (2012) showed toxic effects of CeO_2 and ZnO NPs to nitrogen fixing bacteria (*Sinorhizobium meliloti*) with a change in the extracellular substances of their cell wall. CeO_2 NPs showed a bacteriostatic effect, whereas ZnO NPs showed bactericidal effect to *S. melba* strain.

Soil has high microbial diversity and effects of NPs to the microbial diversity will have detrimental effects on the soil ecosystem function. Studies in bacteria, archaea, and fungi have shown that nanoscale zerovalent iron can make a considerable change in the soil microbial community over a very short period of time (Pawlett et al 2013; Tilston et al., 2013). For example, the abundance of denitrifying bacteria and chloroaromatic mineralizing microbes was decreased by nZVI (Fajardo et al., 2012; Tilston et al., 2013). As a result, nitrogen cycle and biodegradation capability of some functional microbial groups were affected.

Carbon nanoparticles, for example MWCNT showed a considerable alteration of the bacteria community structure (Khodakovskaya et al., 2013; Shrestha et al., 2013). Similar Jin et al., (2014) showed a decrease in gram positive and gram negative bacteria as well as in the fungal biomass by SWCNTs. Moreover, at high concentration, SWCNT showed change in the structure, abundance and activity of the fungal community. Frenk et al., (2013) showed exposure to CuO NPs causes changes to community composition

of microbes and significant decrease in oxidative potential in clay soil. Moreover, addition of CuO to the soil showed negative effects on Rhizobiales and Sphingobacteriaceae which are an important group of soil bacteria. In a mesocosm experiment on wetland sediment microbial communities, one pulse of AgNPs exposure (1 mg kg^{-1}) showed significant effect on sediment microbial communities on short-term (30-60 days) exposure, but not in the long-term (300 days) (Moore et al., 2016).

Arbuscular mycorrhizal fungi are very well known symbiotic soil microorganisms found in the root of most land plants. They do not only help plants acquire mineral nutrients, especially phosphorous but also provide protection from pathogens, heavy metals, agricultural weeds and improve soil structure. Judy et al., (2015) investigation on the effects of Ag_2S-NPs, PVP-Ag NPs and Ag^+ on arbuscular mycorrhizal fungi found that all the tested NPs inhibited microbial community (bacteria, actinomycetes, and fungi) structure and their colonization of tomato (*Solanum lycopersicum*). At environmental relevant concentration (1 mg kg^{-1}) Ag_2S NPs showed detrimental effect to the microbial community and the subsequent ecosystem service they provide. The study on the effects of pristine Ag-NPs on mycorrhizal colonization of sunflower root (*Helianthus annuus*) by Dubchak et al., (2010) showed inhibition of mycorrhizal colonization on sunflower root. In another similar study Feng et al., (2013) showed the effects of both iron oxide (FeO) and Ag-NPs on arbuscular mycorrhizal fungi colonization of clover roots, the result showed considerable reduction in clover biomass due to the exposure to FeO NPs. The decrease in biomass was explained due to reduction in glomalin–glycoproteins which is formed both in the hyphae and spores of the fungi and plays a role in improving soil quality by connecting mineral particles together (Gillespie et al., 2011).

Studies on ecotoxicological effects of NPs under laboratory conditions and natural conditions on plant growth-promoting rhizobacteria are rare (Mishra and Kumar, 2009). Soil borne, free-living rhizobacteria enhance the growth of the plant by a combination of physiological attributes such as asymbiotic nitrogen fixation and phytohormone production, such as, indole-3-acetic acid (IAA), cytokinin, gibberellins (Hayat et al., 2010; Hinsinger et al., 2009). Studies have shown inhibitory and toxic effects by TiO_2, $Al2O_3$, TiO_2, ZnO, and SiO_2 NPs on plant growth-promoting rhizobacteria with high mortality rate e.g. ZnO 100% (Karunakaran et al., 2013). Culturability of engineered strains of bacterial *P. putida* and *P. chlororaphis* were negatively affected by Ag, CuO,

and ZnO NPs. Those effects were reflected on primary metabolism that reduced light emitting as well as in secondary metabolites reducing the production of indole-3-acetic-acid (IAA) and the antifungal compound, phenazine (Gajjar et al., 2009; Fang et al., 2013; Dimkpa 2014; Dimkpa et al., 2015). Gurunathan, (2015) demonstrated antibacterial activity graphic oxide NPs against soil rhizobacteria of multiple *Bacillus species*. Differential effects were shown based on dose, time and strain type and *B. megaterium* and *B. marisflavi* were most and least toxicological affected respectively.

2.4 Nanoparticles characterization and risk assessment.

Nanomaterials behave differently with respect to their bulk counterpart. This is because of their small size, high surface area and potential to modify their surface composition especially with regard to their interaction with biological systems (Hassellöv et al., 2008). Therefore, it is necessary to characterize their physical and chemical properties which include stability, bioavailability and dissolution within biological media. This will add to an implementation of an adequate design and detailed investigation on how NPs interact with the environment and the biological systems (Hinderliter et al., 2010; Meißner et al., 2010). Moreover, handling of samples with NPs can be critical when risk assessment is made. This is because NPs could be adsorbed to the walls of analytical instruments including bottles and tubes (Hassellöv et al., 2008).

Even if NPs are found to be non-toxic, the internalization and distribution patterns as well as the quantification of NP amounts within organisms and cells can give information on NPs biological mechanism (Praetorius et al., 2014). The application of environmentally relevant concentrations has advantages in avoiding the occurrence of non-specific accumulation effects. That can be related to an overload of the biological system with NPs. This can lead to mechanical effects such as the attachment of NPs onto the cell or organism surface and subsequent penetration is reduced (Ma and Lin, 2013). With environmentally relevant concentrations that are usually very low and consequently more sensitive analytical detection and quantification techniques are required.

A number of techniques have been used to assess effects of NPs on soil microbes. Some of the techniques include phospholipid fatty acid (PLFA) fluorescent in situ hybridization (FISH), next generation sequencing (NGS), terminal-restriction fragment

length polymorphism (T-RFLP). Detection and quantification of NPs in environmental samples including water, air, soil or in consumer products is challenging due to lack of technological and analytical techniques. As an alternative, NPs consumption or sink and accumulation is analyzed (Fabricius et al., 2014).

Various characterization techniques are used for characterization of NPs chemical and physical properties of which can vary, depending on whether the particle is in powder, an aqueous particle suspension, or particles suspended in other related media. Currently the absence of proper procedures to characterize NPs in the environmental matrix and the lack of improved characterization techniques poses a challenge for the ecotoxicological study of NPs in the soil. Though dynamic light scattering (DLS) and microscopy techniques including atomic force microscopy, scanning and transmission electron microscopy can be used to analyze NPs in aqueous solutions, characterization of NPs in soils matrix has proven to be difficult. For ecotoxicological studies, NPs properties that are considered to have a control on NP stability and consequent transport and availability to organisms are proposed to be characterized. Those characteristics include size, surface area, charge, surface chemistry, agglomeration, aggregation and dispersibility. Quantifying and characterization of these properties as they interact with soils can give better assessment. However, the majority of the available techniques are limited to aqueous solutions (Tiede et al., 2009). The other challenge of characterization is that several nanoparticles, for example, Ag-NPs and ZnO-NPs release ions over time. The dissolved ionic fraction in the suspension or the test media can be determined by ICP-MS which in turn requires a separation of the particular fraction beforehand by centrifugation, filtration or dialysis (Misra et al., 2012).

For particle powders, the morphology and primary particle size can be investigated by electron microscopic techniques. Investigation with scanning electron microscopy (SEM) produce three dimension image while with transmission electron microscopy (TEM) produce a two-dimension image (Hassellöv et al., 2008). Furthermore, surface area of the NPs in powder form can be obtained by gas adsorption using for example, Brunauer-Emmet-Teller method (Meißner et al., 2010). Similar techniques can also be adapted for characterization of NPs in suspension. However, unlike in powder the preparation of a NP suspension involves dispersion procedures to ensure homogeneous and stable suspensions.

Most of ecotoxicological studies of NPs on soil microorganisms have used pristine NPs at concentrations that are higher than the environmentally relevant but also often with artificial soils. In toxicological testing of NPs, it is necessary to consider investigation of NPs effects under environmentally relevant conditions such as concentration and the possibility of agglomeration of NPs over time and subsequent sedimentation which could reduce their toxic effects. On the other hand, the application of stabilized and mono-disperse NP suspensions could represent worst-case conditions (Kroll et al., 2013). For biological testing NPs stabilization is done, for example, by adding coating substances, to prevent the agglomeration of the NPs which could lead to sedimentation. However, the influence of the stabilizing substance needs to be excluded by using appropriate controls.

Particle size distribution and the agglomeration behavior are other important particle characteristics in suspension and these parameters can be obtained by DLS (Hassellöv et al., 2008). DLS is used to determine zeta potential of NP suspensions, which measures the magnitude of the repulsion or attraction of electrostatic charge between particles. Charged NPs form double electrical layers, one layer is the charged surface sites while the second layer is with the ions in the solution as they are attracted towards particle surface responding to the charge. The value of zeta potential is the measure of the electrical potential at the edge of the two layers. One layer which is the diffused circular and second layer the bulk solution.

Zeta potential is among the factors determining the stability of nanoparticles, the value of zeta potential can explain the dispersion or aggregation of nanoparticles. High absolute values of the zeta potential, more than ±40, indicate strong electrostatic repulsion forces corresponding to a higher stability of the suspension, while low zeta potential, less than ±20, imply weak electrostatic repulsion forces thus a rapid aggregation or less stable particle suspension (Greenwood et al., 1999 ; Bohme et al., 2014).

2.5 Factors affecting nanoparticles microbial toxicity

Once in the environment there are multiple factors and properties such as size, charge, density, surface-coating, as well as agglomeration rate that play major role in promoting NP’s toxicity (Neal 2008; Fabrega et al., 2009; Dinesh et al., 2012). Fate and

bioavailability of NPs determine the potential hazards of NPs to the environment. Bioavailability is the concentration of NPs that are available in the environment to which organisms will be exposed that may cause effect to the organisms (Nowack and Bucheli, 2007). Most of the time, the bioavailability of NPs particularly in soil is lower than its initial concentration in the natural environment. This is because there are different chemicals in the environment especially in soil in which the NPs can interact and be transformed before they can be bioavailable to affect organisms (Soni et al., 2015). As a result, the fate of NPs in the environment will eventually be determined by multiple factors (Böhme et al., 2014).

NPs physical and chemical properties such as size, shape, solubility, acid–base are the main properties that can determine their behavior, fate and ecotoxicity. Those characteristics will eventually determine the degree to which NMs undergoes transformation including aggregation, agglomeration, sorption and dissolution.

2.5.1 Size and surface charge

The small size and high surface area of NPs makes it possible to pass through the biological barriers and potentially enhance their biotoxicity. Small sized nanoparticles are more inclined to cellular internalization and show more toxicity than the larger ones (Hsiao and Huang 2011; Shang et al., 2014). Surface charge of particles affects nanoparticles characteristics such as agglomeration, aggregation and stability. For example, due to the hydroxyl group, metal-based NPs will get the net surface charge. The magnitude of the charge will be based on the surface groups and solution properties mainly pH and concentrations.

Under empirical investigation, the stability of NPs is an important characteristic to predict the mechanism and impact of the nanoparticle. Change in zeta-potential can be used to correspond to the change in particle surface charge. At low pH, zeta potential value for metal or metal-oxide is positive but as the pH increases, zeta potential value changes to negative. At isoelectric point, a point where zeta potential approach zero, the aggregation rate increases due to reduced repulsive force between particles. At zeta potential greater than 30 mV charged particles will be stable (Guzman et al., 2006; Jiang et al., 2009). For example, uncoated TiO_2 NP aggregate size changed with the pH of the

solution. However, coated silver NPs didn't show change in its aggregate size over a range of pH and this was suggested to be due to the narrow variation in zeta-potential at the pH range (Fabrega et al., 2009). El Badawy et al. (2010) investigation using different types of silver NPs showed significant effect of surface coating as compared to uncoated on the properties of NPs. Therefore, characterization such as how long coatings will be maintained by the particles without changing the manufactured surface properties is needed. Moreover, properties such as reversibility and biodegradability of the coating material may influence coating stability over time.

2.5.2 Aggregation and agglomeration

Aggregation and agglomeration have great effect on NPs fate and behavior in the environment. Aggregates are clusters of particles joined with chemical bonds that are strong whereas agglomerates are joined together with weaker forces that can be reversed for example (Van der Waals forces) (Illustration 2.1). The use of those terminologies is important for evaluating effects of NPs in terms of toxicity (Hartmann et al., 2014). However, in many NPs exposure studies the term aggregation is often used in cases where permanent bonding of particles does not exist but only where agglomeration has occurred.

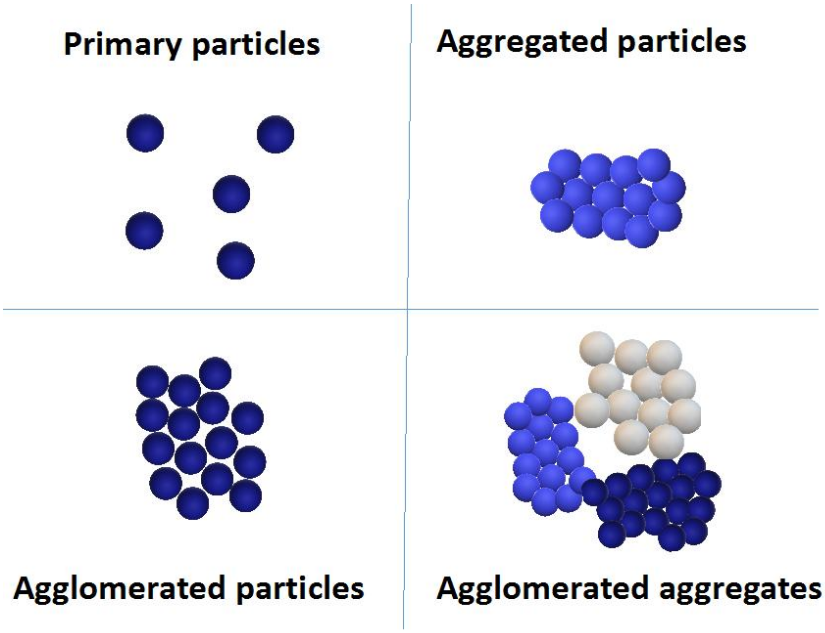

Illustration 2.1 Difference between primary particles, aggregates, agglomerated, and agglomerated-aggregates.

Physical forces such as Brownian motion, gravity, fluid motion and NPs properties such as surface charge and size affect agglomeration and aggregation of NPs. For agglomeration to occurs particles move by Brownian motion and the attraction energy among particles need to be greater than the energy of repulsion. However, for aggregations to occur the cores of particles should collide to make a contact. Aggregation may form sufficient size depending on the original particle size and concentration in which it could sediment in solution with gravitation. High concentrations and bigger original size particles were found to result in higher aggregation rates and size (Phenrat et al., 2006; Tourinho et al., 2012).

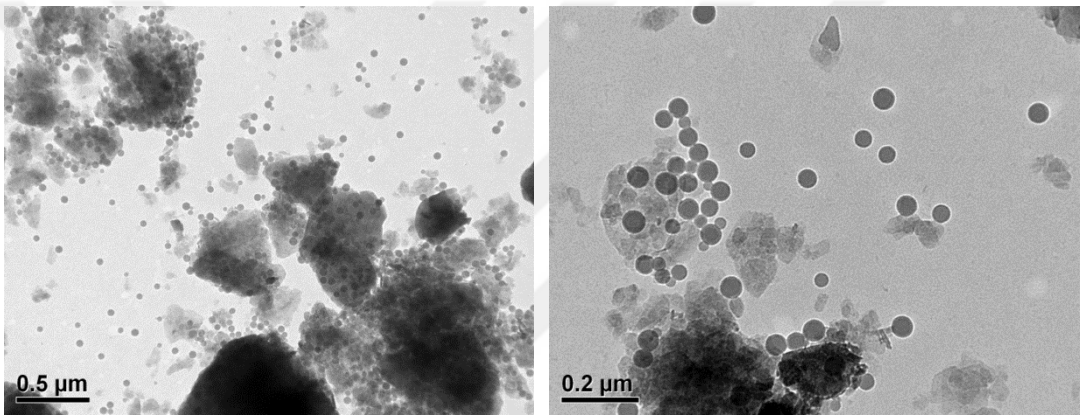

Figure 2.1 Reflection electron microscope (REM) image of PS NP in soil. (Tsegai et al., 2018)

Aggregation and agglomeration will also depend on NPs type (Wang et al., 2009; Lin and Xing. 2008). For example the size distribution of aggregates of ZnO-NPs that were non aggregated in aqueous solution eventually formed aggregates as big as tenfold bigger than the original NP (Pipan-Tkalec et al., 2010). While titanium dioxide NPs were evenly distributed with agglomeration (Jemec et al., 2008).

2.5.3 Dissolution and transformation

Studies have shown that the fate and the chemical nature of NPs, even the pristine materials, changes in the environment. Dissolution is the commonly known transformation reaction as the solubility of particles increases with the decrease in size. In natural environment the effect of size is probably coupled with other process such as

deposition, aggregation, coating and/or transformation. The dissolution of comparatively soluble NPs such as AgNPs, ZnO most probably will enhance in natural soils. Those particles will likely be transformed to stable compounds in the environment including soil. For example, silver transforms into Ag_2S forming uniform size Ag_2S NPs as compared to the original Ag NPs. Unlike the more soluble particles the relatively less soluble NPs, will likely either accumulate or leach out of the soil. On the other hand, for NPs for example ZnO, as Zn becomes associated with iron oxides during aging, they lose their NP character. Moreover, carbon-based NPs in soil possibly undergo biodegradation and their rates of degradation will depend on the available enzymes in soil.

Over time both the properties of the core material and coating of NPs are likely to change in soils, such changes possibly occur abiotically such as by oxidation followed by dissolution. Moreover, reactive oxygen species could contribute to the degradation of carbon based NPs. Removal of coating materials are expected to reduce NPs mobility in soils. However, using naturally occurring organic matter for coating may prevent degradation (Cornelis et al., 2014; Karimi et al., 2017).

CHAPTER 3

POLYSTYRENE NANOPARTICLES

3.1 Polystyrene and applications

Polystyrene is a synthetic aromatic polymer and it is made by polymerization of styrene monomers. Industrially, styrene monomers are produced from ethylbenzene through the process of catalytic dehydrogenation.

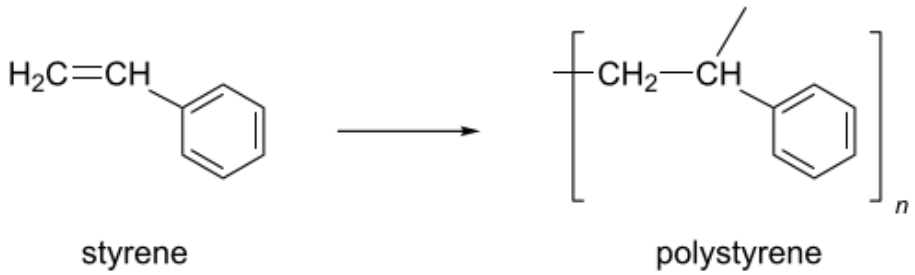

Figure 3.1 Polymerization of styrene to polystyrene.

Polystyrene is one of the most extensively used types of plastic. Between 1950 and 2014 production of plastics globally expanded from 1.5 to 311 million tons (PlasticsEurope, 2015). Polystyrene makes up top five of produced types of plastics, which account for approximately 90% of the total demand volume. The use of polystyrene has increased over the past 20 years mainly driven by consumerism and convenience along with the comparatively low price. Polystyrene, is commonly used in packaging, storage, disposable cutlery, CDs, in construction as insulator, in medical products and toys (PlasticsEurope, 2014; Maul, 2007).

PS NPs have found a broad range of application because of their nanoscale size distribution that ranges from 50 to 300 nm and their uniform shape, They are being used in different fields including biomedicine, optical sensors, paint coating, food, and textile industries. Polystyrene nanoparticles have application industrially such as drug delivery

in nano-medicine. In drag delivery, PS NPs modified with vancomycin-antibody were produced as probes to capture biomolecules (Kell, 2007). Similarly, PS NPs are used in drag delivery to deliver antimicrobial peptide (antibiotic) as polystyrene-sulphonate in treating drug resistant gram-negative bacteria (Insua, 2017). Moreover, PS NPs are used in cosmetics such as personal care, in detergents and related products. Furthermore, PS NPs are incorporated in fluorescent dyes and polymer matrixes.

3.2 Polystyrene nanoparticles in the environment

Nanoparticles affect living organisms distinctly compared to their bulk materials (Ward et al., 2009). Polystyrene is commonly considered as non toxic. However, nano size polystyrene nanoparticles were shown to have a toxic effect. Due to the smaller size, which may enable them to easily pass membranes and internalize, the environmental impacts could be extensive. The concerns are that the environmental and ecotoxicological properties of nanoplastics will be different to their precursors microplastics (Lambert et al., 2014; Mattsson et al., 2015). Investigating toxicity of NPs to bacteria in the soil matrix is difficult because of the complexity of the soil environment. For example, a study by Masrahi et al., (2014) on the impact of Ag+ and polyvinylpyrrolidone-coated size 50 nm AgNPs and uncoated AgNPs of size 15 nm showed much more high toxicity by AgNPs than Ag+ basically due to complexation of Ag+ with soil organic and inorganic components. The interaction between soil matrix and Ag+ at this concentration possibly caused chelating with soil ligands such as thiol functional groups that have decreased its toxicity that is unlike AgNPs, which normally do not form complex with such ligands. Similarly, coated AgNPs had more detrimental effect on the nitrification process than Ag+, possibly due to the oxidative dissolution of AgNPs, however, toxicity of Ag+ was lower than the AgNPs under the same concentration. Moreover, in a natural ecological study, the toxicity of Ag+ on nitrification process at lower concentration showed no significant difference from that of the control. This is likely due to the interaction of Ag+ and AgNPs with the soil matrix. Further, comparing the dose effect of NPs at ecosystem scale and under laboratory-pure culture media showed much lower toxicity of Ag+ and AgNPs in ecosystem than that observed under laboratory pure culture media. For example under laboratory pure culture media 0.08 mgL^{-1} and 1 mgL^{-1} of Ag^{+} and PVA-coated AgNPs respectively

decreased nitrification Ag^+ by 50% and AgNPs by 86 %. This is likely due to the interaction of Ag^+ and AgNPs with the soil matrix.

Polystyrene nanoparticles can enter the terrestrial environment not only through direct release from products (e.g. biosensors) and applications (e.g. in photonics, in research and medical) during its life cycle but also through fragmentation of macro and micro plastics (Loos et al., 2014). Release of PS NPs from polystyrene floats used in aquaculture facilities through aging and biological (boring crustaceans) corrosion has been reported (Davidson, 2012).

Pollution by plastic particles in the environment has been described by many studies as an emerging worldwide threat. Though plastics can be recycled, recycling however remains insufficient. Most of plastic wastes are disposed in landfills with growing accumulation of plastic waste in the environment. As a result, millions of tons of plastics end up in landfills (North, 2013). Despite its durability, polystyrene products are usually made as use-and-throw to be used for a short time because of their low cost.

Once in the environment (e.g. landfills), plastics can undergo weathering through UV radiation, mechanical abrasion, biological degradation, and disintegration that result into smaller sized microplastics which could eventually fragment to nanoplastics (Singh, 2015). Many reports suggest a subsequent degradation of microplastics into nanoparticles (Andrady, 2011, Lambert et al., 2013; Mattsson et al., 2015). As a result, nanoplastics are expected to increase consistently with time in the environment (Corsi et al., 2014).

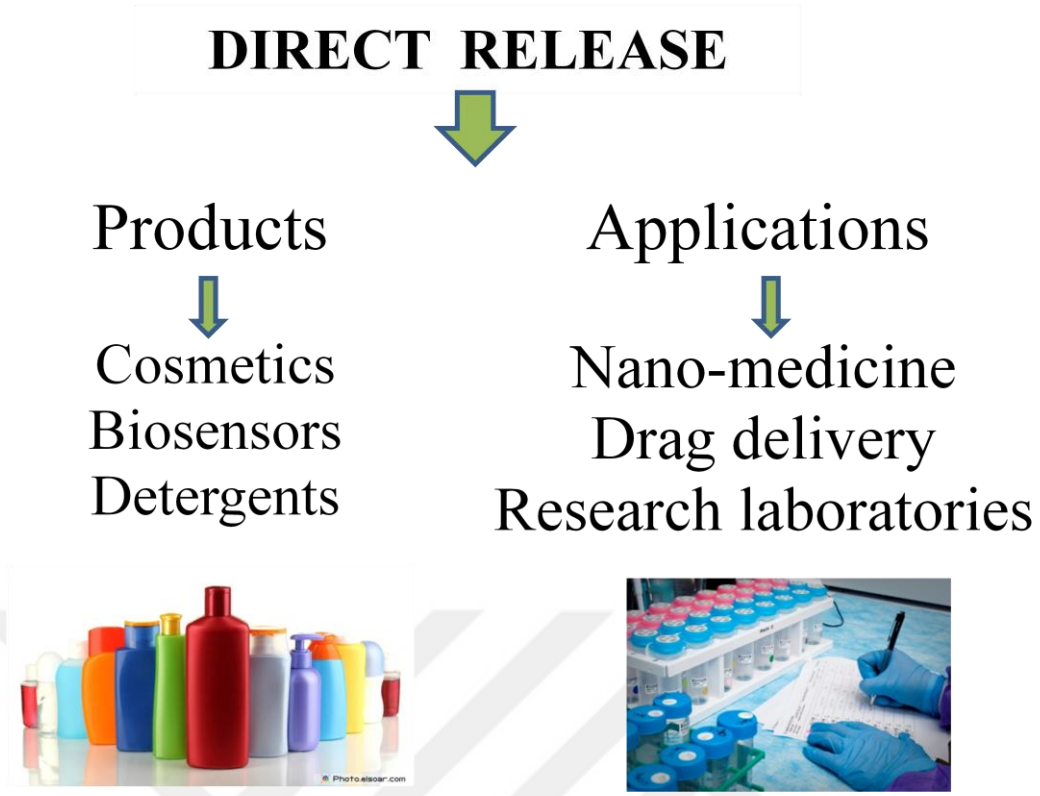

Illustration 3.1 Polystyrene nanoparticles release into the environment through fragmentation and direct release

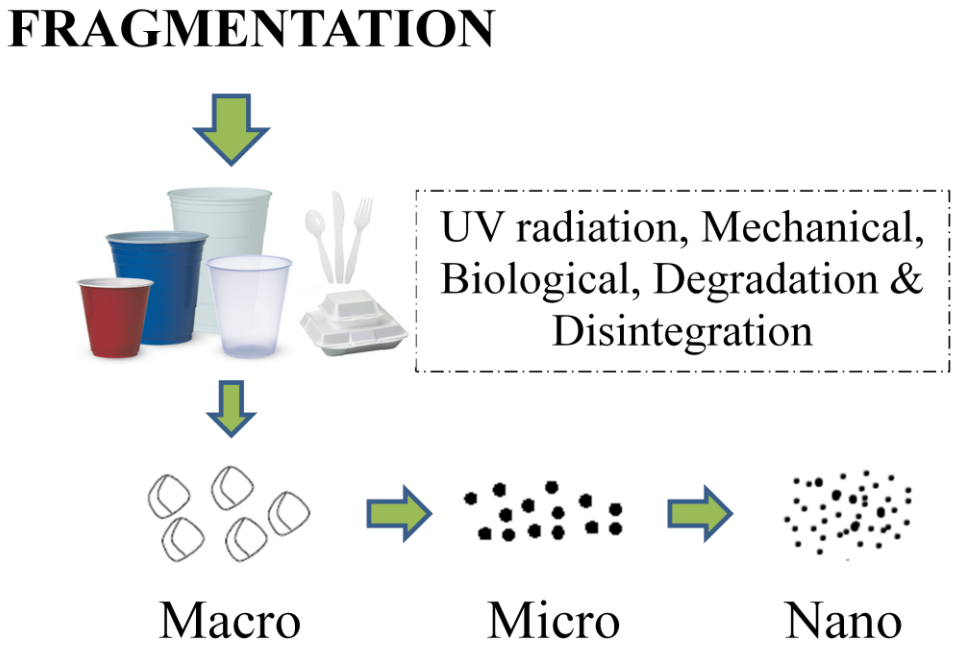

Illustration 3.2 Polystyrene nanoparticles release into the environment through fragmentation.

Lambert and Wagner (2016), investigated the effect of degradation on the formation of polystyrene nanoparticles using weathering chamber. In their investigation polystyrene disposable coffee cup lid were cut into small pieces (1 cm^2) and the sample, which were placed in a weathering chamber of temperature 30 ^oC were exposed for 24 hrs to both visible and ultra-violet lights. The formation of PS NPs was determined using nanoparticle tracking analysis at seven days interval over a period of 56 days. Their result showed an increased formation of nanoplastics over time. Moreover, there are reports of PS degradation by microorganisms (*Rhodococcus ruber*) (Mor, 2008), mandibulate insects and larva of mealworms (Riudavets, 2007; Yang, 2015). Furthermore, geophagous soil fauna, particularly earthworms, could contribute to fragmentation of plastics as ingested and broken down by their gizzard. Thermal cutting of polystyrene foam has been shown to emit nano-sized polymer particles, in the range of 22 - 220 nm that can enter the soil through atmospheric deposition (Zhang, 2012).

3.3 Impact of polystyrene nanoparticles in the environment

Polystyrene nanoparticles may well be a potential pollutant in the environment especially soil since soil is the major sink for nanoparticles. PS NPs can be toxic as well as bioaccumulate to impact higher trophic levels and the ecosystem as whole. In a molecular simulation, Rossi et al., (2013) used PS NPs to determine NPs effects on a model biological membrane. Their finding reveled potential effects of PS NPs on the biological membrane. That is PS NPs were able to pass easily into lipid membranes and dissolved in the membrane, thus, altering lipid membrane structure, softening the membrane and severely affecting membrane's lateral organization and significantly reducing diffusion. Change in membrane property and organization potentially disturb membrane proteins activities thus affecting cellular function. Similarly, carboxylated PS NPs of size between 40 and 50 nm were able to enter cells irreversibly (Salvati et al. 2011). PS NPs transfer has been reported in a food chain from algae to zooplankton and ultimately in fish causing behavioral and metabolic changes (Cedervall et al., 2012). Moreover, PS NPs were reported to have negative effects on development of sea urchin embryos, algal growth, and reproductive success of *Daphnia magna* (Mattsson et al., 2015). It was also demonstrated that polystyrene nanoplastics suppressed body size and number of neonates in Daphnia as well as change in reproduction with increased percentage in neonate malformations (Besseling et al., 2014).

The available studies suggest several effects of PS NPs mainly on aquatic environment but as far as the current study there are no previous studies on the effects of PS NPs on soil environment. Soil matrix is a complex medium which unlike the air and aquatic environment, it constitute minerals and heavy metals, dissolved organic matters. Therefore, the potential toxic effects of PS NPs on soil microorganisms deserves attention. PS NPs can interact with soil matrix and can pose serious danger to soil organisms and subsequent soil ecological functions. Not only that, once inside a cell NPs can potentially interact with variety of biomolecules inside the cell, which could possibly change the physicochemical attributes of the NPs (Nam et al., 2013). For example, polystyrene NPs of size 50 and 100 nm both amine- and carboxyle-functionalized and unmodified were all able to aggregate in-vitro, suggesting a varying toxicological mechanism (Sanfins et al., 2014; Smyth et al., 2015).

3.4 Factors affecting polystyrene nanoparticles toxicity

Physicochemical properties, including size, charge and surface coating are the key properties that are believed to determine PS NPs environmental behavior. According to their surface charge, which depends on the surface coating, PS NPs can be categorized into three main groups; these are cationic, anionic functional groups and non-functionalized (unmodified) PS NPs. The frequently used functional groups are $NH2^-$ and $COOH^-$ as cationic and anionic surfaces respectively.

Surface modification has a profound effect on toxicity of nanoparticles. Different functional groups of NPs form different charges over NPs surfaces. The functional groups change the property of PS NPs. They can give it similar molecular structure to proteins, allowing the PS NPs to pass through the cell membrane. For example, –COOH and $–NH_2$ functional groups are generally considered to be positively and negatively charged NPs respectively. Those functional groups change not only the charge of NPs but also their cytotoxicity. Functionalized NPs have greater potential to react with biological molecules as compared to their neutral ones (Deng et al., 2012). Positively charged PS NPs were shown to induce greater toxicity as compared to negatively charged ones. Positively charged NPs are reported to adsorb to and to internalize through the cell membrane as compared to the negatively charged or neutral NPs. Consequently, positively charged PS NPs are reported to induce more toxic, unlike the

neutral and negatively charged PS NPs (Fröhlich, 2012). Xu et al., (2010) demonstrated that positively charged polystyrene nanoparticles induced greater cytotoxicity including DNA damage while negatively charged nanoparticles showed non-significant effects. Similarly, it was reported that positively charged PS-NH_2 NP induce cytotoxicity whereas negatively charged PS-COOH NP showed less or no toxicity (Liu et al., 2011; Della et al., 2014; Mattsson et al., 2014). Since surface charge was shown to affect nanoparticles toxicity, oxidative stress is considered as a potential mechanism for toxicity.

Bhattacharjee et al., (2014) investigated in-vitro PS NPs surface charge cytotoxicity and the possible role of reactive oxygen species as a mechanism of oxidative stress as well as the role of membrane disturbance on toxicity. In their investigation monodisperse, fluorescent, amine-terminated cationic polystyrene nanoparticle (PSNP-NH_2), and anionic acid-terminated polystyrene nanoparticles (PSNP-COOH) of size 50 and 100 nm were tested for reactive oxygen species production and cytotoxicity. The result showed detrimental effect by amine-functionalized cationic PS NPs. It showed cytotoxicity that was followed by the production of intracellular reactive oxygen species and a decrease in intracellular ATP content and an increase in cytoplasmic calcium. Comparing the effect of size, PS NPs of size 50 nm showed more pronounced effect than 100 nm PS NPs. While comparing between cationic and anionic effects, though both increase the cell membrane roughness, cationic effect were greater than anionic PS NPs. The defense mechanism produced by cellular antioxidants towards positive PS NPs was similar to protection against mitochondrial electron transport disrupting agents (e.g. 2,4-dinitrophenol). The response mechanism was not similar to that of oxidative stress that would normally be triggered by hydrogen peroxide. Based on these findings, it was concluded that PS NPs toxicity was mainly through interacting with the membrane and the disruption in electronic transport chain as the main principal cause of toxicity. Following oxidative stress, it may have caused reactive oxidative species production.

Clift et al., (2008) by comparing the uptake of carboxylic acid functionalized polystyrene beads size of 20 nm and 200 nm revealed that smaller size PS NPs were internalized much quicker than 200 nm. To compare effect of size on mode of entry, an uptake experiment with carboxylated PS NPs of size 40 and 200 nm was performed. The results showed that regardless of the size, PS NPs were able to enter the cells through

active transport that requires energy. Moreover, PS NPs of 44 nm and 100 nm showed energy dependent mechanism of internalization while 44 nm PS NPs accumulate faster in the cytoplasm than 100 nm PS NPs, affecting cell morphology and viability and gene expression. Similarly, accumulation of PS NPs on the blue mussel (Mytilus edulis) showed increase with the decrease in particle size (Ward and Kach, 2009; Browne et al., 2008).

Wegner et al., (2012) investigated the effects of different concentrations of 30 nm PS NPs on blue mussel (Mytilus edulis) feeding behavior. In their investigation, the result showed the formation of pseudo feces in the treatments that contained PS NPs. Pseudo feces are means of getting rid of rejected particles. The quantity of feces was higher with higher concentration of PS NPs. Moreover, the removal of PS NPs from the water was confirmed by the decrease of PS NPs concentration in the water as well as a bioassay of M. edulis.

An investigation by Nolte et al., (2017) on the effects of surface functionalized polystyrene on green algae *P. subcapitata* showed a higher adsorption of functionalized PS NPs in the algal cells which was attributed to the change in cell wall as well as zeta potential. These toxic effects were possibly due to the interaction of PS NPs with the cell cytoplasm components (Tussellino et al., 2015). Symens et al., (2011) demonstrated that nuclear enclosure of PS NPs was dependent on the physical properties of size and charge of the polystyrene beads. Unmodified PS NPs of 48 nm exposed to X. laevis during early phase of larval development showed dose dependent toxicity, and affect embryonic development.

CHAPTER 4

METHODS AND MATERIALS

4.1 Nanoparticle characterization

Polystyrene nanoparticles (Cat. Nr. PL-PS-F) were provided by PlasmaChem (Berlin Germany) and were used as supplied. Characteristics of PS NPs were as fellows. Size (TEM) was 32.6 nm ±11.9 nm hydrodynamic diameter, DLS z-average was 69.5 ± 0.5 nm; PDI (DLS) = 0.036 ± 0.005; gyration diameter calculated from AF4-MALS (Peak maximum) = 46.4 nm ± 0.3 nm; hydrodynamic diameter calculated from AF4-DLS (Peak maximum) = 72.3 nm ± 1.2 nm; Zeta-Potential: -45.9%, 1.04 mV (in ultrapure water) (Tsegai et al, 2018).

4.2 Soil preparation

The soil used for this experiment was collected at Helenenberg, Northwest of Trier, Germany (49.8526°N, 6.5417°E). Soils are deeply developed haplic Stagno-Luvisols derived from Pleistocene eolian loess covering Middle Triassic limestone. Land use at this site was winter wheat. Prior to the experiment, soil was thoroughly sieved < 2mm. For soil microbial properties, according to Alef and Nannipieri (1995) maximum water holding capacity (WHCmax= 59.9%) the moisture content of the soil was adjusted between 40% and 60%. The water holding capacity were computed as stored water by percolation test.

4.3 Experimental design

To investigate the eco-toxicological effects of PS NPs on soil microbial community, microbiological soil properties namely microbial biomass, respiration, and enzyme activities were assessed. Prior to use, PS NPs stock solution (63.4 mg mL^{-1}) was bath sonicated at 42 W/L for 15 minutes. From the stock solution, 1000 µg L^{-1} dilution was

prepared for subsequent use. Samples of 100 g soil were spiked with 0 (control), 10 (PSNP-10), 100 (PSNP-100) and 1000 (PSNP-1000) µg PS NP kg^{-1} dry soil, respectively (Illustration 4.1). Each treatment was carried on a quadruplicate. Soil spiking was done by drop-wise addition of PS NP dilutions into the soil contained in a beaker followed by manual homogenization with a spatula.

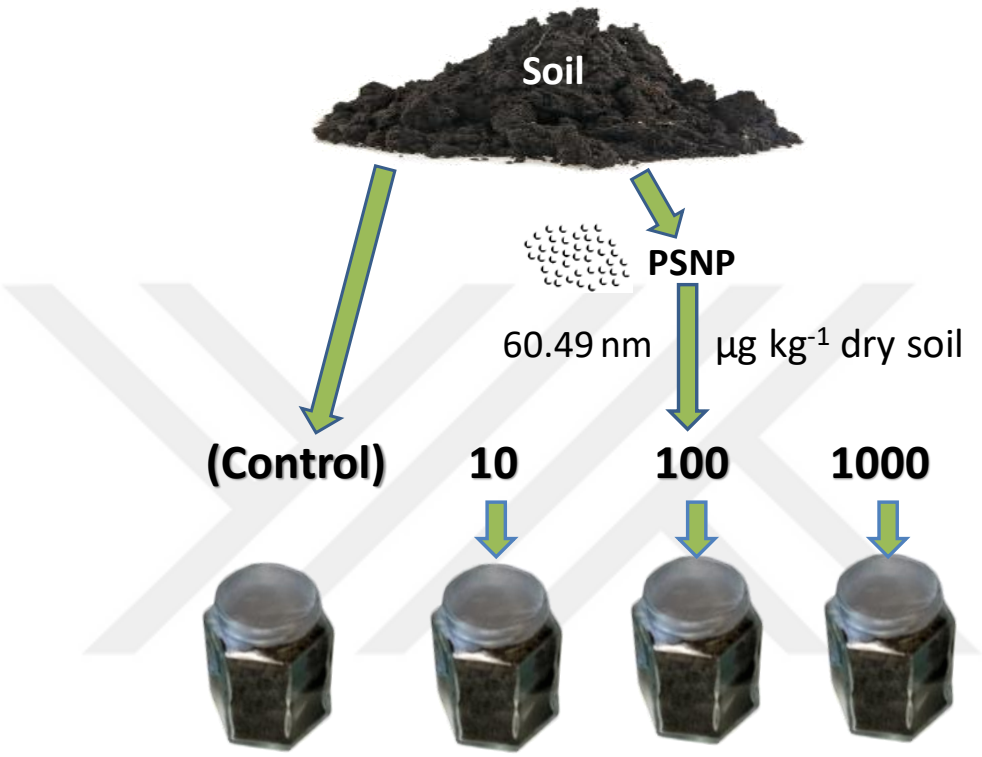

Illustration 4.1 Experimental design treatments and control.

4.4 Chemical and microbial analysis

The soil pH was determined potentiometrically in a 0.01 M $CaCl_2$ solution with a glass electrode. The water content was determined after drying the soil samples at 105°C for 24 h. Microbial biomass carbon (MBC) was determined by the chloroform fumigation extraction method (Vance et al., 1987). For MBC analyses, a 0.01 M–$CaCl_2$ solution used for extraction and a kEC of 0.45 was used for calculations (Joergensen, 1996). TOC- TN analyzer (Shimadzu TOC-V+TNN) was used to determine total organic C of the extracts. The dehydrogenase activity was measured by triphenyl tetrazolium chloride (TTC) method (Thalmann, 1968). Soil-respiration was measured according to

Heinemeyer et al., (1989) with an infrared gas analyzer using 30 g (55% WHC) subsamples.

4.4.1 Determination of the soil water content

Initially constant mass of samples were dried at 105 ^{o}C in an oven. The difference between the initial and final (drying) mass were used to determine the water content in soils.

Procedure: Soil samples were sieved to < 2 mm . Approximately 10 g of the soil sample from all pre-treated samples (for 10, 100, 1000 ppm PSNP treatments and one for control) were weighed into the glass vessels. The glass vessels containing the soil samples were placed in an oven at 105°C over night. After cooling down the vessel and its content were weighed and water content was calculated.

Water content based on dry soil material

$$H_2O\ (\%) = \frac{(WM - DM) \times 100}{DM}$$

WM = Wet mass DM = Dry mass

H_2O-Factor = $(100+H_2O(\%)) / 100$

Determination of the net mass = designated dry mass x H_2O-Factor

4.4.2 Dehydrogenase activity

TTC method (Thalmann, 1968)

Triphenyltetrazolium chloride (TTC) method is commonly used procedure for dehydrogenase activity determination that was initially developed by Casida et al., (1964). In this method, dye is used as indicator to flow of electrons in the electron transport system. That is, a water soluble and colorless TTC substrate is reduced to

insoluble and red colored triphenylformazan (TPF) in the presence of dehydrogenases in the soil environment. TPF color intensity corresponds to the activity of dehydrogenases and it is measured with spectrophotometer at wave length of visible light (546 nm) (Figure 4.4). For TTC method, the pH needs to be maintained at around pH 7 and for buffering of the soil system, calcium carbonate is used. The intensity of the red color obtained from the spectrophotometric analyses is directly proportional to DHA in the soil sample, thus, soil samples without or less intense color are corresponds to lower DHA values and vise versa. Even though dehydrogenases can utilize other electron acceptors including oxygen, soil DHA level is higher under anaerobic conditions, because most of the enzymes are produced by anaerobic microorganisms.

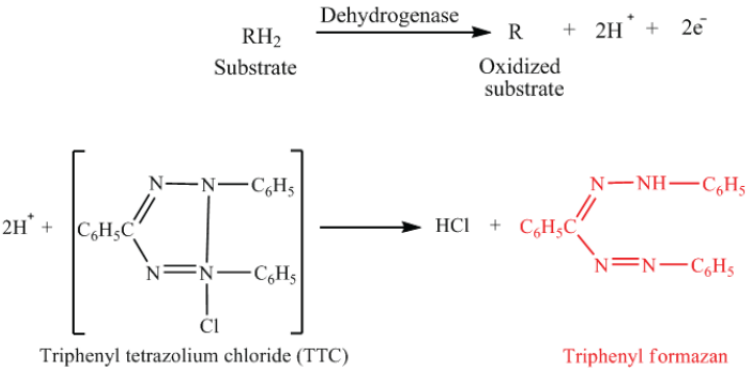

Illustration 4.2 Mechanism depicting dehydrogenase activity in the reduction of triphenyl tetrazolium chloride (TTC) to triphenyle formazan (TPF)

Chemicals and solutions

Tris-HCL buffer (100 mM) was first prepared by mixing 12.1 g of Tris hydroxylmethyl aminomethane with 700 ml of distilled water. The pH was adjusted to 7.6 and total volume was brought up to 1000 ml.

TTC solution was prepared by mixing 0.7 g of TTC with 80 ml of Tris-buffer and brought up to 100 ml.

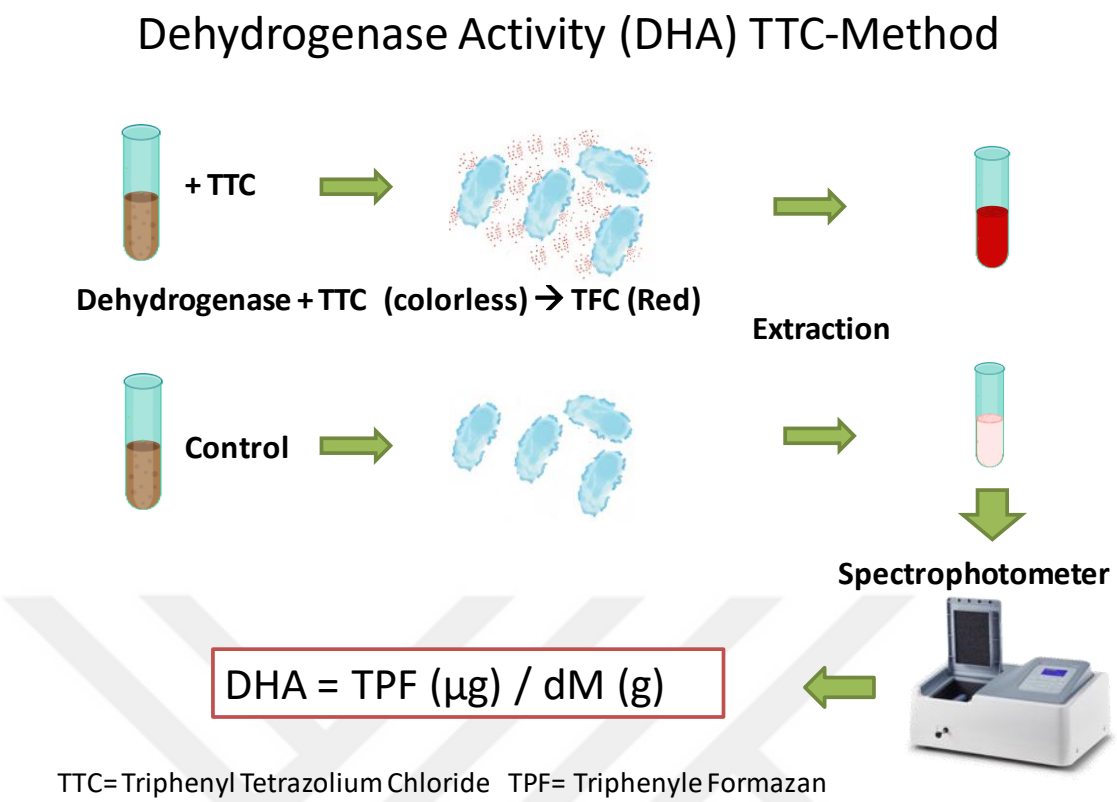

Illustration 4.3 TTC- method to measure dehydrogenase activity.

Procedure: Tris (hydroxymethyl aminomethane) was used as a buffer to maintain the pH of samples. For dehydrogenase test a tetrazolium salt, triphenyl tetrazolium chloride (TTC) was used.

In to 5 ml of TTC solution field moist soil samples (5 g) were weighted and mixed in test tubes. Test samples were incubated at 30 ^{o}C for 24 hrs after that tube were sealed with rubber stoppers. For the control, 5 ml of Tris-buffer without TTC were used. Following the incubation to each test tube containing samples, 40 ml acetone was added and the mixture was shaken to mix thoroughly and extract TPF from the cells. Subsequently, the samples were further incubated for 2 hrs in the dark at room temperature while being shaken at intervals. All procedures were performed under diffused light since both TTC and TPF are light sensitive.

Finally the soil suspension (15 ml) was filtered and the optical density of the supernatant was measured against the blank at 546 nm with a Victor3 MultiLabel Reader (Perkin Elmer, Germany).

Figure 4.1 Acetone treated soil samples in measure dehydrogenase activity with TTC method.

Calculation

Dehydrogenase activity (DHA)

= TPF (µg) / dM (g)

= (TPF (µg) / ml x 45) / (dM x 5)

Where dM is the dry mass of 1 g of moist soil, 45 is the volume of solution added to the soil sample in the assay and 5 is the moist soil used in grams.

4.4.3 Extracellular enzyme activity

Fluorimetric microplate enzyme assay measures the rates of soil extracellular enzyme activities in soil by adding synthetic substrates that are bound to a fluorescent dye. Enzyme-catalyzed reaction release fluorescent dye from the substrate, the intensity of the dye corresponds to enzyme activity. Higher fluorescence indicates more substrate degradation thereby higher enzyme activity.

Procedure: Fluorimetric microplate enzyme assay was employed for all extracellular enzymes after Marx et al., (2001) method. Four enzyme substrates that are based on

methylumbelliferone (MUB) and 7-amino-4–methylcoumarin (AMC) were examined. They represent major pathways of C-, N- and P-cycling in soil. Theses are L-leucin-AMC for leucine-aminopeptidase (EC 3.4.11.1), MUB-ß-D-cellobioside for ß-cellobiohydrolase (EC 3.2.1.91), MUB-ß-D-glucopyranoside for ß-glucosidase (EC 3.2.1.3), and MUB-phosphate for alkaline phosphatase (EC 3.1.3.2) activities.

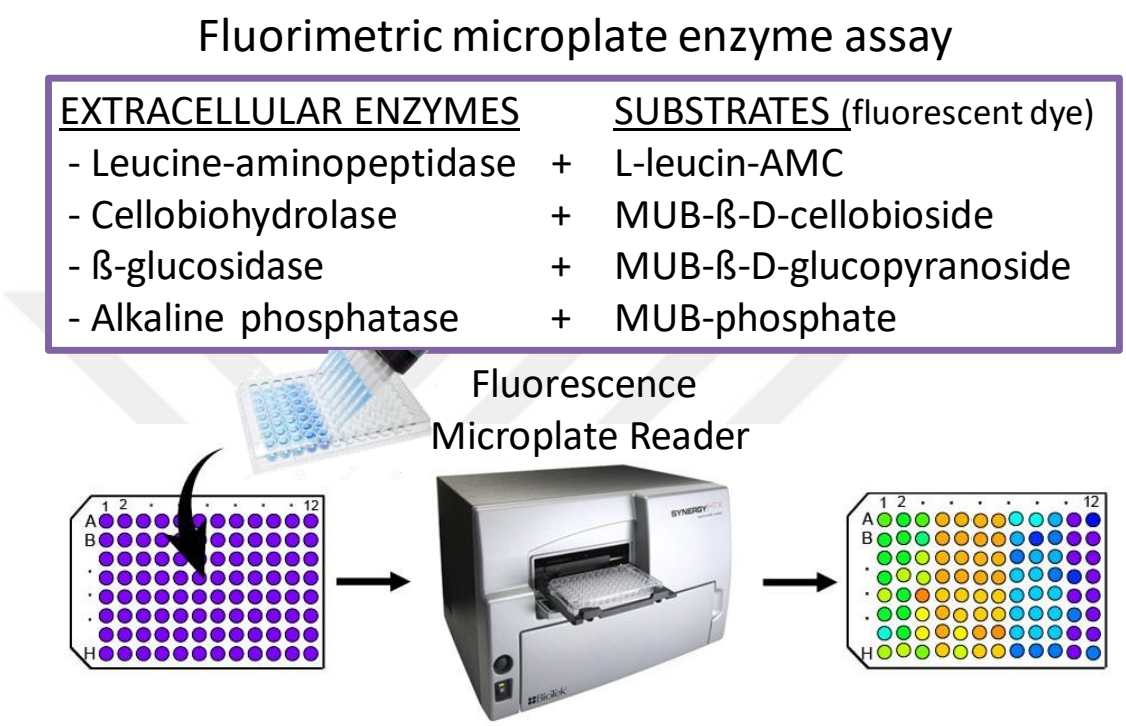

Illustration 4.4 Fluorimetric microplate enzyme assay for measuring extracellular enzymes.

Stock solution for a subsequent use of each substrate as well as calibration solutions for MUB and AMC were prepared. Moist soil sample dry mass weight of 0.5 g was prepared in a sterile glass jar and was vigorously stirred for 2 min with 50 mL autoclaved water to achieve a homogenous suspension. Subsequently, 50 mL of the aliquot were distributed in triplicate for each sample into black 96-well plates microtiter (Biozyme Scientific, Hess. Oldendorf, Germany) . Then, 50 mL of 50 mM TRIZMA, pH 7.8 and 100 mM MES buffer, pH 6 were added into each well for leucin-aminopeptidase and carbohydrolases respectively according Marx et al., (2005). Eventually, to achieve a final volume of 200 mL, 100 mL substrate solution was added per microtiter. Chemicals used were all bought from Sigma Aldrich Chemicals (Taufkirchen, Germany). Plates were placed in the dark at temperature of 30 ^{o}C and

fluorescence measurement was done in 30 min intervals for 120 min with an excitation and emission wavelength of 355 nm and 460 nm respectively using Victor3 MultiLabel Reader (Perkin Elmer, Germany).

4.4.4 Estimation of microbial biomass carbon

Fumigation Extraction Method

Soil samples are first fumigated using chloroform afterward organic carbon is extracted with calcium chloride and after filtering microbial biomass carbon is determined from the organic carbon in the filtrate.

The principle behind this method is that fumigated soil samples will have much greater microorganisms killed than non-fumigated samples during the incubation period, thus releasing more organic carbon than non-fumigated. Since fumigation do not affect non-living parts of the soil organic matter, the difference between the extracted organic carbon of the fumigated and non-fumigated samples corresponds the microbial biomass carbon (Illustration 4.5).

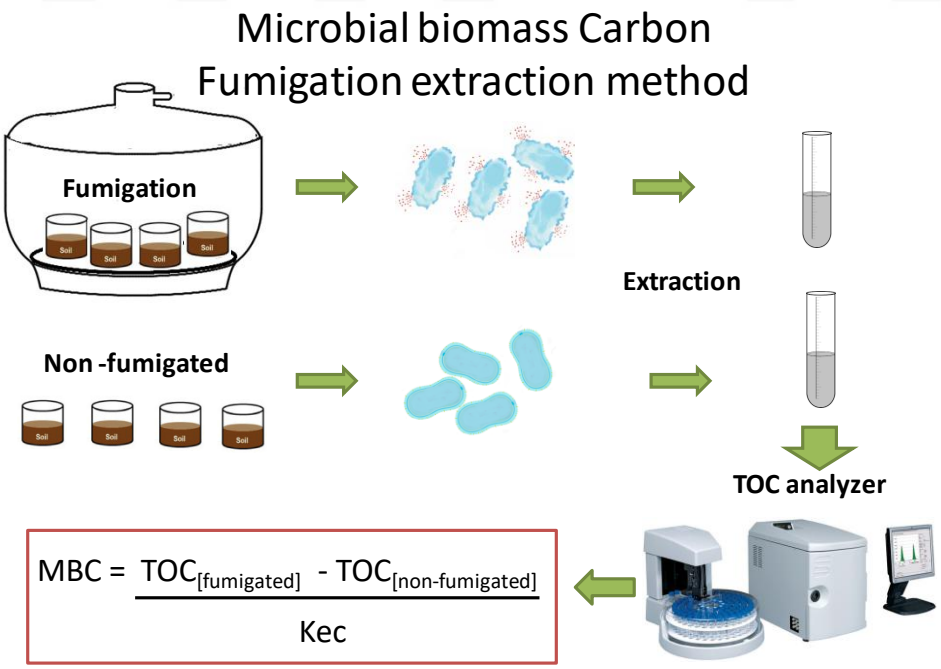

Illustration 4.5 Fumigation extraction method for measuring microbial biomass carbon.

Materials and equipment

- Calcium chloride, 0.01 M

- Chloroform

- Dehydrator and pump to receive low-pressure (vacuum)

- TOC analyzer

Procedure: First the soil sample was divided into two sub samples. One sub-sample of 30 g of field-moist soil (40-60% MaxWHC) was fumigated with chloroform for 24 hrs. Subsequently vacuum was used to remove the chloroform. The fumigated soil was mixed with calcium chloride solution (1 : 5 parts) then was shaken for 30 min, and eventually filtered (Figure 4.6). This procedure was repeated for the second sub-sample but without fumigation as a control. Finally total organic carbon (TOC) content in both extracts was analyzed with a TOC analyzer.

Calculation:

Microbial Biomass Carbone (MBC) = Kc / Kec

$Kc = \text{Total Organic Carbon}_{[\text{fumigated}]} - \text{Total Organic-Carbon}_{[\text{non-fumigated}]}$

Kec = 0.45; Kec converts organic C flush into microbial C

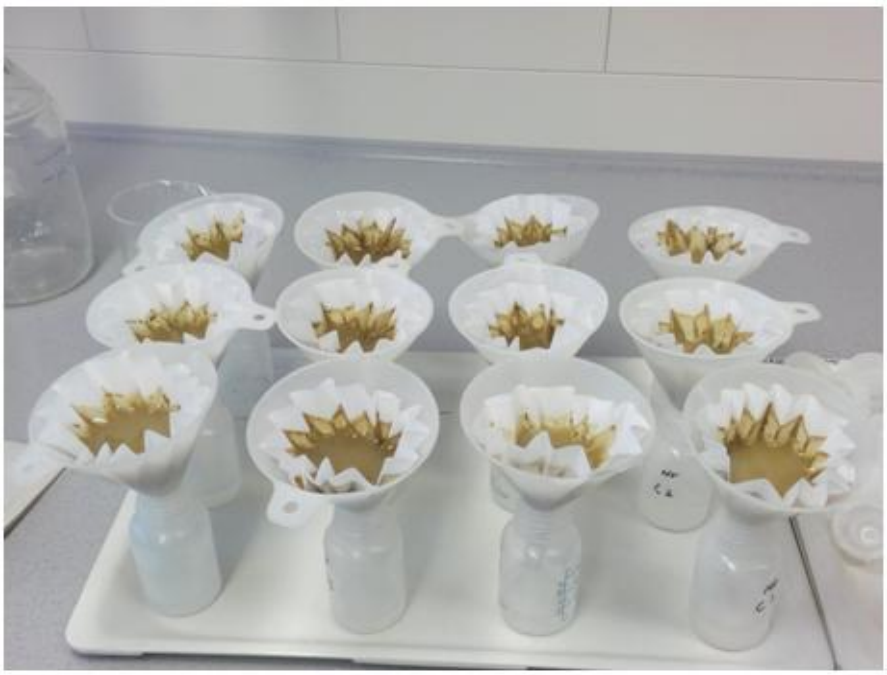

Figure 4.2 Filtration of fumigated soil samples after mixing with CaCl2 for TOC-analysis

4.4.5 Microbial respiration rate

Infrared gas analysis: This technique uses an infrared gas analyzer (IRGA) to automatically estimate CO_2 released during respiration. IRGA measures the CO_2 by detecting the absorption of emitted infrared light. Air is made to pass through soil samples, in order to prevent diffusion of gas and CO_2 accumulation.

Procedure: Soil sample of 30 g (55% of the WHC) was analyzed with IRGA and the complete set up was being kept at 22 ^{o}C.

Figure 4.3 Infrared gas analysis of basal respiration rate of soil samples.

4.5 Statistical analysis

The mean values of enzyme activity, basal respiration and metabolic quotient were calculated. Moreover, the metabolic quotient was calculated as the ratio of soil basal respiration (CO_2-C) and microbial biomass C (MBC). One-way ANOVA and post-hoc Tukey-B test was used to compare between treatments and control and $p<0.05$ was taken as significant cut-off. For statistical analyses, SPSS version 22 (SPSS, IBM Corporation, NY) was used.

CHAPTER 5

RESULTS

5.1 Soil characterization

The experimental soil is characterized by a pH value of 7.2, amount of total organic carbon (TOC) was 28.8 mg g^{-1} dry mass and total nitrogen (TN) of 2.0 mg g^{-1} dry mass, and a subsequent C to N ratio was 14. The water holding capacity (WHC) as well as the cation exchange capacity (CEC) of this soil was very high (Table 5.1).

Table 5.1 Characterization of the soil used for the experiments

Parameters	**Units**	**Properties**
Soil type		Haplic Stagno-Luvisol
Soil texture		Lu (silty loam)
WHC	L m^{-2}	135
pH	0.01 M $CaCl_2$	7.2
TOC	mg g^{-1} dM.	28.8
TN	mg g^{-1} dM.	2.0
C/N		14
CEC	$mmol_c$ kg dM.	193.3

5.2 Soil water content

For the four PSNP treatments soil water content was calculated and the consistency water content in all soil samples was confirmed (Table 5.2).

Table 5.2 Mean percentage of soil water content (± S.D., n =12) before PS NP concentrations treatment of 10 (PSNP-10), 100 (PSNP-100), and 1000 (PSNP-1000) μg kg^{-1} dry soil.

Soil Water Content (%) before PSNP treatment			
	Mean ±SD		%
Soil Samples	Wet Mass	Dry Mass	SWC
PSNP-0	4.58 ±1.32	3.78 ±1.09	21.25 ±1.27
PSNP-10	4.80 ±1.47	3.94 ±1.19	21.63 ±1.40
PSNP-100	4.59 ±1.35	3.76 ±1.09	21.69 ±1.51
PSNP-1000	4.82 ±1.36	3.98 ±1.10	20.79 ±1.42

5.3 Microbial biomass carbon

PS NPs showed detrimental effects on the investigated soil microbial biomass. Microbial biomass carbon (MBC) in the treatment of high PS NPs concentration application (PSNP-100 and PSNP-1000) was lower than in the treatment of low concentration application of PSNP-10 and the control throughout the experiment (Figure 5.1). Though at day 1 the difference was not statistically significant, at day 14, it was statistically significant. Moreover, at day 28 the decrease for the same treatments was statistically significant for PSNP-10 but not for the control. High concentration treatment (PSNP-1000) showed the lowest MBC during the incubation period with lowest value at day 28. On the other hand when compared to control, low concentration treatment (PSNP-10) tends to gradually increase in MBC with significant increase at day 28.

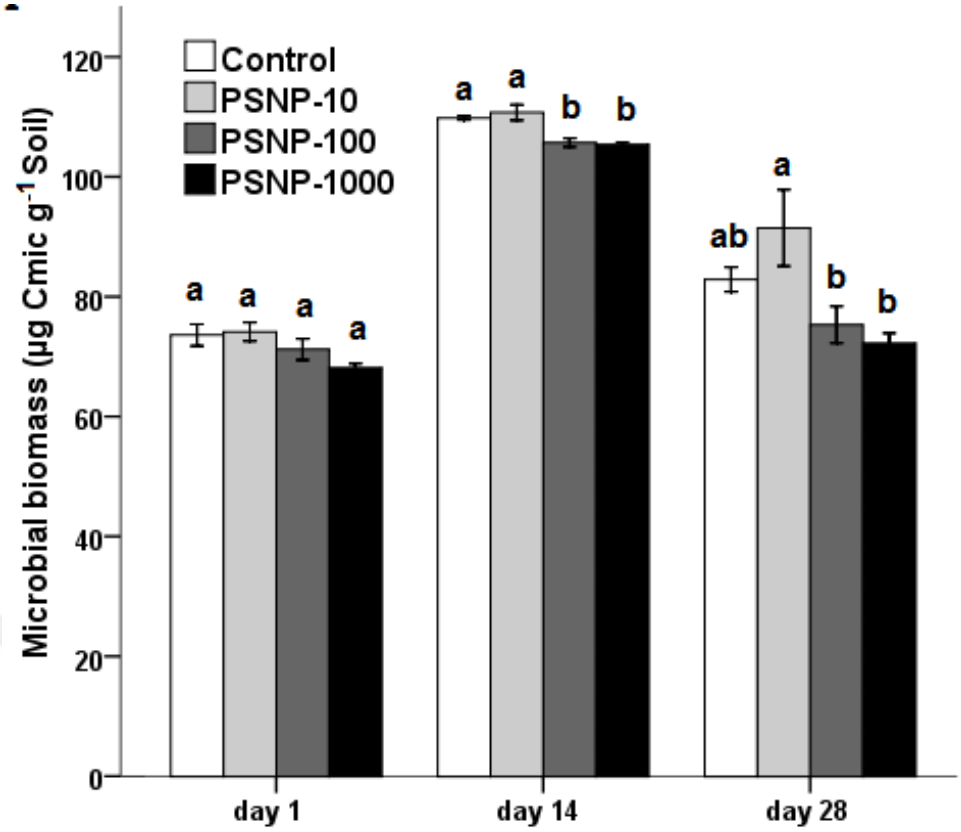

Figure 5.1 Mean (± S.E., n =4) of microbial biomass carbon (MBC) under different PS NP concentrations treatments of soils during 28 days of incubation.

5.4 Enzyme activities

Overall, PS NPs showed detrimental effect on enzymes activity both for the intracellular and extracellular enzymes. The effects were more evident toward the end of the incubation period with statistical significance. The result showed a negative impact for all fluorogenic substrates due to PS NPs application at day 28 with statistical significance for cellobiohydrolase, β-glucosidase, and alkaline phosphatases activities as well as to intracellular dehydrogenases activity.

5.4.1 Dehydrogenases activity

At day one, microbial activity, measured as dehydrogenase activity, showed no statistically significant difference but there was a slight decrease in dehydrogenase activity for low concentration application of PSNP-10 (Figure 5.2). At day 14, despite increased application of PS NPs, dehydrogenase activity increased notably in treatments

of high concentration application PSNP-100 and PSNP-1000, with statistical significance for PSNP-100 as compared to PSNP-10 and the control. At day 28 however, in all treatments of PS NPs applications, there was statistically significant decrease in dehydrogenase activity as compared to control.

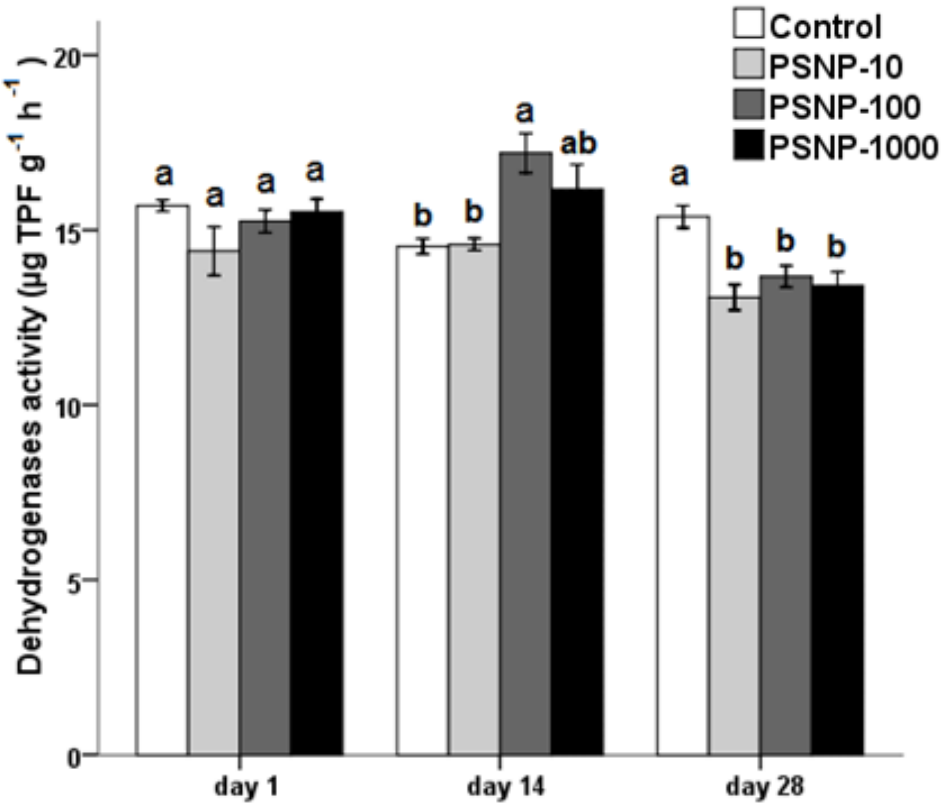

Figure 5.2 Mean (± S.E., n =4) of dehydrogenases activity under different PS NP concentration treatments of soils during 28 days of incubation.

5.4.2 Extracellular enzyme activity

The negative effects of PS NPs on β-glucosidase were more prominent toward the end of the incubation period. That is, at day 28 all treatments of PS NPs applications (PSNP-10, PSNP-100 and PSNP-1000) reduced β-glucosidase activities with statistically significant difference as compared to the control. At day 1, activities of β-glucosidase was not statistical significant, however, the lowest enzyme activities were shown at treatments of high concentration application of PSNP-100 followed by PSNP-10 and control respectively. Interestingly, highest β-glucosidase activity was observed by concentration treatments of PSNP-1000. Similarly, at day 14, treatments of high

concentration applications PSNP-100 and PSNP-1000 showed higher β-glucosidase activities as compared to the low concentration treatment PSNP-10 and control, however, the difference were not statistically significant.

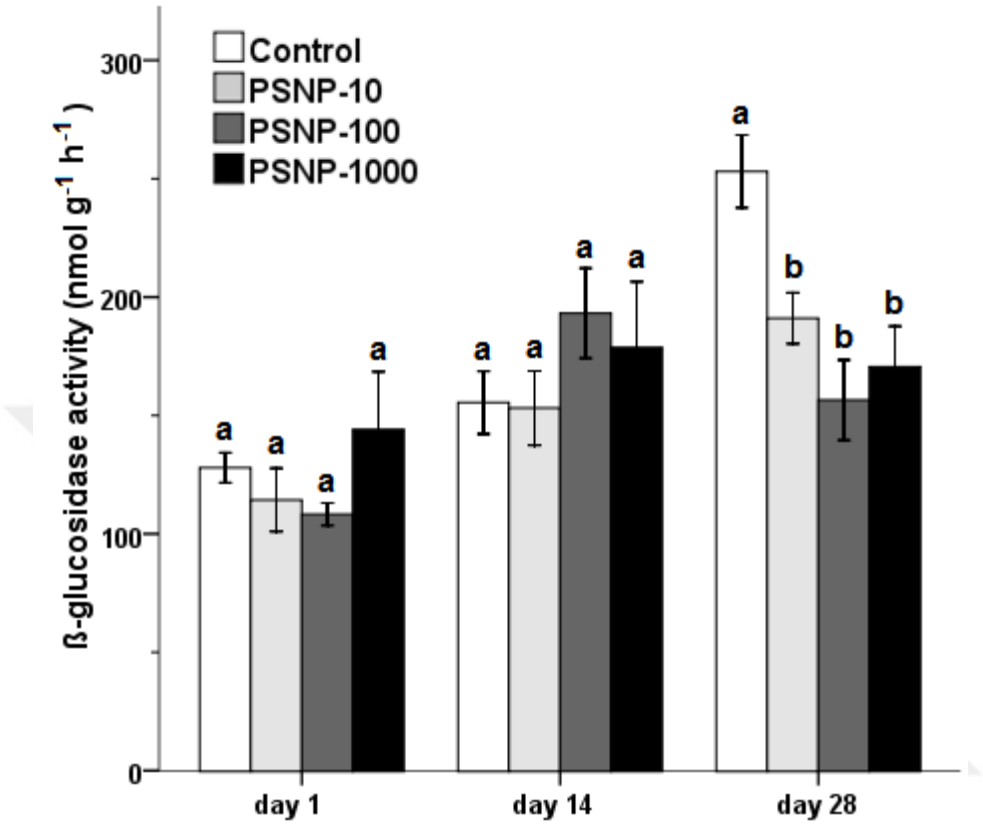

Figure 5.3 Mean (± S.E., n =5) of extracellular enzyme activity of 1,4-β-glucosidase under different PS NP concentration treatments of soils during 28 days of incubation.

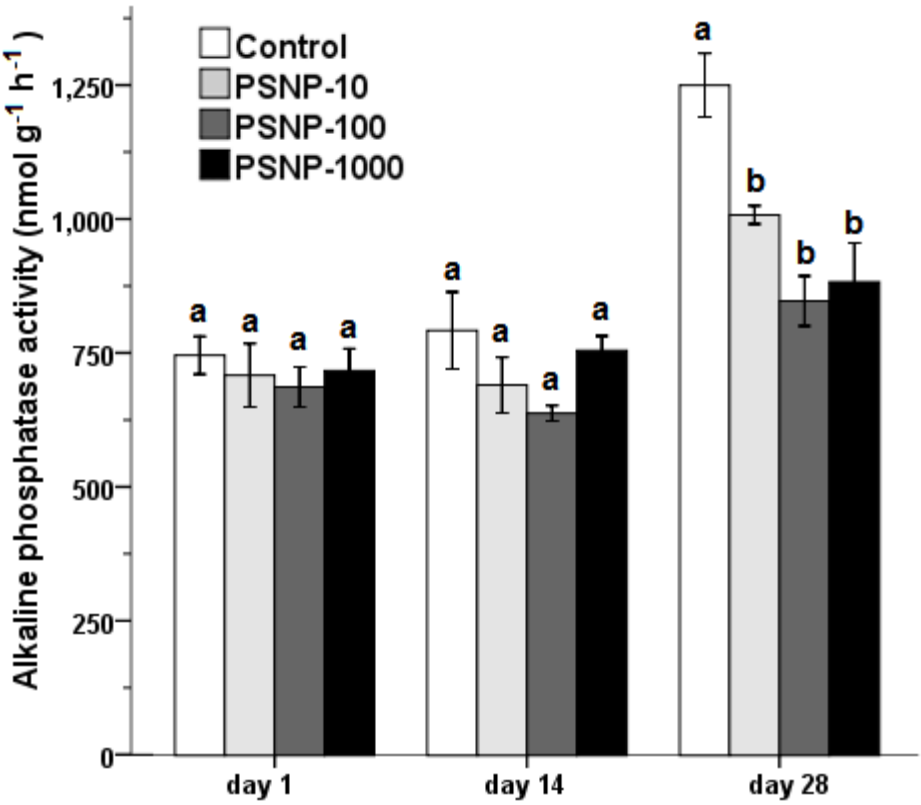

Figure 5.4 Mean (± S.E., n =5) of extracellular enzyme activity of alkaline phosphatase under different PS NP concentration treatments of soils during 28 days of incubation.

Leucine-aminopeptidase and alkaline phosphatases activities tended to decrease in all of the treatments throughout the incubation period with pronounced negative effects at the end of the incubation period. When compared to control, at day 1, β-glucosidase and cellobiohydrolase activity decreased at lower concentrations treatments of PSNP-10 and PSNP-100 and increased at high concentration treatment of PSNP-1000. However, due to high standard error, the change was not statistically significant.

Effects of PS NPs on alkaline phosphatase were consistently detrimental throughout the incubation period, though it was not statistically significant in all the days. In all treatments of PS NPs application alkaline phosphatases activity were lower as compared to the control. At day 28, all treatments of PS NPs application (PSNP-10, PSNP-100 and PSNP-1000) were lower as compared to the control with statistically significant difference. Among all the treatments PSNP-100 concentration application showed the lowest alkaline phosphatase activity throughout the incubation period.

Leucin-aminopeptidase showed no statistically significant difference among all the treatments and the control. Though at days 1 and 14 the difference was small, at day 28 however, there was significant reduction in Leucin-aminopeptidase activity at high PS NPs concentration treatments of PSNP-100 and PSNP-1000 as compared. The absence of statistical significant difference at the end of the incubation period was largely due to high standard errors.

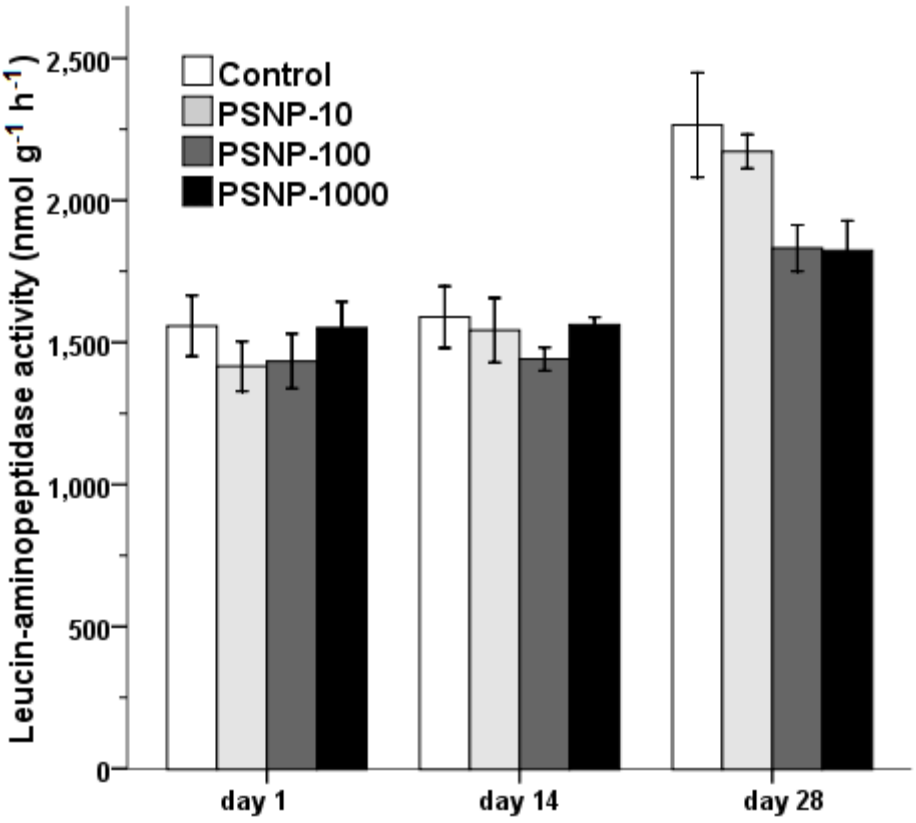

Figure 5.5 Mean (± S.E., n = 5) of extracellular enzyme activity of Leucin-aminopeptidase under different PS NP concentration treatments of soils during 28 days of incubation.

Similar to the rest of extracellular enzymes, effects of PS NPs application to cellobiohydrolase activity was not statistically significant at the beginning of the experiment but toward the end of the incubation period, the detrimental effects were statistically significant. At day one, treatments of high concentration applications of PSNP-100 and PSNP-1000 showed an increased cellobiohydrolase activity as compared to low concentration treatment PSNP-10 and the control. However, towards the end of the incubation period, at days 14 and 28, the effects were reversed. That is, treatments of

high concentration applications of PS NPs showed detrimental effect on cellobiohydrolase activity as compared to the low concentration application PSNP-10 and control. At day 28, all treatments of PS NPs application were statistically significant as compared to the control.

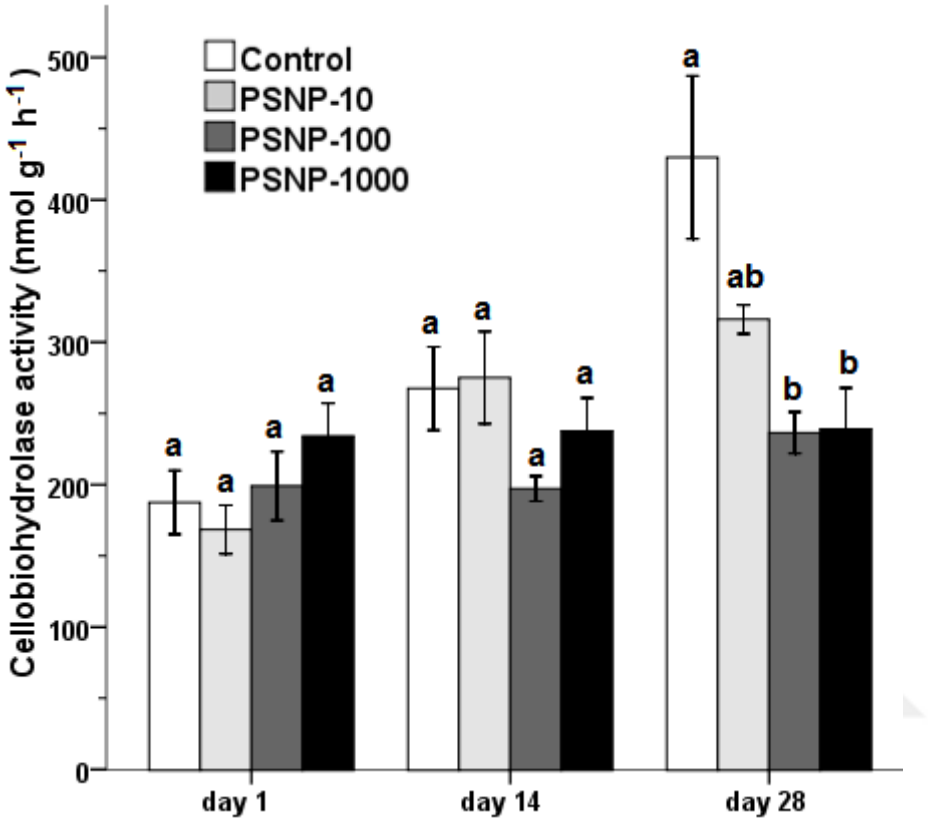

Figure 5.6 Mean (± S.E., n =5) of extracellular enzyme activity of cellobiohydrolase, under different PS NP concentration treatments of soils during 28 days of incubation.

5.5 Basal respiration

Basal respiration rate showed a trend of increase with increasing PS NP application as compared to control (Figure 5.7). At day one, basal respiration for high concentration treatments of PSNP-100 and PSNP-1000 showed significant increase as compared to PSNP-10 and the control. Treatments at the concentration application of PSNP-10 were with lowest basal respiration rate. At day 14, all the treatments showed an increase in basal respiration rate as compared to the control. The increase was statistically significant for the high concentration treatment of PSNP-1000. Similarly, at day 28, all

treatments showed an increase in basal respiration compared to control though due to high standard error the increase was not statistically significant.

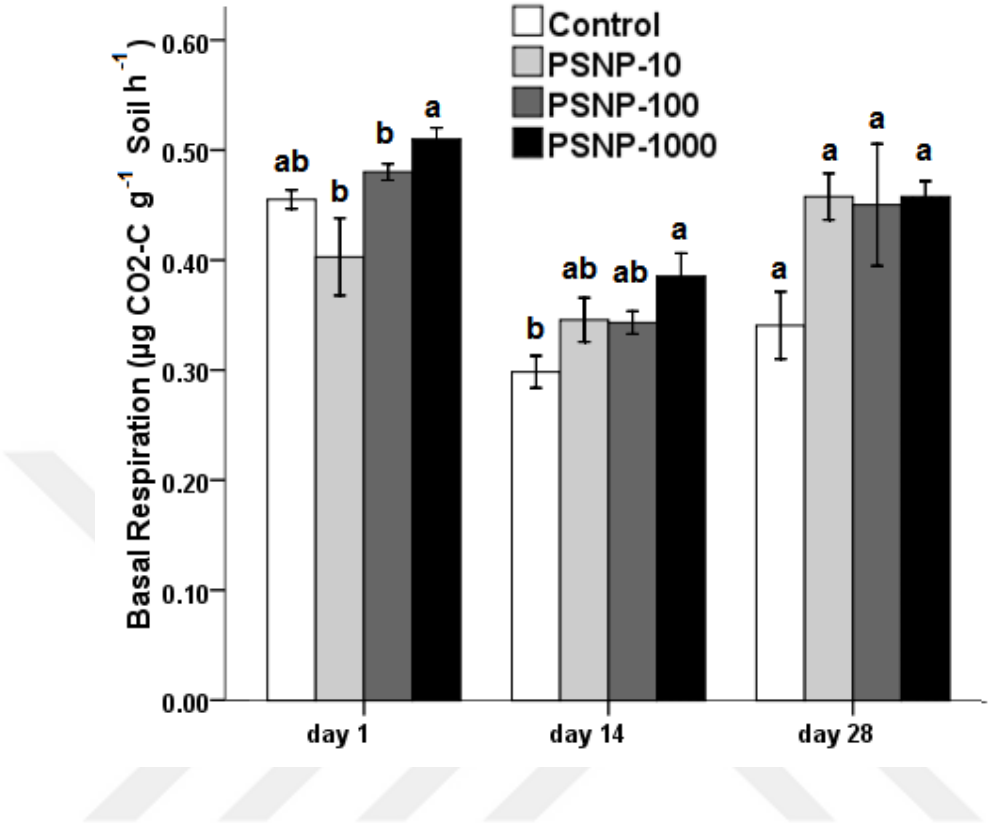

Figure 5.7 Mean (± S.E., n =4) of basal respiration under different PS NP concentration treatments of soils during 28 days of incubation.

5.6 Metabolic quotient

Similar to basal respiration rate, metabolic quotient (qCO_2) showed a trend of increase with increasing PS NPs application as compared to control (Figure 5.8). The trend of increase was more prominent for qCO_2 than for basal respiration, where metabolic quotient showed statistically significant difference between the control and the highest concentration (PSNP-1000) in all of the treatments.

At the beginning of the experiment metabolic quotient was high for treatments of high concentration applications PSNP-100 and PSNP-1000 as compared to low concentration application of PSNP-10 and the control. Moreover, treatment of low concentration application of PSNP-10 was with the lowest of metabolic quotient value and was statistically significant as compared to treatments of the high concentration applications

PSNP-100 and PSNP-1000. Furthermore, treatment of highest concentration application PSNP-1000 has the highest metabolic quotient value and was statistically significant as compared to the low concentration treatment PSNP-10 and the control. At days 14 and 28, all the treatments have the same trend, with dose dependent increase in metabolic quotient values. At day 14, all PS NPs treatments were higher as compared to the control with statistical significance, at day 28, however, only the highest concentration treatment PSNP-1000 was statistically significant due to high standard error.

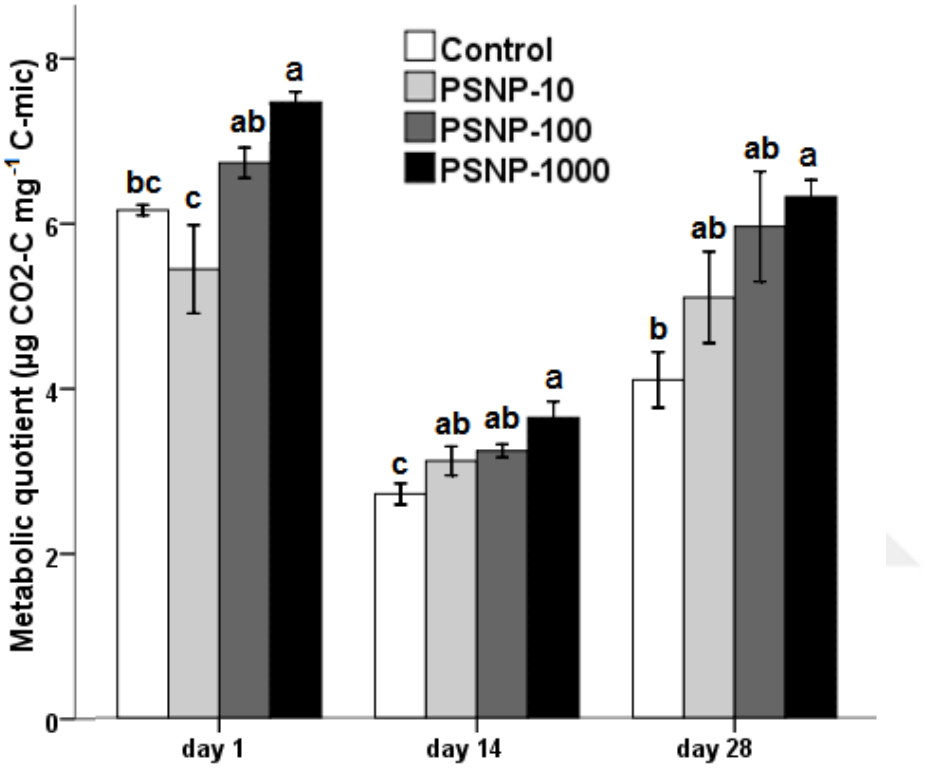

Figure 5.8 Mean (± S.E., n =4) of metabolic quotient under different PS NP concentration treatments of soils during 28 days of incubation.

CHAPTER 6

DISCUSSION

The results of this study demonstrated for the first time that PS NPs could significantly lower soil microbial biomass and enzyme activities. Soil microbial biomass carbon decreased with increasing application of PS NPs, which was also accompanied by a decrease in both intracellular and extracellular enzyme activities with prominent effects towards the end of the incubation period suggesting broad antimicrobial effect of PS NPs on soil microbiota and their enzyme activities. PS NPs inherent nano specific properties, such as small size and high surface area to volume ratio, can allow it to closely interact with the microbial cell and the sub-cellular structures but also with soil matrix that could lead to multidimensional antimicrobial activities (Brown et al., 2013). NPs antimicrobial effects though may vary the mechanisms are often multidimensional which could be through direct effects such as deactivating the membrane bound enzymes and damaging the cell membrane and/or through indirect effects such as damages caused by reactive oxygen species inside the cells (Klaine et al., 2008; Li et al., 2010; Marambio-Jones and Hoek, 2010).

Although neutral PS NPs are generally considered non-toxic, studies have shown that surface charge, size, and aggregation of NPs could be altered in environmental media (Liu et al., 2014; Lowry et al., 2012). Unlike other environments (e.g. marine), soil presents a more complex environment and nanoparticles are known to interact rapidly with soil particles including minerals, heavy metals, dissolved organic matters and toxic chemicals (Singh, 2015). The interactions of PS NPs with soils could contribute a big part to its toxicity of microbes in the soil.

The biological effects of nanoparticles have been shown to depend greatly on the surface chemistry of nanoparticles. The interactions between PS NPs and soil could have potentially changed PS NPs physicochemical properties to cause antimicrobial effect. Soil solid phase components such as humic molecules and clay particles potentially

affect NPs behavior within soil systems. The charge on the surfaces of those soil components will affect the interaction of NPs with the solid-phase and could form colloids in the aqueous-phase, which will further interact with NPs. In such circumstances, the characteristics or the stability of NPs could be affected such as when desorbed humic molecules in the aqueous phase attach to the surface of NPs (Gimbert et al., 2007).

Strong sorption affinity of NPs for toxic compounds in soil due to their high surface area could potentially contribute to the cumulative toxicity effect of PS NPs to soil microbes (Velzeboer et al., 2014). Investigation by Nomura et al., (2015) on the toxicity and behavior of surface-functionalized polystyrene nanoparticles with various functional groups including amine-, carboxyl-, sulfate-, and non-modified toward yeast *Saccharomyces cerevisiae* showed altered effects of the nanoparticles. That is, after the yeast were exposed to PS NPs at the concentration of 40 mgL^{-1} in NaCl solutions for 1 hr, the results showed no or little toxic effect by the negatively charged nanoparticles. However, positively charged PS NPs with amine functional group were highly toxic at 5 mM NaCl but were non toxic in 154 mM NaCl. Further analysis with a confocal microscopy revealed that the internalization of amine- functionalized PS NPs at 154 mM but not at the concentration of 5 mM NaCl. At 5 mM NaCl, the nanoparticles were covering cell surfaces, which suggested that cell death due to nanoparticles adhesion to cells rather than internalization. Similarly, other NPs were also reported to cause damage to cell membranes by binding to them. For instance, silicon nanoparticles and fullerene derivatives such as carboxyl fullerene could be embedded in the membrane leading to membrane rupture that result to cell death (Tsao et al., 1999; Jang et al., 2003). Similar mechanism could also be a contributing factor for PS NPs antimicrobial activity.

6.1 Microbial biomass carbon

Generally, high amount of microbial biomass corresponds to the total organic matter and is considered as an advantage for soil quality and fertility (Sparling, 1997). Decrease in soil microbial biomass for example due to pollutants including nanoparticles is an indication of soil degradation and a subsequent indicator of long-term trend of organic matter content (Powlson et al., 1987). In this result, the dose dependent decrease in

microbial biomass could be a combination of multiple factors. Nanoparticles with detrimental effects can directly or indirectly kill microorganisms. Indirectly could be through reducing availability of substrates that could be eventually reflected by the decrease in microbial biomass (Brookes, 1995). Such changes could have further detrimental effects on community activity (e.g. enzyme activities) and function (e.g. nutrient cycle) (Bour et al., 2015). The decrease in MBC at high concentrations of application of PSNP-100 and PSNP-1000, could be due to direct antimicrobial effects of PS NPs.

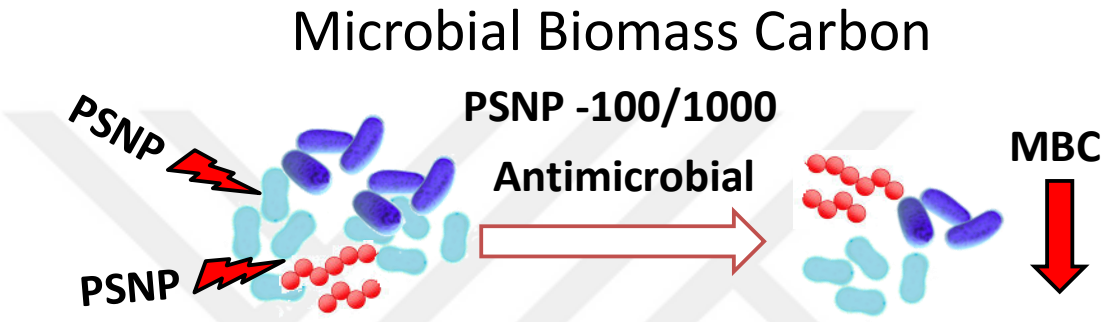

Illustration 6.1 Antimicrobial effect of PSNP at high concentrations.

Towards the end of the incubation period, at day 28, the pronounced increase of MBC at PSNP-10 might be due to increased PS NPs antimicrobial activity to some microbial genera over time. As a result, dead cells might have provided readily available substrate for the resistant microbes that led to cryptic growth (Postgate, 1967). Cryptic growth is a hidden growth of some individual cells in a starving bacterial culture in a death phase. The intensity of cryptic growth is mostly inferred rather than experimentally measured. In cryptic growth, the majority of cells do not grow and as the result the biomass measured in starving culture declines, but few bacteria are able to multiply by using products of lysis of dying cells.

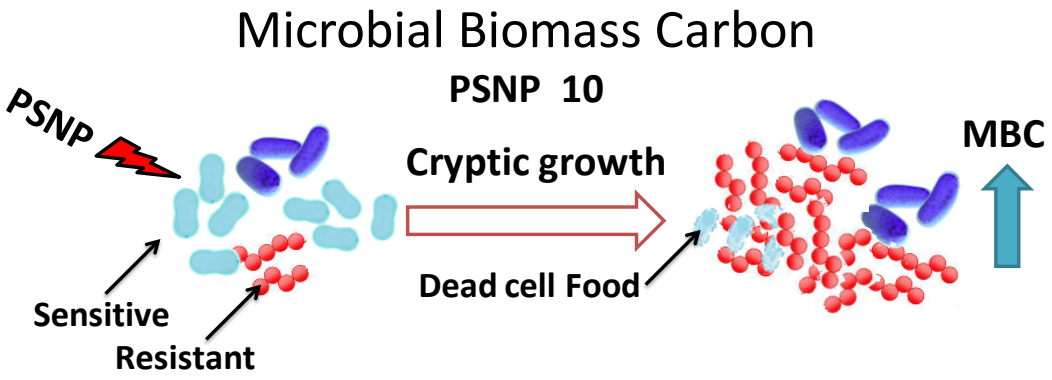

Illustration 6.2 Cryptic growth of microbes at low PSNP concentrations.

Similarly a change in microbial community composition with negative effect on the broader range of soil microbial groups while positive effect on other microbial groups were reported. Single and multi-walled carbon tubes have been shown to selectively reduce or increase certain phyla and genera of the soil microbial population (Chung et al., 2011; Jin et al., 2014). Jin et al., (2014) showed SWCNTs effects on soil microbial communities, where the changes in soil microbial community that were analyzed by phospholipid fatty acid (PLFA) showed a negative relationship between concentration of SWCNT and the biomass of the dominant groups such as gram positive and gram negative bacteria, as well as fungi. However, there was a positive relationship between SWCNT concentration and overall abundance of bacteria community.

Interestingly, careful observation at PSNP-10 reveals a gradual increase in MBC with time that was significant at day 28. At day 1, MBC at concentration treatment of PSNP-10 remained almost unchanged while enzyme activities, basal respiration rate and metabolic quotient (qCO_2) decreased. This suggests a sublethal effect of PS NPs at low concentrations on day 1.

Even though the organism specific effects of NPs are still not clear, there are reports of selective reduction of certain bacterial populations. For example investigation on the impact of nZVI showed substantial shift in the soil microbial community structure and composition by exerting selective pressure on certain groups of microbial community while promoting other groups. As a result, β- and γ-Proteobacteria and subclasses decreased while Archaea, α-Proteobacteria increased. Similarly fullerene NPs as well induced slight modification in Eubacteria and protozoans but not microbial diversity (Tong et al., 2007; Johansen et al., 2008). TiO_2 as well as ZnO were also shown to have

changed the soil microbial diversity after 60 days of incubation, the effect was dose dependent affecting different taxa with both increasing and decreasing effects that resulted in reduced biodiversity (Ge et al., 2011, 2012).

6.2 Dehydrogenases enzyme activity

The results showed statistically significant decrease in the activities of dehydrogenases towards the end of the experiment. Dehydrogenases are intracellular enzymes that are found in all living microbes, therefore, the change in their activity is generally a good indicator of overall microbial activity in the soil (Gu et al., 2009; Moeskops et al., 2010; Zhao et al., 2010; Salazar et al., 2011; Yuan & Yue, 2012). One of their important characteristics is, dehydrogenases are highly linked to microbial oxidoreduction processes and they do not accumulate extracellularly in the soil (Moeskops et al., 2010). That is, in the biological oxidation of soil organic matter dehydrogenases transfer H atoms from organic substrate to inorganic acceptor and a positive correlation was reported between dehydrogenase activity and organic matter (Chodak & Niklińska, 2010; Moeskops et al., 2010; Romero et al., 2010; Zhao et al., 2010; Yuan & Yue, 2012). Moreover, dehydrogenases activities and organic matter were correlated in their spatial distribution (Zhang et al., 2010). A positive relation was also reported between carbon cycling and activities of dehydrogenases in different forest (Salazar et al., 2011). Since soil dehydrogenases are an integral part of intact cells and are only present in viable cells (Skujins, 1978; Trevors, 1984), the decrease in dehydrogenases activity accompanied by decrease in microbial biomass at day 28 suggests direct effect of PS NPs on metabolic activities of soil microorganisms.

At day 14, dehydrogenase activities increased at PSNP-100 and PSNP-1000 despite a decrease in MBC. Dehydrogenase activities correspond to the metabolic activities in the soil and this is often regarded proportional to the soil microbial biomass. Nevertheless, such a relationship between individual biochemical properties, in this case, dehydrogenase activity, and the overall microbial activity are not obvious especially in complex systems like soils. This is because of the high diversity of microbes in the soil but also the complexity of the processes involved in breaking down of the organic compounds (Salazar et al., 2011). Moreover, inconsistent behaviors of dehydrogenases have been reported depending on type (McCarthy et al., 1994) and concentration

(Barnah and Mishra, 1986) of pollutant, and soil type (Doelman and Haanstra, 1979; Kandeler et al., 1997), which has raised a doubt on its reliability (Dick, 1997).

The exact mechanism of antimicrobial activity of PS NPs may have multidimensional effects. Cell membrane disruption by direct contact, physical piercing, and oxidative stresses have been reported as possible mechanisms of antimicrobial activity (Kim et al., 2013; Xia et al., 2006; Wang et al., 2013). PS NPs have been shown that it could pass through lipid membranes and could damage intracellular proteins and nucleic acids as well as affect membrane activity and thereby cellular functions (Kloepfer et al., 2005; Xu et al., 2004; Rossi et al., 2013). Using molecular simulations, Rossi et al., (2013) has shown that PS NPs could permeate easily into lipid membranes, which could severely affect membrane activity and thereby cellular functions. It was reported that NPs could generate reactive oxygen species (ROS) that can damage intracellular protein, nucleic acids and eventually causing damage and deformation of DNA strand through cross-linking, strand break, and adduction of nitrogen bases and sugars (Takenaka et al., 1999; Imlay 2003; Green and Howman, 2005; Cabiscol et al., 2010).

6.3 Extracellular enzyme activities

Extracellular enzymes play a key role in the soil ecosystem function including nutrient cycle and microbial metabolism. They catalyze rate-limiting steps in decomposition and nutrient cycling and thus they play a key part in ecosystem function of the soil where soil microorganisms subsequently obtain energy for their metabolic activities. Analyses of extracellular enzymes are used to demonstrate the effects of soil contaminants. Change in the activity of extracellular enzymes has been used to demonstrate the effects of soil contaminants such as heavy metals and antimicrobial agents on soil microorganisms (Kuperman and Carreiro, 1997; Hinojosa et al., 2004; Chaperon and Sauve, 2008; Liu et al., 2009). Except few microorganisms, majority of microorganisms do not produce all required enzymes (Sinsabaugh, 1994). Therefore, many enzymes are produced dominantly by certain groups of microbes. For example, fungi are the major producers of soil cellulases, whereas bacteria are the predominant producers of protein degradation enzymes (Caldwell, 2005). Hence, the reaction of soil microbial communities to the change in the environment is reflected in the activities of various enzymes.

In this study, the decrease in activities of extracellular enzymes involved in N (Leucine-aminopeptidase), P (alkaline phosphatase) and C (β-glucosidase and cellobiohydrolase) cycles in the soil at day 28 was consistent with the decrease in MBC and dehydrogenase activity, clearly showing detrimental effects of PS NPs on soil microbes. An increased disruption of intercellular interactions may have contributed to the more pronounced changes in MBC and enzyme activities towards the end of the incubation period. Moreover, high concentrations of PS NPs lowered the extracellular activity of enzymes that play key roles in C, N, and P cycling as well as in microbial biomass. This suggested that PS NPs could have caused a wider range of detrimental effects on soil microbial activities and nutrient cycle. In a similarly research high concentrations of MWCNTs was also shown to have a negative effect on the activity of enzymes that play key roles in C, N, and P cycling and soil microbial biomass. Moreover, investigations have shown a decrease in extracellular activities of soil enzymes involving in the nutrient cycle such as C, N, P with application of low concentration (few mgs per gram of soil) of heavy metals such as Cd and Pb (Kuperman and Carreiro, 1997; Moreno et al., 2001; Hinojosa et al., 2004). Further, soils treated with SWCNTs have demonstrated a direct relation between microbial biomass and various enzyme activities, where a decrease in enzyme activity was followed by a lower microbial biomass (Jin et al., 2014).

B-glucosidase is commonly found in the environment and it plays an important role in catalyzing cellulose degradation, which release glucose, an energy source for metabolic activities in soil. Moreover, B-glucosidase has been actively detected in soil as it plays an important part in energy availability that it is associated with carbon content in soil. Therefore, B-glucosidase can play an important role in stabilizing soil organic matter by reducing the change due to season (Knight et al., 2004). In this research, unlike the activity of the other enzymes, at day one β-glucosidase and cellobiohydrolase showed increased activity at high PS NPs concentrations (PSNP-1000) which also persisted for β-glucosidase at day 14. PS NPs might have induced cytotoxicity to some microorganisms, for example, fungi, which have high carbon storage mainly due to chemical composition of their cell wall and are also the predominant source of cellulase enzyme in the soil (Caldwell, 2005). The dead microorganisms might have increased the availability of carbon in the soil that caused an increased activity of both enzymes β-glucosidase and cellobiohydrolase which are related to the C-cycle. However, the

increased activity of cellobiohydrolase at day one could be due to its prior presence in soil, while its subsequent significant decline could be due to the negative effect of PS NPs to fungi or related sources. The persistence of β-glucosidase activity at day 14 might be due to the availability of substrates, possibly the byproducts of cellulase activities. Like other extracellular enzymes β-glucosidase activity is inhibited by the presence of contaminants and pollutants such as heavy metals (Makoi, 2008). Normally extracellular enzymes in soil solution are short lived due to degradation and denaturation or irreversible inhibition; however, some of these free enzymes may be adsorpted or incorporated into humic material and minerals in the soil which could possibly enable enzyme activities to endure in soil for a longer period of time (Marx et al., 2005; Burns et al., 1982).

Interestingly, in this work, the activity of β-glucosidase showed similar pattern to dehydrogenase activity throughout the incubation period (Figure 5.3). β-glucosidase is an extracellular enzyme that plays an important role in the carbon cycle by producing glucose, which is an important energy source for microbes (Tabatabai, 1994). Dehydrogenase activity is positively correlated with the number of microorganisms and with the organic content (Fontaine et al., 2003; Zhang et al., 2010). In agreement to the latter, our result suggests that the change in dehydrogenases activity could be in response to the availability of organic matter, in this case glucose, the byproduct of β-glucosidase.

The impact of PS NPs on microbial community could contribute to microbial physiology as well as functions that could affect cell to cell interactions such as genetic exchange and production of metabolites. NPs have been reported to show an inhibitory effect on secondary metabolites suggesting a potential negative impact of NPs on intercellular interactions in microbial communities (Dimkpa et al., 2012; Maurer-Jones et al., 2013). Allison (2005) suggests that the unparallel result of enzyme activity from the microorganisms that produced them would allow other microorganisms that do not produce enzymes to potentially destabilize the system.

Soil microorganisms produce extracellular enzymes such as phosphatases that are involved in phosphate solubilization. Phosphatases are a group of enzymes that contribute an important part in hydrolysis of esters as well as anhydrides of phosphoric acid. From phosphatases phosphomonoesterases were the most investigated enzymes

which are active both in acidic and alkaline conditions, moreover catalyze low molecular phosphate compounds such as sugar phosphates, polyphosphates, nucleotides which make them key soil quality indicators (Makoi, 2008). A strong correlation was shown between the activity of phosphatases and soil properties, nitrogen, phosphate and clay content as well as pH (Turner and Haygarth, 2005).

In this study, the persistent decrease in alkaline phosphatase and Leucine-aminopeptidase throughout the incubation period and the significant decrease in activity of all enzymes at day 28, which was also accompanied by decrease in microbial biomass, suggests that PS NPs act as stressors to soil microorganisms with a broad impact on nutrient cycling mediated by soil microorganisms.

6.4 Basal respiration

Soil basal respiration rate of microbes is one the key characteristic that is related to soil fertility and is commonly used as indicator of soil quality (Nannipieri et al., 1990; ISO, 2002). In this study, soil basal respiration rate was used to determine the effect of PS NPs on soil microbiological activity. Pollutants including nanoparticles in soils can significantly affect the decomposition of substrates by the microbe, and the amount of CO_2 released can be used to measure the effect of contamination on microbial activity (Burkhardt et al., 1993; Rost et al., 2001; Nwachukwu and Pulford, 2011; Kaplan et al., 2014). The values of basal respiration, therefore, are valuable indicators of stress to soil microbes (Azarbad et al., 2013; Dai et al., 2004). However, the complexity of soil systems might bring varying behavior of microorganisms to pollutions, which might affect the indicators including respiration.

Basal respiration rate showed an increasing trend with increasing PS NPs application (Figure 5.7). As dose increased, possible antimicrobial effects of PS NPs might have selectively decreased the abundance of some microbial genera. As cells die, the surviving bacterial genera may have grown using the readily available remains of the lysed cells that resulted in substrate-induced respiration. Some reports showed no evidence of decrease in soil respiration as the dose of pollutant increased. While other reports showed that contaminated soils produced higher respiration than the controls and not only that but also an increase in respiration rates was observed with an increase in soil contamination (Scelza et al., 2008 , Wakelin et al., 2010; Zornoza et al., 2015;

Dinesh et al., 2012). The change in soil basal respiration in response to PS NPs application could be based on the soil microbial community composition. This can reflect soil microbial communities resistance and resilience to different types of contaminants, for example, this could relatively be due to variation of fungi and bacteria proportion (Scelza et al., 2008; Allison and Martiny, 2008; Hänsch and Emmerling, 2010).

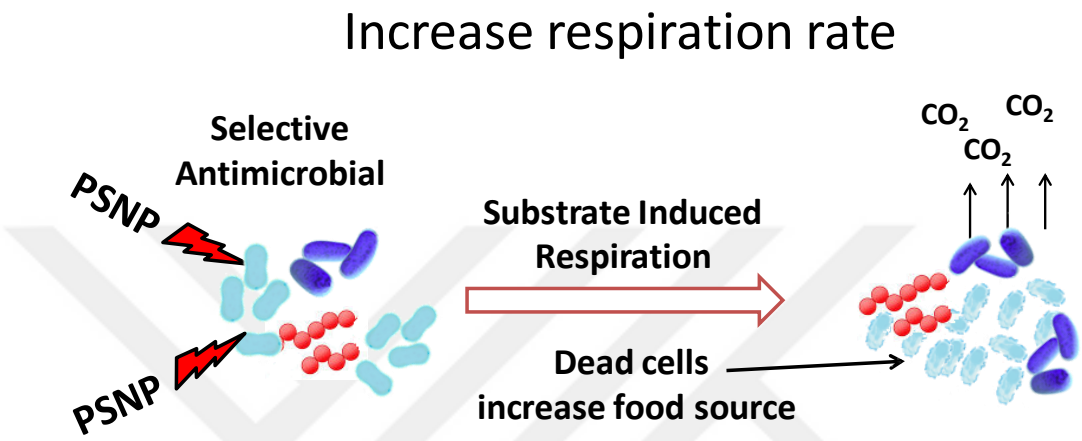

Illustration 6.3 Substrate induced respiration.

6.5 Metabolic quotient

A change of metabolic quotient could be due to either a change of community composition or changes in substrates used by unchanged community. It could be also due to change in the physiological status of the community to maintain damages. Reports on metabolic quotient response to contamination, however, are contradictory. There are reports of increase in qCO_2 response with application of pollutants (Brookes and McGrath, 1984; Fließbach et al., 1994; Ortiz et al., 1993) and some reported a decrease of the qCO_2 with increase application of pollutants (Bååth et al., 1991). In agreement to the former, the results of this study on metabolic quotient showed a clear increase with increased application of PS NPs throughout the incubation period (Figure 5.8). This result was positively correlated to the respiration rate but negatively correlated with microbial biomass. The decrease in microbial biomass is related with increased application of pollutants (Bardget and Saggar, 1994; Khan and Scullion, 2000; Akmal et al., 2005). High values of qCO_2 indicate a stress induced respiration where energy is diverted from growth and production to repairing damage due to disturbances (Odum,

1985). This is a clear indication of the detrimental effects of PS NPs on soil microbes that could lower substrate use efficiency by diverting energy from growth to repair (Hänsch & Emmerling, 2010).

CHAPTER 7

CONCLUSIONS

This study demonstrated for the first time that PS NPs can negatively affect soil microbes. PS NPs significantly lowered soil microbial biomass and enzyme activities suggesting broad antimicrobial effect of PS NPs on soil microbiota and their enzyme activities.

Overall soil microbial biomass carbon and both intracellular and extracellular enzyme activities decreased with increasing application of PS NPs. The persistent decrease in extracellular enzymes alkaline phosphatase and Leucine-aminopeptidase throughout the incubation period and the significant decrease in activity of all enzymes towards the end of the incubation period, which was also accompanied by decrease in microbial biomass, suggest a broad antimicrobial effect of PS NPs on soil microbiota and their enzyme activities. Moreover, dose dependent increase of metabolic quotient (qCO_2) clearly indicate detrimental effects of PS NPs on soil microbes that lowered substrate use efficiency by diverting energy from growth and reproduction to damage repair.

Although neutral PS NPs are generally considered non-toxic, soil presents a more complex environment and PS NPs could interact with soil particles including minerals, heavy metals, dissolved organic matters and toxic chemicals that could alter PS NPs physicochemical properties such as surface, size, and aggregation. Those changes could have introduced novel properties that possibly contributed to the toxicity of presumably non-toxic PS NPs to negatively affect soil microorganisms.

These findings provide evidence that PS NPs pose a potential hazard to soil microorganisms. Future work needs to investigate a possible change in soil microbial-community composition due to PS NPs application. Investigating effects of PS NPs on different types of soils could shade more light on the potential interaction of PS NPs that was suggested by this research as a source of the presumably non toxic PS NPs toxicity. Moreover, studying the effects of PS NPs on longer timescales in conditions that have

relevant field conditions and ecological setting will be essential. Furthermore, focus need to be laid on the development of analytical strategies, which are able to determine critical physicochemical properties of the PS NPs such as Zeta potential and agglomeration state directly in or after extraction from soil matrices.

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Appendix A, SUPPLEMENTARY DATA

TABLE 1 Microbial biomass carbon (MBC), basal respiration, dehydrogenase activity and metabolic quotient, under different PS NPs concentrations of 10 (PSNP-10), 100 (PSNP-100), and 1000 (PSNP-1000) μg kg^{-1} dry soil at day 1, 14, and 28 during 28 days of incubation.

DAY	PSNP (μg kg^{-1} dry soil)	Replica	Microbial biomass (μg C-mic g^{-1} soil)	Basal respiration (μg CO_2-C g^{-1} soil h^{-1})	Dehydrogenase activity (TPF g^{-1} h^{-1})	Metabolic quoteint (g CO_2-C g^{-1} C-mic h^{-1})
1	0	1	77.89	0.48	15.63	6.16
1	0	2	76.04	0.46	16.12	6.01
1	0	3	69.74	0.44	16.16	6.31
1	0	4	72.31	0.45	15.52	6.22
1	10	1	76.29	0.30	16.16	3.93
1	10	2	77.02	0.43	14.51	5.58
1	10	3	70.08	0.45	12.75	6.42
1	10	4	74.74	0.44	14.77	5.89
1	100	1	72.91	0.49	14.54	6.72
1	100	2	75.65	0.49	15.47	6.48
1	100	3	67.32	0.49	16.16	7.28
1	100	4	70.51	0.46	15.48	6.52
1	1000	1	69.55	0.54	16.16	7.76
1	1000	2	69.82	0.51	15.86	7.30
1	1000	3	67.64	0.49	16.12	7.24
1	1000	4	67.14	0.51	14.57	7.65
14	0	1	109.48	0.27	14.69	2.47
14	0	2	110.56	0.28	14.39	2.53
14	0	3	110.87	0.32	14.35	2.89
14	0	4	110.46	0.33	15.32	2.99
14	10	1	111.75	0.39	14.86	3.49
14	10	2	112.99	0.30	14.94	2.66
14	10	3	107.41	0.33	14.23	3.07
14	10	4	112.98	0.37	14.94	3.27
14	100	1	107.48	0.37	15.96	3.44
14	100	2	104.85	0.32	17.53	3.05
14	100	3	105.10	0.34	18.74	3.24
14	100	4	107.58	0.35	17.27	3.25
14	1000	1	106.25	0.43	17.05	4.05
14	1000	2	105.01	0.39	18.00	3.68
14	1000	3	106.32	0.40	14.97	3.76

14	1000	4	106.15	0.33	15.32	3.11
28	0	1	88.93	0.39	15.33	4.39
28	0	2	81.67	0.40	14.74	4.90
28	0	3	83.30	0.28	16.16	3.36
28	0	4	79.29	0.30	15.94	3.78
28	10	1	73.69	0.49	12.12	6.65
28	10	2	88.76	0.49	13.51	4.83
28	10	3	92.29	0.46	13.47	4.98
28	10	4	100.31	0.40	13.75	3.99
28	100	1	80.60	0.59	14.68	7.32
28	100	2	77.28	0.32	13.33	4.14
28	100	3	66.73	0.43	13.39	6.44
28	100	4	78.29	0.47	13.86	6.00
28	1000	1	69.56	0.48	13.34	6.90
28	1000	2	69.87	0.42	12.72	6.01
28	1000	3	75.33	0.48	14.63	6.37
28	1000	4	75.78	0.46	13.53	6.07

TABLE 2 Extracellular enzymes activity under different PS NPs concentrations of 10 (PSNP-10), 100 (PSNP-100), and 1000 (PSNP-1000) μg kg^{-1} dry soil at day 1, 14,and 28 during 28 days of incubation Supplementary data extracellular activity

DAY	PSNP (μg kg^{-1} dry soil)	Replica	Leucin-aminopeptidase activity (nmol g^{-1} h^{-1})	Alkaline phosphatase activity (nmol g^{-1} h^{-1})	Cellobiohydrolase activity (nmol g^{-1} h^{-1})	ß-glucosidase activity (nmol g^{-1} h^{-1})
1	0	1	1634.47	806.59	164.31	134.83
1	0	2	1605.92	772.31	276.52	116.67
1	0	3	1360.35	734.30	153.93	117.02
1	0	4	1340.64	729.33	164.33	127.07
1	0	5	1931.70	935.07	189.45	151.26
1	10	1	1117.13	539.14	121.10	75.21
1	10	2	1477.78	722.51	142.49	110.03
1	10	3	1650.17	861.83	175.43	160.13
1	10	4	1400.23	755.94	210.52	117.38
1	10	5	1510.95	900.30	202.94	115.69
1	100	1	1364.36	656.00	181.47	112.10
1	100	2	1424.66	714.29	146.05	116.87
1	100	3	1768.12	876.96	214.21	107.42
1	100	4	1176.30	667.15	176.37	119.09
1	100	5	1514.32	748.23	288.87	92.12
1	1000	1	1749.79	805.63	264.19	205.24
1	1000	2	1607.33	906.46	151.97	89.02

1	1000	3	1261.80	677.52	276.14	123.64
1	1000	4	1471.90	669.54	222.42	107.41
1	1000	5	1748.82	764.78	269.06	203.30
14	0	1	1790.80	933.70	304.00	169.58
14	0	2	1939.33	1069.79	371.23	166.11
14	0	3	1390.84	644.88	222.98	195.69
14	0	4	1494.11	708.46	216.45	132.39
14	0	5	1415.46	867.42	237.58	121.98
14	10	1	1202.51	569.53	183.20	101.76
14	10	2	1472.79	758.51	295.76	143.82
14	10	3	1907.69	913.27	332.73	154.82
14	10	4	1625.77	696.65	223.05	190.45
14	10	5	1587.86	743.64	355.77	183.24
14	100	1	1550.95	653.48	214.20	190.62
14	100	2	1462.62	681.07	191.99	223.53
14	100	3	1317.24	665.83	168.96	217.16
14	100	4	1428.52	739.44	203.52	122.43
14	100	5	1525.41	661.95	218.50	222.38
14	1000	1	1532.25	888.87	253.05	211.23
14	1000	2	1646.31	764.89	315.75	197.40
14	1000	3	1510.77	723.28	240.35	109.35
14	1000	4	1626.66	820.22	172.35	259.99
14	1000	5	1579.42	829.40	220.25	125.26
28	0	1	2544.50	1264.90	547.52	255.63
28	0	2	2654.33	1298.55	571.60	238.70
28	0	3	2554.46	1197.18	349.44	220.56
28	0	4	1738.62	1332.62	267.96	312.69
28	0	5	1947.05	1569.84	434.13	250.59
28	10	1	2304.35	1128.19	304.35	164.53
28	10	2	2323.42	1050.14	340.54	193.07
28	10	3	1993.09	1059.65	294.50	184.49
28	10	4	2213.27	1030.96	311.57	231.34
28	10	5	2136.48	1104.75	346.32	191.91
28	100	1	1704.85	786.38	233.03	111.72
28	100	2	1913.56	1018.16	294.31	176.28
28	100	3	1784.01	875.15	239.14	205.31
28	100	4	2142.49	1022.00	215.81	170.89
28	100	5	1710.21	815.87	213.41	126.86
28	1000	1	1895.53	824.67	177.12	148.74
28	1000	2	2158.40	930.70	246.62	206.92
28	1000	3	1780.77	1025.00	238.06	212.61
28	1000	4	1504.12	741.33	201.12	121.19

28	1000	5	1873.47	1184.65	346.16	172.36

Appendix B, CURRICULUM VITAE

PERSONAL INFORMATION

Name and Surname: Awet Tekeste TSEGAI

Nationality: Eritrean

Email: awet2c@yahoo.com

Phone: +905445293077

EDUCATION

Ph.D.	**2014– 2019**	Gaziantep University, Gaziantep, TURKEY.
		Ph.D. Thesis: effects of polystyrene nanoparticles on soil microbiota.
	2015 -2016	Research at Trier University, Trier, GERMANY.
		Ph.D. Research: effects of polystyrene nanoparticles on soil microbiota
M.Sc.	**2010 - 2012**	Northeast Normal University, CHINA.
		M.Sc. Thesis: Effects of spatial distribution on plant associational defense against herbivory.
B.Sc.	**1996 - 2001**	University of Asmara, ERITREA.
		Project: Extirpated mammals of Eritrea.

WORK EXPERIENCE

2001 - 2010	**University of Asmara**
	Biology Department - Graduate Assistant.

Freshman program - Database manager.

2004 - 2010 **Asmara Theologian Pavoni Institute**

IGCSE Biology Instructor

2008 - 2010 **Einstein Information Technology Center**

IT training and computer maintenance

PUBLICATIONS

Awet, T. T., Kohl, Y., Meier, F., Straskraba, S., Grün, A. L., Ruf, T., ... & Emmerling, C. (2018). Effects of polystyrene nanoparticles on the microbiota and functional diversity of enzymes in soil. *Environmental Sciences Europe*, **30(1)**, 11.

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Tsegai, A. T., Wang, L., Wang, D., Huang, Y., Lin, H., Li, J., & Liu, C. (2013). Effects of spatial distribution on plant associational defense against herbivory. *Basic and Applied Ecology*, **14(8),** 680–686.

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Tsegai, A. T., Grun, A., Straskraba, S., Kohl, Y., Meier, F., Drexel, R., Tunç, E., & Emmerling, C. (2017). Effects of polystyrene nanoparticles on soil microbiotas. In *ECOLOGY 2017* (p. 144).

Çelik, M. A., Tunç, E., & **Tsegai, A. T.** (2017). An investigation of the relationship between drought and ecology in Barak plain. In *2nd International Energy & Engineering Conference* (pp. 59–60).

Tunç, E., **Tsegai, A.T.**, & Emmerling, C. (2017). Effects of Monoculture and Mixed-Culture Agricultural Fields of Pistachios and Olive Trees on Soil Microbial Biomass and Activity in Gaziantep Province. In *ECOLOGY 2017* (p. 543).

Tunç, E., Gençdal, S., & **Tsegai, A. T.** (2017). Investigation of Some Microbiological Properties of Gaziantep / Araban Agricultural Soils. In *ECOLOGY 2017* (p. 204).

FOREIGN LANGUAGE

English: Excellent (YDS= 88.75)

Turkish: TOMER (C1)

German: Basic (A1)

Chinese: Basic

COMPUTER SKILLS,

Statistical Package for the Social Science (SPSS),

Ms-office (Advanced level) including access database,

Graphics (Adobe Photoshop).

Web design (HTML).