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IMPACT OF METAL AND METAL OXIDE ENGINEERED NANOPARTICLES IN SOIL AND PLANT SYSTEMS

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Abbreviations and definitions

AR	*Aqua regia*: 2 mL HNO_3 plus 6 mL HCl
DAPI	4', 6-DiAmidino-2-PhenylIndole
DL	Detection limit
DTPA	Diethylene-triamine-penta-acetic acid
EDTA	Ethylenediaminetetraacetic acid
ENMs or MNMs	Engineered nanomaterials or manufactured nanomaterials; they may include many objects composed of materials with the requisite characteristics of having at least one dimension of 1-100 nm and displaying novel properties. See also definition in Introduction. In this thesis, the abbreviation ENMs could be used as synonym of NPs/ENPs.
FEG-ESEM-EDS	Field Emission Gun - Environmental scanning electron microscope - Energy Dispersion Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
ICP-OES	Inductively coupled plasma - optical emission spectroscopy
NPs or ENPs	Nanoparticles or engineered nanoparticles; a discrete entity that has all three dimensions in the nanoscale (<100nm). In this thesis, the abbreviation NPs/ENPs could be used as synonym of ENMs.
nZVI	Zero valent Fe nanoparticles
PLFA	Phospholipid-derived fatty acids
PVP	Polyvinylpyrrolidone
qCO_2	Metabolic quotient; defined as the basal respiration to microbial biomass ratio, which is associated to mineralization of organic substrate per unit of microbial biomass.
SMB	Soil microbial biomass
TBARS	Thiobarbituric acid reactive substances
TUNEL	Terminal deoxy nucleotidyl transferase-mediated nick end labelling
WHC	Water holding capacity

1. Introduction

1.1. Nanotechnology and Nanomaterials

Nanotechnology is a recent branch of applied science defined by National Nanotechnology Initiative (NNI) USA as "the understanding and control of matter at dimensions of roughly 1 to 100 nanometres, where unique phenomena enable novel applications [...] At this level, the physical, chemical and biological properties of materials differ in fundamental and valuable ways from the properties of individual atoms and molecules or bulk matter".

Nanotechnology refers to a set of techniques and processes requiring a multidisciplinary approach and enabling the creation and utilization of materials, devices and systems with dimensions of the nanometre level. The prospects associated with nanotechnology derive from the fact that, at this scale, behaviours and characteristics of materials change drastically (Hristozov & Malsch 2009).

Nanotechnology can be applied in all industries. Many products arising from the use of nanotechnology are already available on the market (Woodrow Wilson Nanotech Inventory http://www.nanotechproject.org/inventories/consumer/) — or are about to be — and their number is steadily growing.

Among them we can mention nanoparticles for cosmetics, coatings and paints, crease and odour resistant fabrics, sporting goods, but also nanocomposites, "hard disks" with nanostructured surfaces for recording data, memory "chips" with dimensions below 100 nm, photonic devices, self-cleaning surfaces, systems for medical diagnosis based on, for example, the principle of "lab-on-a-chip" (Ghallab & Badawy 2010).

Finally, some properties of the nanoparticles, such as increased chemical activity and the ability to cross tissue barriers, are useful for the development of new techniques in the field of pharmacology. In the future, a nanoparticle or a group of nanoparticles can be designed to search, locate and destroy a single cell pathology. Particularly, within the next few years advanced systems for targeted delivery of drugs and medical implants that are more durable and that have improved biocompatibility are expected. (Naahidi et al. 2013).

Nanomaterials are defined as "natural, incidental or manufactured materials 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" (European Commission Recommendation 2011/696/EU http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:275:0038:0040:EN:PDF).

Natural nanomaterials are ubiquitous in nature, in air they are usually named ultrafine particles, whilst in soil and water they are known as organic and inorganic colloids, with a slightly different size range (Lead & Wilkinson 2006). These NPs are generated continually by geological processes, such as the weathering of minerals, volcanic eruptions and biological processes involving redox reactions. Clays, organic matter (carbohydrates, proteins, humic materials), iron and aluminium oxides are the soil's natural NPs. These components have been studied for decades because they play an important role in soil development (pedogenesis).

Incidental nanomaterials are unintentionally generated by human activities such as fires, explosions, combustion engines, welding fumes, power plants, incinerators (Klaine et al. 2008).

Finally, engineered nanomaterials (ENMs) are intentionally built nanoproducts designed with the purpose of improving materials and devices in current use. The peculiarity of ENMs is the high surface/volume ratio that render them highly reactive, as the nanosize enhances or creates even more unique properties with respect to the non-nanoscale materials with the same chemical composition (Auffan et al. 2009). The discovery of size-dependent electric, magnetic, optical, thermal and electronic properties has been exploited in recent decades to develop new manufactured or engineered nanomaterials in a wide range of industries such as electronics, textile industry, construction, sensors, chemical industry, automobile industry, medicine and many others (Klaine et al. 2008).

The potential benefit of these new technologies is great, however the production of ENMs should go hand in hand with the assessment of the potential risks to human health and the environment, and should ensure that risks do not outweigh benefits.

1.2. Engineered nanoparticles in the environment

Nanotechnology promises huge benefits for society, therefore capital invested in this new technology is steadily increasing, moreover there is a growing number of nanotechnology products on the market (http://www.nanotechproject.org/inventories/consumer/) and inevitably ENMs will enter the environment. Some ENMs can be intentionally released, like zero-valent metals employed to remediate contaminated soils or groundwater (Li et al 2006) and some may be unintentionally released. The latter is proportional to ENM use: in the case of production facilities releasing ENM in the atmosphere or in solid or liquid waste, the emission can be associated with wear and erosion from general exploitation or recycling and disposal of ENM-containing products. Some studies

have already shown the release of nanoparticles (NPs) from commercially available antiodour socks and self-cleaning façades (Benn & Westerhoff 2008; Kaegi et al. 2008 - 2010). Without doubt widespread release will have an impact on living organisms; predicted environmental concentrations (PEC) based on a probabilistic material flow analysis were applied in some research to estimate the environmental exposure levels of Ag NPs, carbon nanotubes, TiO_2 NPs and ZnO NPs (Gottschalk et al. 2009). In addition, a study conducted in the United Kingdom was able to estimate the future concentrations in soil and water from fuel additive cerium oxide ENPs (Johnson & Park 2012). To our knowledge, environmental exposure levels for Co NPs and SnO_2 NPs are not yet available.

1.3. Engineered nanoparticles in soil system

The interest of NPs effects on the environment is growing day by day, as demonstrated by the increasing number of published works. Aquatic systems are the most studied and several reviews (Moore 2006; Nowack & Bucheli 2007; Scown et al. 2010; Fabrega et al. 2011) analyzed the behaviour and effects of NPs in this environments. Instead, soil or aquatic sediments are poorly investigated, in addition the related information can be fragmentary and conflicting**.**

As shown by the simulation conducted by Gottschalk et al. (2009) a relevant portion of the ENMs released in the environment could run out in soils directly or indirectly as air deposition or application of sewage sludge on soils.

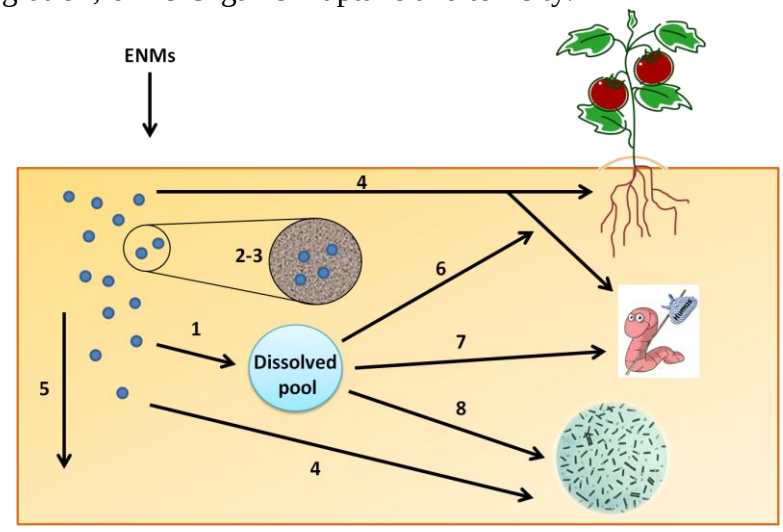
Soil is a fundamental component of the environment: it supports vegetation, the primary producer, which constitutes the first ring of the food chain for all ecosystems, but also is the basis of all economic and social functions of human beings (European Commission COM(2002)179 http://www.eea.europa.eu/policy-documents/com-2002-179-final) and soil has a prominent role for long-term sustainability (Herrick 2000). Soil is an open system in dynamic equilibrium with the other components of the environment, in continuous evolution and regulated by complex metabolic mechanisms, which are still not fully understood (Nannipieri et al. 2003). Unfortunately, soil is a non-renewable resource, and the formation and regeneration processes are extremely slow while the rate of degradation is potentially rapid. In this regard, it became essential to carefully evaluate the impact of anthropogenic activities on soil quality that is "the capacity of a soil to function, within ecosystem and land use boundaries, to sustain productivity, maintain environmental quality, and promote plant and animal health" (Doran & Parkin 1994; Bridges & van Baren 1997; Karlen et al. 1997). For this reason, in order to have a global comprehension of the impact of NPs' detrimental activity (i.e. release of ENMs into the environment), their effects on soil functions, plant biomass production, soil invertebrates, terrestrial vertebrates, and accumulation through the food chain

should be all taken into account (European Commission COM(2002)179 http://www.eea.europa.eu/policy-documents/com-2002-179-final).

Particularly, the toxic effect of ENMs is proportional to the bioaccessibility and bioavailability of these materials. According to Semple et al. (2004), bioaccessibility has been defined as the "fraction available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical", whilst bioavailability has been defined as the "fraction freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time" (Semple et al. 2004). For this reason, we differentiate between bioaccessibility, assessed by chemical extraction techniques, and bioavailability, assessed by quantifying the concentration of ENMs in organisms.

Most NP types in current use are metal-based NPs, such as nanosilver, zinc oxide, titanium dioxide and iron oxide (27th Report, RCEP, London 2008); in addition, frequently metallic NPs are produced with surface coatings which could modify their characteristics. The environmental behaviour, fate and ecotoxicity of metallic NPs is basically controlled by physical characteristics, such as size and shape, and chemical characteristics, particularly the metal solubility and the surface's acid-base character. These properties control the NPs stability and the likelihood to which NPs undergo transformations such as aggregation and agglomeration, surface sorption and release of metal ions.

Figure 1.1 Adapted from Klaine et al. (2008). The fate and bioavailability of manufactured NPs in the soil system: 1 Dissolution; 2-3 Aggregation/agglomeration - sorption; 4 Direct particle uptake; 5 Particle migration; 6-7-8 Organism uptake and toxicity.



According to Klaine et al. (2008) (Fig. 1.1) the following processes are likely to affect the fate and bioavailability of NPs in the soil environment:

1. **Dissolution**: it occurs when a thermodynamically unstable ion separates, leaves the particle surface and migrates into the solution (Borm et al. 2006). The ionic species released

from metallic NPs may be toxic. Thus, the dissolution amount and the relative toxicity of NPs should be assessed to understand the potential effects on living beings over time. A few studies assessed the NPs dissolution in soil due to the lack of suitable techniques. In addition, a very small number of metal-based NPs have been studied: after 28 days, the dissolution of Au NPs varies with the size (Unrine et al. 2010); CeO_2 NPs show a very low dissolution (Cornelis et al. 2011) but they can change in different types of soil (Cornelis et al. 2010); Ag NPs of different size change in Ag(I) from 10 to 17% in one month, probably due to oxidative dissolution to ions (Shoults-Wilson et al. 2011a), Ag dissolution depends on the content of organic matter or clay present in soil (Cornelis et al. 2010); ZnO NPs exhibit behaviour similar to non-nanosized ZnO (200nm<Ø<1μm) (Milani et al. 2010; Kool et al. 2011).

2. **Aggregation/agglomeration:** aggregation is defined as the association of primary particles by strong bonding, whereas agglomeration is defined as the association by weak bonding caused by Van der Waals forces (Jiang et al. 2009). In literature this terminology has been used indiscriminately which causes some confusion. The agglomeration and aggregation of NPs are influenced by physical forces (e.g. gravity, Brownian motion and fluid mechanics) and the properties of NPs (e.g. size, shape, surface charge) (Farré et al. 2009). When aggregation or agglomeration occur, particle flocks can be formed and can sediment due to gravity (Lin et al. 2008; Rosická & Šembera 2011). Studies conducted in solution showed that the NPs' aggregate size depends on initial particle size (Wang et al. 2009; Pipan-Tkalec el al. 2010), NP concentration (Phenrat et al. 2006), and vary among particle types (Jemec et al. 2008; Wang et al. 2009). However, individual NPs were also found in suspension with aggregates (Lin & Xing 2008; Wang et al. 2009).

3. **Sorption:** NP behaviour is complicated by the presence of clay particles and humic molecules. These natural nanoparticles have their own electrically charged surfaces which interact with NPs and can influence their stability in the aqueous phase. For example, Fang and co-workers (2009) studied the aggregation rate of TiO_2 in soil suspension and found that it is negatively correlated to soil properties like dissolved organic matter and clay content, while it is positively correlated to ionic strength, zeta potential and pH. Other studies confirmed the influence of ionic strength on NP aggregation (French et al. 2009; Jiang et al. 2009). Conversely, coating materials could protect NPs from increasing ionic strength to the steric repulsion (e.g. Ag NPs polyvinylpyrrolidone coated El Badawy et al. 2010). Humic substances could influence NPs in different ways. At environmental pH, humic acids and NPs will form a negatively charged agglomerate (Ghosh et al. 2008) which may be more

stable in soil solution determining lower aggregation and sedimentation (Fang et al. 2009; Ben-Moshe et al. 2010). On the other hand, the presence of organic matter and low pH could destabilize particle dispersions and cause aggregation (Ghosh et al. 2008; Kool et al. 2011). Soil pH and humic substances are important factors in particle stability and bioavailability in soil systems.

4. **Direct particle uptake**: Huang and co-workers (2008) showed that ZnO has been internalized by some microorganisms causing a biocidal and bacteriostatic effect. The mechanisms by which NPs penetrate cell walls and the Casparian strip in plants are still subject to debate. Zhang and co-workers (2011) showed that CeO_2 NPs (25nm) were internalized in root and shoots and suggested that the root apical meristematic tissues are the most likely path of entry for NPs. Conversely, NPs may be absorbed on the root surfaces and partially dissolved with the assistance of the root's exudates, inducing cellular toxicity (Zhang et al. 2012). NPs may be ingested by soil invertebrates through contaminated food and some studies suggest that NPs may be internalized intact (Unrine et al. 2010; Shoults-Wilson et al. 2011a).

5. **Particle migration:** the transport of metallic NPs along the soil profile, as sorption, is a result of the interaction of different properties. Indeed, an important factor is the surface charge of NPs and soil surfaces: the migration along the soil profile is enhanced if there is a electrostatic repulsion between particles and soil (Darlington et al. 2009). The soil pore water characteristics have also been noted to affect the partitioning of NPs: when pore water pH is higher than the point of zero charge, NPs will be more mobile (Dunphy Guzman et al. 2006; Jiang et al. 2009), in addition higher ionic strength could increase aggregation and sorption (Fang et al. 2009; Ben-Moshe et al. 2010). Another key property is NP size; effectively smaller particles could move about in the soil porosity avoiding retention and are more likely to penetrate to groundwater depth (Darlington et al. 2009). The mobility may also be affected by the solution flow rate: Jeong and co-workers (2009) showed that if the flow rate is scarce, the CuO NPs mobility is also reduced and can have an effect on depositing and aggregation in a porous media.

1.3.1. Soil and soil microbial biomass (SMB) system

Soil quality is closely linked with soil microorganisms, indeed they influence soil ecosystem processes through the decomposition of soil organic matter and the cycling of nutrients (Kennedy & Smith 1995). Accordingly, soil microbial biomass and diversity should be preserved to maintain nutrient turnover (Torsvik & Øvreås 2002) as well as the capability of the soil to suppress disease

(Janvier et al. 2007). It is well known that the presence of contaminants can alter the soil environment (Richardson 2002), however little is known about the influence of ENMs on microbial biodiversity, especially under field conditions (Dinesh et al. 2012; Tilston et al. 2013). Soil microbial biomass concentration could be negatively affected by the toxic effect of NPs, whose mechanisms are not well understood, however. Two main types of impact were proposed: a direct effect (toxicity) and an indirect effect resulting from changes in bioavailability of nutrients or toxins, or from interactions with natural or toxic organic compounds which would intensify or mitigate their toxicity (Simonet & Valcárcel 2009). According to Klaine et al. (2008) "possible mechanisms include disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation of reactive oxygen species (ROS), and release of toxic constituents". Raghupathi et al. (2011) suggest that NPs are toxic due to the combination of two factors: ROS production and accumulation of these materials in the cytoplasm or on the outer membranes. Indeed, structural changes in the cell membranes due to close contact with NPs could lead to cell death (Suresh et al. 2010). ENM toxicity towards microorganisms has been assessed mainly through *in vitro* studies. For instance, Ag, CuO and ZnO NPs may inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* (Jones et al. 2008) and *Pseudomonas putida* (Gajjar et al. 2009); Al_2O_3, SiO_2, TiO_2 and ZnO NPs were toxic to *Bacillus subtilis*, *E. coli* and *P. fluorescens* (Jiang et al. 2009). Conversely, little is known about their toxicity towards soil microorganisms that promote plant growth and those involved in nutrient cycling. Studies carried out in pure culture conditions or aqueous suspensions of rhizobacteria, such as *P. aeruginosa*, *P. putida*, *P. fluorescens*, *B. subtilis* and soil N cycle bacteria showed varying intensity inhibition when exposed to ENMs (Mishra & Kumar 2009). Unlike the *in vitro* experiments, only a few studies investigated the effects of ENPs on soil microorganisms in soil systems. To our knowledge, the metal and metal oxide that have been examined to date are Ag-, Al-, Au-, Cu-, Pd-, Si-, TiO_2-, Zn- and ZnO-NPs. The antibacterial capability of Ag ions has been verified also in soil systems: Murata et al. (2005) demonstrated that soil dehydrogenase activity may be negatively affected and bacterial colony growth was inhibited with a dose between 0.1 and 0.5 mg of Ag kg^{-1} soil. Hänsch and Emmerling (2010) carried out a medium-term experiment (four months), spiking soil with 3.2, 32 and 320 μg Ag kg^{-1} soil and found a decrease in microbial biomass concentration and an increase of basal respiration with an increase in Ag NPs, even though microbial biomass N, fluorimetric enzymes (Leucine-aminopeptidase, β- cellobiohydrolase, acid phosphatase, β-glucosidase, chitinase and xylosidase), soil pH and organic C were not influenced. The metabolic quotient was higher in the treated soil compared to the control soil, highlighting a decreased substrate efficiency in the samples contaminated by Ag NPs. Also, metal oxide were found to have a negative impact on soil

bacterial communities. Ge and co-workers (2011) carried out a microcosm experiment where a grassland soil was exposed to TiO_2 and ZnO NPs at increasing doses over 60 days, showing that NPs reduced microbial biomass, bacterial diversity and composition; in addition, at the same exposure concentration (0.5 mg g^{-1} soil) the effect of nano-ZnO was stronger than that of nano-TiO_2. Contrarily, other metal NPs have been found to influence soil microorganisms to a lesser degree. Soil respiration studies were carried out in soil amended with 5000 mg kg^{-1} soil of Al NPs (with aluminum oxide, or carboxylate ligand coating, Alex and L-Alex, respectively); the presence of nano-aluminum particles had no effect on soil respiration (Doshi et al. 2008). Shah and Belozerova (2009) studied the impact of Si, Pd, Au and Cu NPs on soil microbial communities at a final concentration of 0.013% (w/w) or 0.066% (w/w). At the end of fifteen days of incubation the influence of ENMs was assessed by several biological methods, but no significant influence was noted, not even at the highest concentration.

As shown, the effect of ENMs on soil microorganisms has been assessed in various studies, but most of the findings are based on incubation or microcosm studies, thus emphasizing the need for experiments in field conditions or using models that simulate the natural soil environment exactly, and that take into consideration the fact that impact depends on the kind of metallic NPs.

1.3.2. Soil and plant system

The information about production and release volumes of ENMs complicate the assessment of the impact of these materials in the soil (Hendren et al. 2011); moreover the amount of ENMs is convoluted by the interference from natural nanomaterials, such as phyllosilicates and organic carbon. The physical, chemical and biological properties of soil can modify and address the quantity and quality of crops, however they are also influenced by environmental and anthropogenic changes. Indeed, plants are in close contact with soil, water and atmospheric environmental compartments which can convey ENMs (Miralles et al. 2012). For this reason, plants were used as bioindicators to evaluate the bioavailability and mobility of pollutants in the soil (Andén et al. 2004). In addition, the employment of plants can be useful to assess the effects of a xenobiotic compound in the terrestrial trophic chain.

Plant toxicity and bioaccumulation have been observed with NPs present in the soil solution or adsorbed in soil, which can interact with plant roots. To date, a wide variety of effects of ENMs on plants have been observed and several endpoints were applied: germination, seedling growth, cytotoxicity and genotoxicity (Miralles et al. 2012). El-Temsah and Joner (2012) showed that Ag NPs, at different particle size, may inhibit seed germination of *Linum usitatissimum, Lolium perenne, Hordeum vulgare* at 10 mg L^{-1}. Conversely, Ma and co-workers (2010) reported that rare

earth-oxide ENMs (CeO_2, La_2O_3, Gd_2O_3 and Yb_2O_3 NPs) did not affect the germination of *Cucumis sativus*, *Brassica oleracea*, *Brassica napus*, *Rapganus sativus*, *Lactuca sativa*, or *Lycopersicon esculentum;* whereas seedling elongation of all tested species was inhibited by La_2O_3 and Gd_2O_3 NPs. Phytotoxicity studies performed at early seedling stages (i.e. germination and seedling elongation) showed that NPs can induce oxidative stress in roots and determine cell membrane damage (Wang et al. 2011; Song et al. 2012). DNA damage was observed in the roots of *Allium cepa* and *Nicotina tabacum* treated with TiO_2 NPs after 3, 6 and 24h of exposure and at 4mM and 2mM, respectively (Ghosh et al. 2010). ENM uptake and bioaccumulation were investigated mainly in crop species, such as *Triticum aestivum* (Wild et al. 2009), *Oryza sativa* (Lin et al. 2009), *Cucurbita pepo* and *Cucurbita maxima* (González-Melendi 2008; Zhu et al. 2008; Corredor et al. 2009). Zhu et al. (2008) reported that *C. maxima* could absorb, translocate and accumulate in the aerial organs Fe_3O_4 NPs after 20 days of growth in a medium containing 0.5g NPs L^{-1}, without developing phenotypic defects. Indeed, ENMs interact with plants' penetrating root cells, but the exact uptake mechanisms are not fully elucidated. On the other hand, the transfer of ENMs in terrestrial trophic chains has been scarcely investigated. Au NPs showed biomagnification in *Manduca sexta* fed with contaminated *N. tabacum;* unfortunately, it was unclear if Au NPs were internalized by the plant or absorbed only superficially (Judy et al. 2010). Most of the studies assessing the phytotoxicity of ENMs in plants have been conducted with an *in vitro* model (Schwabe et al. 2013; Lee et al. 2008), which is very useful for understanding ENM behaviour in a standardized media, but it can be misleading since the amount of nanoparticles available to soil biota and crops is affected by soil properties (Vittori et al. 2011; Rico et al. 2011; Vittori Antisari et al. 2013). In addition, experiments carried out in aqueous suspension or Hoagland solution applied high rates of NPs, ranging from 1000 to 4000 mg L^{-1} (Rico et al. 2011), which exceed the environmental concentrations that will likely range from ng L^{-1} to low mg L^{-1} for most ENMs (Mueller & Nowack 2008), and showed inhibition of germination and root growth of various plant species (López-Moreno et al. 2010) or caused death of almost all living cells at the root tip (Lin & Xing 2008).

To sum up, further experimental studies applying a relatively low concentration of ENPs for long periods are needed to assess the risk for human and environment health.

1.3.3. Soil and earthworm system

Soil invertebrates play an important role in soil ecosystem function (e.g., decomposition and nutrient recycling), and thus addressing NP effects on these organisms is crucial to the understanding of the potential impact of NPs on the soil environment (Tourinho et al. 2012). The

environmental concentrations of ENPs are still unknown, however PEC suggest that the disposal of sewage sludge may be the major source of NPs in soil (Mueller & Nowak 2008). The possible route of exposure for soil invertebrates is dermal uptake (Wang et al. 2009) and ingestion of contaminated particles and food (Hu et al. 2010). The toxicity of metal-based NPs to earthworms has been conducted with different exposure media (e.g. soil, food, water), applying a wide range of concentrations (from 0.1 to 10,000 mg g^{-1}) and using several endpoints (Tourinho et al. 2012). According to Tourinho et al. (2012), tests conducted in aqueous media are likely to be quite unrealistic as compared to the soil system.

Survival, growth, reproduction and avoidance have been employed to assess the toxicity of Ag, Al_2O_3, Au, CeO_2, Cu, TiO_2, ZnO NPs to different species of soil invertebrates (*Caenorhabditis elegans*, *Eisenia fetida* and *andrei*, *Folsomia candida*). Various effects of metallic NPs have been observed, but conflicting results were often observed, therefore NPs toxicity remains a troubling point that requires further assessment.

Silver is one of the most studied ENMs due to its bactericidal properties. Tests conducted on *C. elegans* showed that Ag NPs caused reproductive toxicity due to the formation of reactive oxygen species, while no effect were seen in survival and growth (14-20 nm up to 0.5mg L^{-1}in K-media) (Rho et al. 2009). Shoults-Wilson and co-workers (2010, 2011a, 2011b) widely investigated the impact of Ag NPs on *E. fetida;* they found that soil type is more important than particle size and that avoidance behaviour is more sensitive than mortality, growth and reproduction. However, extended x-ray absorption fine structure spectroscopy analysis suggested that Ag NPs accumulation was not caused only by the NPs ionic form. Only a few authors examined gene expressions (Unrine et al. 2010; Unrine et al. 2010), apoptosis (Lapied et al. 2011), immune activity (Hooper et al. 2011), and extended the duration of the experiment (McShane et al. 2012). The toxicity is strictly correlated with the intrinsic chemistry of NPs: for example TiO_2 was found to be less toxic than Ag (McShane et al. 2012) and ZnO NPs (Cañas et al. 2011). Conversely, TiO_2 has a negligible dissolution so it can accumulate in soil and water compartments (Baun et al. 2008) and in the long-term bioaccumulation may occur (Unrine et al. 2008; French et al. 2009).

As a matter of fact, different effects of NPs on soil invertebrates have been reported, therefore studies should be made that focus on the link between the development of NPs over time and toxicity at realistically low doses, applying innovative microbiological approaches (e.g. analysis of phospholipid-derived fatty acids, enzyme activities) and other sensitive endpoints.

As shown, soil complexity induces fragmentary comprehension of physicochemical behaviour, accumulation and toxicity of manufactured NPs in this system and its related organisms.

1.4. INESE project

As a result of the above mentioned need for further study, The Italian Institute of Technology (IIT) funded the project “Impact of Nanoparticles in Environmental Sustainability and Ecotoxicity” (INESE), which supported this work. The basic concept of the project was to assess possible risks for the ecosystem due to unintentional release of engineered and transformed/secondary NPs during the disposal of nanoproducts. The dispersion can cause new pollution in air, soil and water environments. For this purpose this work focused on the assessment of the impact of NPs after repeated and chronic exposure, at lab and greenhouse scale, in the following simulated ecosystems: tomato-bumble bees, rice-bacteria, soil-worms, algae-sea urchin. Among the available commercial nanoproducts and engineered NPs, a selected group of them was investigated. The sample reflected materials present in the market, as the most commonly used are silver, titanium, silica and iron-oxide, and the innovative production, like cerium, cobalt, nickel and tin.

The nanoparticles studied here are significant to the field of environmental nanotechnology and nanotoxicology. Indeed, Ag, CeO_2, Fe_3O_4 and SnO_2 nanoparticles are produced by nanotechnology and are already present in the market in several products, in addition Co NPs occur in the environment as degradation products and pollutants:

- *Silver (Ag)*: the application of this metal for medical use has been documented since 1000 B.C. for its antimicrobial properties (Lea 1889; Chen & Schluesener 2008). In addition, silver nanoforms exhibit antifungal, antiviral, anti-inflammatory properties, surface plasmon resonance, plasmonic heating and fluorescence properties that make it suitable for consumer products (El-Badawy et al. 2010).
- *Cerium dioxide (CeO_2)*: this rare-earth metal has specific optical properties and the global market for the nanomaterial is around one thousand tonnes (Commission staff working paper 3/10/2012). CeO_2 NPs have been introduced into gasoline as a fuel additive to enhance the combustion process Cassee et al. (2011) and recently has been examined as a free radical scavenger.
- *Cobalt (Co)*: is an interesting material for its magnetic properties, it has potential use in medicine as a contrast agent and hyperthermia treatment of tumours. In addition, it has promising applications in the separation of various catalytic solids, fuel cells, catalyses (Legrand et al. 2001; Yang et al. 2006; Fernandez-Garcia et al. 2011).
- *Magnetite (Fe_3O_4)*: extensively investigated for its magnetic properties, these materials are extremely useful in biomedical applications. They can be "driven" with an external magnetic field in order to recover particles (to isolate specific compounds from

complex matrices for diagnostic purposes) and to drive particles to a target tissue (to concentrate particles in biological organs of interest). This type of particle can be employed as contrast agent for nuclear magnetic resonance and for hyperthermia treatment. In addition, magnetite has been tested and used to remediate contaminated soil and groundwater (Huber 2005).

- *Nickel (Ni):* the characteristics exhibited at the nanoscale level (high level of surface energy, high magnetism, low melting point, high surface area, and low burning point) have led to its experimentation and use in industry, such as medicine (Ban et al. 2011) and electronics (Magaye & Zhao 2012). It is worth noting that the bulk Ni is a chemical known to cause cancer or reproductive toxicity but little is known about the toxicity of nickel and nickel-based nanoparticles.

- *Tin dioxide (SnO_2)*: is a semiconducting metal oxide extensively used for gas leak detection and environmental monitoring (Liu et al. 2009). It has widespread applications: anti-static coatings, catalysts, electrodes and antireflection coatings in solar cells, energy-conserving coatings, liquid crystal displays, optoelectronic devices, resistors, transparent heating elements.

- *Titanium dioxide (TiO_2)*: very durable, lightweight, heat resistant to corrosion even in drastic chemical conditions, they have high photocatalytic activity and a relatively low cost. They are extensively used as pigment in paints, in addition the bulk material was classified as non-toxic and used in medicine to build joint replacements and in cosmetics as UV filter. Conversely, TiO_2 NPs may be released due to the extensive mobility of joint replacements; moreover when injected into rat tracheas, nanostructures of rutile (TiO_2 sometimes produced inflammatory effects in the lungs (Nemmar et al. 2008). Thanks to recent studies, TiO_2 has been registered under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals, Regulation (EC) No 1907/2006) but has not been classified as hazardous by the registrant due to lack of information (Commission staff working paper 3/10/2012).

1.5. Aims of the study

As a part of the INESE project, this study set out to extend the comprehension the impact of metallic NPs on one of the most important environmental compartments contaminated by NPs, the soil system, through the use of chemical and biological tools. For this purpose experiments were carried out to simulate believable environmental conditions of wet and dry deposition of NPs while considering ecologically relevant endpoints. In detail, the aim of this thesis involved the study of the following model systems and the evaluation of related issues:

- NPs and bare soil: could NPs affect functions of soil microbial communities?
- NPs and plants: could NPs exert chronic toxicity and accumulate in edible tissues?
- NPs and invertebrates: could NPs affect earthworms, damaging their functionality?

In the first experiment, the impact of NPs on soil microbial communities was assessed in lab-scale conditions in a medium-term experiment. The test aimed at determining several biochemical parameters, such as soil microbial biomass, soil respiration, ecophysiological indices like metabolic quotient (qCO_2), and the evaluation by DNA profile of the microbial communities' structure.

In the second experiment, the influence of NPs on plants was evaluated in a greenhouse environment, employing a chronic dose of NPs. Such assays allowed us to investigate phenotypic responses, from seedling to fruit maturity, and the plant uptake.

Finally, the effect of NPs on earthworms was estimated in a laboratory incubation experiment at both short and medium-term. Analysis focused on NP uptake and biological endpoints (i.e. PLFA).

In addition, at the end of each experiment ENM bioaccessibility in the soil was assessed by chemical extraction techniques as well as ENM presence in soil, plant and worms were investigated by environmental scanning electron microscope (ESEM) in order to identify and characterize the NPs and/or secondary nanoscale structure in soil and in the biological matrices.

2. Materials and methods

2.1. Nanoparticles

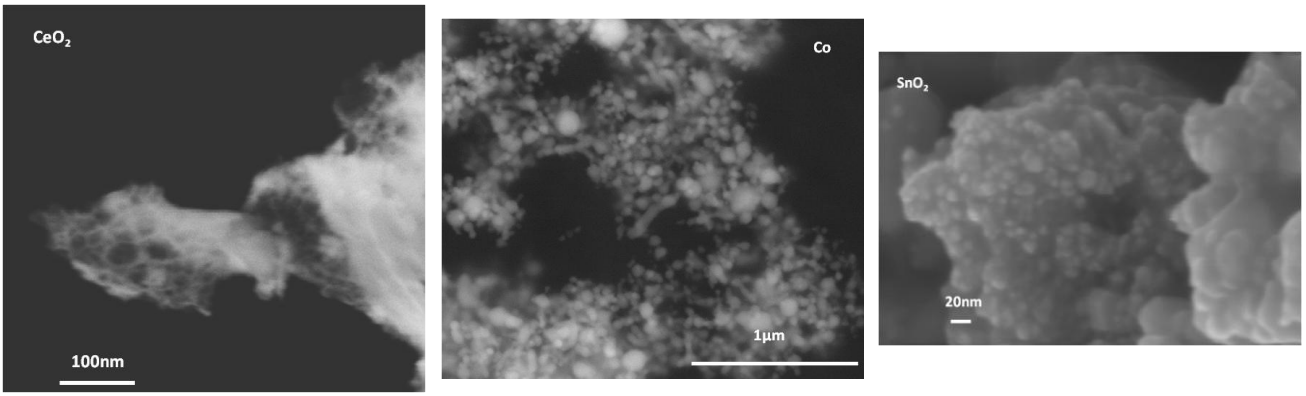
The nanoparticles examined in this study were: Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2, and TiO_2. Ag NPs were obtained from Polytech (Germany, type WM 1000-c), as a 1000 mg L^{-1} suspension in deionised water with polyvinylpyrrolidone (PVP) coated metallic silver (Ag); the NP size ranged between 1 and 10 nm. CeO_2, Co, Fe_3O_4, Ni, and SnO_2 powders were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, USA) with at least 98% purity. TiO_2 powder was purchased from Tal Materials, INC, USA. Table 2.1 reports the NPs' characteristics.

NP suspensions were freshly prepared before material spiking as follows: NPs were weighed with an analytical scale, suspended in deionised water to bring them to the required concentrations (see the following experimental design sections) and dispersed by ultrasonic vibration (100W, 40kHz; S100, Elmasonic, Germany) for one hour. The Ag solution did not need further sonication, as it was very stable. Fig. 2.1 shows some examples of pristine NPs observed through an Environmental Scanning Electron Microscope (ESEM, 200 QUANTA, FEI Company, The Netherlands).

Table 2.1 Selected characteristics of NPs used in the study.

Material	Shape	Purity (%)	Nominal particles size (nm)	Specific surface area (m^2 g^{-1})	Average hydrated diameter (nm)	Z-potential (mV)
Ag	-	-	10	-	60.3	-32.5
CeO_2	spherical	99.9	15-30* 50-105	30-50 8-15	133.1 178.3	44.5 43.0
Co	spherical	99.8	28	40-60	102	24.6
Fe_3O_4	spherical	99.0	20-30	>40	1407	10.6
Ni	spherical	99+	62	6.2	682.2	27.9
SnO_2	faceted	99.5	61	14	40.2	-47.7
TiO_2	-	-	20-160	-	999.0	-11.6
*Employed in Plant and NPs experiment II						

Figure 2.1 CeO_2, Co, SnO_2 NPs observed in the ESEM.



2.1.1. Hydrodynamic diameter and zeta potential

The hydrodynamic diameter and zeta potential of nanoparticle fresh suspension was obtained with the technique of Photon Correlation Spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, UK). The samples were measured 3 times, and the analysis were performed at 25°C with an angle of 90°, the data are shown as a function of on the number.

The zeta potential gives an idea of the stability of NPs in suspension: particles with a zeta potential at pH 7 below -30 and over +30 mV are considered stable (i.e., no aggregation over time), while particles with zeta potential between -30 mV and +30 mV at pH 7 have a tendency to aggregate over time (Zuin et al. 2011).

2.1.2. Ultrafiltration

To assess the NP ion release, 10mL of 100mg L^{-1} of the NP suspensions were centrifuged for 40 minutes at 4000 g in centrifugal filter devices (Amicon Ultra-15, Millipore, USA) with a 3kDa cut-off. The metal concentration in filtrates was measured by an inductively coupled plasma optical emission spectrometer (ICP-OES, Arcos, Ametek Spectro, Germany). According to Sasaki et al. (2006), 3kDa cut-off corresponds to approximately 1.5nm, for this reason the amount of metal found in the filtrates was in the ion form or small complex (Coutris et al. 2012).

2.2. Soils

In order to verify the possible impact of NPs on the soil system, different soils types were employed depending on the experiment.

The interaction between NPs and soil microbial biomass was assessed in natural soil collected at Monghidoro, Apennine in Northern Italy, from beneath an oak forest, is an Epileptic Cambisol (IUSS 2007). A1 (M1), A2 (M2) and AB (M3) horizons were sampled, dried, sieved (<2 mm) and then homogenised in the laboratory. The main biochemical characteristics are shown in Table 2.2.

Table 2.2 Biochemical characteristics of soils.

Soil type	Texture (USDA)	pH (H_2O)	TOC (g kg^{-1})	TKN (g kg^{-1})	CEC ($cmol_{(+)}$ kg^{-1})	Cmic (g kg^{-1})	Nmic (g kg^{-1})	Experiment
M1	Sandy clay loam	6.6	41.9	3.2	26.7	1083.5	97.7	SMB&NPs: Exp I
M2	Sandy clay loam	6.5	22.2	2.1	12.5	423.5	70.9	SMB&NPs: Exp I
M3	Sandy clay loam	6.5	20.4	1.4	16.5	448.4	29.2	SMB&NPs: Exp II
Mixture soil:peat	-	7.4	59.0	9.0	35.2	863.2	95.7	Worm&NPs

The experiment conducted to evaluate the influence of NPs on plants was carried out in a model soil made of 10% carbonate sand 10% silica sand and 80% neutral sphagnum peat which represents an excellent growth medium due to high moisture and nutrient-holding capacity with an uniform and slow breakdown-rate of physical structure (Ball et al. 2000).

Finally, earthworm breeding and the toxicological tests were performed in a mix of neutral sphagnum peat and natural soil in 1:1 v/v ratio to provide an optimal source of organic matter to annelids. See Table 2.2 for the main characteristics of the soil mixture.

2.3. Test organisms

The interaction between soil and plants was evaluated examining *Lycopersicon esculentum* Mill (tomato) and *Ocimum basilicum* L. (basil) as model plants. Tomato is one of the most important vegetables worldwide because of its high rate of consumption (Ouzounidou et al. 2008) and is commonly used in phytotoxicity studies (Ma et al. 2010). Basil is a culinary herb normally used fresh in Mediterranean area cuisine; unlike the tomato, the edible part of the basil plant is the leaf.

The response of invertebrates to NP exposure was assessed employing earthworms as the bioindicator (Paoletti et al. 1998). *Lumbricus rubellus* was chosen as a test species being an epigeic earthworm which lives on the soil surface in leaf litter. *Eisenia fetida* is most frequently used in ecotoxicity testing (OECD, 2004) but it lives in compost bin (warm and moist environments) and thus is less relevant for exposure in soil (Lapied et al. 2011). *L. rubellus* lives in and feeds on the leaf litter where NPs most likely end up after wet and dry deposits or sewage sludge disposal.

2.4. Chemicals

Chemicals and reagents used in the present study were analytical-grade and purchased from Sigma Aldrich (Italy), unless otherwise indicated. Ultrapure water (18MΩ cm^{-1}; Milli-Q, Millipore, USA) was used in all experiments, unless otherwise stated.

2.5. Interaction between the soil system and NPs

The aim of the research was to evaluate the interaction between soil systems and Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 NPs. For this purpose three model systems were prepared and the equivalent experimental designs are described in the following sections.

Table 2.3 Overview of the experimental design of the thesis.

	NPs	Species	Concentrations mg/kg	Matrix	Duration	Analysis
SMB&NPs Exp I	CeO_2, Fe_3O_4, SnO_2		0, 10, 100	Natural soil	7d, 30d, 60d, 90d	Bioaccessibility, SMB-C and N, Soil respiration, Viable count, DNA quantification, DGGE ESEM FTIR
SMB&NPs Exp II	Ag		0, 10, 100	Natural soil	30d, 60d, 90d	SMB-C and N, Soil respiration, Viable count, DNA quantification, DGGE Sequencing
Plant&NPs Exp I	Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2, TiO_2	*L. esculentum*	100	Artificial soil	130d	Growth, Metal uptake, Nutrient content ESEM
Plant&NPs Exp II	Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2, TiO_2	*O. basilicum*	80	Artificial soil	30d	Growth, Metal uptake, Nutrient content Gas exchange Net photosynthesis Pigment content Lipid peroxidation ESEM
Worm&NPs	Ag, Co	*L. rubellus*	10	Artificial soil Food: horse manure	Uptake: 5 wk Excretion:1 mth	Survival, Metal uptake, PLFA, Frequency of apoptosis ESEM

2.6. Interaction between SMB and NPs

Soil microbial biomass-bare soil system was studied by exposing a natural uncontaminated soil to NPs through a suspension in order to reproduce wet and dry depositing. Two experiments were carried out: in the first one short and medium-term incubation were used to assess the toxicity of CeO_2, Fe_3O_4, and SnO_2 NPs on microbial biomass and soil properties. In the second experiment a medium-term incubation was employed to assess the resistance and/or resilience of the soil system to the Ag NP disturbance.

2.6.1. Experiment I: impact of CeO_2, Fe_3O_4, and SnO_2 NPs on soil properties

M1 and M2 soils (100g as dry weight, see Table 2.1 for the main characteristics) were placed in Stericup® (Millipore, USA) to maintain correct gas exchange. The CeO_2, Fe_3O_4 and SnO_2 NP solutions (see section **2.1**) were added at 0 (deionised water, NP0) 10 (NP1) and 100 (NP2) mg of metal-NPs per kg of dry soil (Gottschalk et al. 2009) and soil moisture brought to 60% of its water holding capacity (WHC); each treatment was replicated three times. The Stericups® were incubated for 7 and 60 days in a thermostatic chamber at 25±0.5°C and the moisture was maintained at 60% WHC by gravimetrical method. At the end of the incubation period soil samples were prepared for chemical and biological analysis as described in the following sections. Furthermore, microbial viable counts and bacterial diversity by PCR-DGGE analyses were performed on M2 soils treated with the highest dose (NP2) at 30, 60, and 90 days.

2.6.1.1. Physicochemical characterization of soil

At the end of the experiment an aliquot of fresh soil was sieved at 2mm to perform the biochemical analysis, whereas another aliquot was air dried, sieved (<2mm) and finely ground with an agate mill for chemical characterization.

Soil pH was determined potentially in a soil/distilled water (1:2.5 w/v) suspension with a glass electrode (Compact Titrator, Crison, Spain). The total C and N were analyzed by gas chromatography after combustion at 1100°C using an elemental analyzer (EA1110 CHNS-O, CE Instruments, Italy). The soil cation exchange capacity (CEC) was determined in 0.1M $BaCl_2$ followed by a re-exchange with a 0.1M $MgSO_4$ solution with determination of elements by ICP-OES (Schwertfeger & Hendershot 2009). Texture was determined using a wet sieving and sedimentation method (Day 1965).

The total concentration of elements in soil samples was determined by ICP-OES after mineralization with *aqua regia* (AR: 2 mL HNO_3 plus 6 mL HCl; both Suprapur grade Carlo Erba, Italy) in a microwave oven (Start D 1200, Milestone, USA). The program used for the mineralization of the soil sample has the following characteristics:

- 3 minutes at 250 Watt
- 4 minutes at 450 Watt
- 3 minutes at 700 Watt

ICP-OES calibrations were performed by the standard solution of Bureau of Collection Recovery (BCR-909) and some internal standards were used.

2.6.1.2. Biochemical characterization of soil

Microbial biomass C (Cmic) and N (Nmic) were determined by a chloroform fumigation extraction method (Vance et al. 1987). Sub-samples of fresh soil were divided in two portions; one portion (10g of moist soil) was fumigated for 24 hours at 25°C with ethanol-free $CHCl_3$. After the fumigant removal, the samples were extracted with 40mL of 0.5 M K_2SO_4 for 30 minutes of shaking and then filtered (2.5µm filter paper, Whatman® 42, UK). The other portion (10 g), called "non-fumigated", was extracted similarly without fumigation. The extracts were analysed for both C and N concentrations by the Total Organic Carbon Analyser TOC-V/CPN (Shimadzu, Japan). Both Cmic and Nmic were calculated using a k_{EC} of 0.45 (Jenkinson et al. 2004) and a k_{EN} of 0.54 coefficients, respectively (Brookes et al. 1985; Joergensen & Mueller 1996). The amount of C and N from non-fumigated soil samples extracted by K_2SO_4 form was considered as the labile pool (Badalucco et al. 1992). The results for total and labile content of both elements are expressed as mg of C or N kg^{-1} dry soil.

Soil-respiration was determined in a closed system as described by Isermeyer (1952). Briefly, 20g (dry basis) of fresh sample were incubated in 500mL stoppered glass jars. The CO_2 evolved was trapped, after 1, 3, 7, 10, 14, 21, 28 days of incubation, in 2mL 0.5 M KOH and determined by back titration after adding 0.05M HCl. The metabolic quotient (qCO_2) expressed as ratio between soil respiration rate and microbial biomass carbon, was calculated according to Anderson and Domsch (1993).

2.6.1.3. Bioaccessibility of NPs in soil

The amount of accessible metals in soil samples was determined by soil extraction using deionised water, 1M NH_4NO_3 and a 0.02M ethylene-diamine-tetra-acetic acid (EDTA) solution. The latter was performed with a soil/solution ratio of 10 according to Wear and Evans, 1968. According to Khan et al. 2009 a soil/solution ratio of 2.5 w/v was used to perform the NH_4NO_3 extraction. Water extraction was carried out by shaking the soil-water suspension, with a ratio of 1:10 v/v, for 16 h (Blaser et al. 2000). Both soil suspensions were centrifuged for 15 minutes at 1200 *g*; then the surnatants were filtered through 0.45µm filter HTTP (Millipore, USA) and Whatman® 42 water and EDTA extract, respectively. The concentration of elements in the soil extracts was determined by ICP-OES.

Finally, the partition coefficient (Kp) was calculated according to the following equation:

$Kp = [metal]_{soil\ fine\ earth}/[metal]_{water\ extract}$

where Kp is the solid/water partition coefficient (L kg^{-1}); $[metal]_{soil\ fine\ earth}$ is the total metal concentration in soil determined in AR (mg kg^{-1}) and $[metal]_{water\ extract}$ is the free ion concentration

extracted in water (mg L^{-1}) at equilibrium conditions (after 16 hours) (Blaser et al. 2000). The data are expressed as log Kp (Vittori Antisari et al. 2013).

2.6.1.4. Determination of $CHCl_3$-labile (uptake) metal arising from NPs

The labile metal fraction of NPs stored in microbial cells was extracted after cellular lyses with $CHCl_3$ ($CHCl_3$-labile metal), with the above mentioned fumigation-extraction method (see section **2.6.1.2**) using 1M NH_4NO_3 as the extracting solution with a soil/solution ratio of 2.5 w/v (Khan et al. 2009).

After filtration, the extracts were acidified with HNO_3 Suprapur (Merck, Germany) (1:10 v/v ratio) and stored at 4°C. The metal concentration in the fumigated and non-fumigated extracts was determined by ICP-OES. $CHCl_3$-labile metal content was calculated as follows:

Labile metal = $[metal]_{fumigated\ extract}$ - $[metal]_{non\text{-}fumigated\ extract}$

No conversion values were applied according to Khan et al. (2009).

2.6.1.5. Characterization of NPs in soil

The distribution of NPs (CeO_2, Fe_3O_4, SnO_2) among soil fractions was monitored by scanning electron microscopy (ESEM, see section **2.9**) and infrared spectroscopy (FTIR, Tensor 27, Bruker, USA). After 60 days, soil treated with the highest rate (100 mg NPs kg^{-1}) and the control soil were fractionated into different particle size fractions (500, 125, 53, and 2mm). The fractionation was carried out by the wet sieving method (Day 1965). All fractions were analysed by FTIR, while only the clay fraction (<2 mm) was analysed by ESEM**.**

The different soil fractions were analysed by FTIR without subtracting organic matter. Two milligrams of the sample was mixed with 200mg KBr (FTIR grade) and pressed into a pellet. The sample pellet was placed in the sample holder and FTIR spectra were recorded in the range 4000-450cm^{-1} in FTIR spectroscopy at a resolution of 4cm^{-1} (Hemath Naveen et al. 2010).

2.6.1.6. Microbial cultivable viable counts

The M2 soil microcosms with the highest amount of NPs (NP2) and the relative control without NPs (NP0) were analysed for microbial viable count at time zero (i.e. before experiment start) and after 30, 60, and 90 days of incubation. Soil (10g) was suspended in 90mL of distilled water, and serial dilutions were prepared and plated on Tryptic Soy Agar (TSA) additioned with 2g L^{-1} cycloheximide and Sabouraud Dextrose Agar (SDA, Merck) containing 1g L^{-1} chloramphenicol for the enumeration of bacteria and fungi, respectively. Inoculated TSA plates were incubated for 3 days at 30 ± 1°C, whereas inoculated SDA plates were incubated for 72-120 hours at 25 ± 1°C.

Each soil sample was replicated three times. After incubation, the number of colony forming units (CFU) mL^{-1} was recorded, transformed into log values, and means and standard deviations were calculated.

2.6.1.7. DNA extraction from soil samples

Soil (250mg) was extracted using the PowerSoil DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions with some modifications as described in Gaggìa et al. (2013). Briefly, 5µL of mutanolysin (100U mL^{-1}) and 195µL of lysozyme (50mg mL^{-1}) were added to the soil powder in the bead solution supplied with the kit. The soil suspension was then incubated at 37°C on a rotary shaker for two hours, prior to chemical (with SDS-containing solution supplied with the kit) and mechanical (bead beating on vortex at maximum speed for 10 min) cell lyses. DNA was eluted with 100µL of 10mM Tris-HCl pH 8.0. The purity and quantification of extracted DNA was determined by measuring the ratio of the absorbance at 260 and 280nm (Infinite® 196 200 PRO NanoQuant, Tecan, Switzerland). Extracted DNA was stored at -20°C. Extraction was performed in triplicate samples for each incubation time and DNA obtained was pooled in order to have an average representation of the microbiota, according to the procedure of Smalla et al. (2001).

2.6.1.8. 16S rRNA gene amplification and DGGE analysis

PCR amplification of 16S rDNA extracted from soil was performed with universal primers 357f with GC clamp (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTACGGG AGGCAGCAG-3′) and 907r (5′-CCGTCAATTCCTTTGAGTTT-3′) (Sass et al. 2001). The amplification reaction was carried out in a 50µl volume containing 1.5U AmpliTaq Gold DNA polymerase (Applied Biosystems, USA), 5µL of 10X PCR Gold Buffer (Applied Biosystems), 200µM of each deoxynucleotide triphosphate (Fermentas GmbH, Germany), 1.50mM $MgCl_2$ (Fermentas), 0.50µM of each primer (MWG), 0.5mg mL^{-1} bovine serum albumin (Fermentas), 4µL DNA template (20ng μL^{-1}), and sterile MilliQ water. The PCR reaction was performed on a Biometra thermoblock (Biotron, Germany) under the following thermocycling program: 5 minute initial denaturation at 95°C; 35 cycles at 95°C for 30 seconds, 55°C for 60s, 72 °C for 40s; followed by a final elongation step at 72°C for 7 minutes. The size and amount of the PCR products were estimated by analyzing 2µL samples by 1.5% agarose gel (w/v) electrophoresis and ethidium bromide staining.

The DGGE analysis was basically performed as described by Muyzer et al. (1993), using a DCode System apparatus (Bio-Rad, USA). Polyacrylamide gels [7% (w/v)

acrylamide/bisacrylamide (37.5/1) (Bio-Rad)] in 1X Tris-Acetate-EDTA (TAE) buffer were prepared using a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad), using solutions containing 45–55% denaturant (100% denaturant corresponds to 7M urea and 40% (v/v) formamide). The electrophoresis was run at 55V for 16 hours at 60°C. Gels were stained in a solution of 1X SYBR-Green in 1X TAE for 20 minutes and their images captured in UV transillumination with Gel DocTM 226 XR apparatus (Bio-Rad).

DGGE patterns were analyzed with Gene Directory software (Syngene, UK), a similarity index was determined using the Dice coefficient and the unweighted pair group method with mathematical averaging (UPGMA).

2.6.2. Experiment II: impact of Ag NPs on soil microbiota

After a promising pilot test at short-term incubation, the following experiment was carried out to assess the impact of Ag NPs on soil microbial biomass. Soil M3 (100g as dry weight) were placed in Stericup® (Millipore, USA) to maintain a correct gas exchange. The Ag NP solutions (see section **2.4**) were added at 0 (deionised water, NP0) 10 (NP1) and 100 (NP2) µg of metal-NPs per gram of dry soil and soil moisture brought to 60% of its water holding capacity (WHC); each treatment was replicated three times. The Stericups® were incubated in a thermostatic chamber at 25±0.5°C and the moisture was maintained at 60% WHC by gravimetrical method. After 30, 60, and 90 days, soils were analysed for chemical and biological characterization as described in section **2.6.1.1**, **2.6.1.2**, **2.6.1.3** and **2.6.1.4**. Furthermore, the soils treated with the highest dose (NP2) were analysed for microbial viable counts and bacterial diversity by PCR-DGGE (see sections **2.6.1.6, 2.6.1.7**, **2.6.1.8**).

2.6.2.1. Sequencing of DGGE bands

Selected bands were cut from the gel with a sterile scalpel and DNA was eluted by incubating the gel fragments for 16 hours in 50 mL of sterile deionised water at 48 °C. Two mL of the solution were then used as template to re-amplify the band fragments using the same primers without the GC-clamp and the same PCR conditions. The obtained amplicons were sequenced (Eurofins MWG Operon, Germany) with primer 907r. Sequence chromatograms were edited and analyzed using the Finch TV software programs, version 1.4.0 (Geospiza Inc., USA) and obtained sequences were subjected to taxon classification using RDP classifier, a tool which is available at the RDP-II website (http://rdp.cme.msu. edu/classifier/classifier.jsp).

Moreover, SeqMatch search was used to find the closest match for each 16S rRNA fragment. (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Cole et al. 2009).

2.7. Interaction between plants and NPs

Plant-soil model was studied using an experimental protocol which provides the application of NP chronic doses on soil mixture, through irrigation water, (see section **2.2** for the soil characteristics) to avoid unrealistic concentrations and substrates. Two experiments, using two plant species, were performed to assess the influence of metal- (Ag, Co, Ni) and metal oxide- based (CeO_2, Fe_3O_4, SnO_2, TiO_2) nanoparticles on: i) the morphological parameters (e.g. dry weight, plant height); ii) the amount of metal absorbed by plants from NPs added to soil (namely Ag, Ce, Co, Fe, Ni, Sn, Ti); iii) the content of major nutrients (Ca, Mg, K, Na, P and S) in different organs; iv) the physiological parameter (e.g. gaseous exchange, Chlorophyll a/b).

2.7.1. Experiment I: tomato model

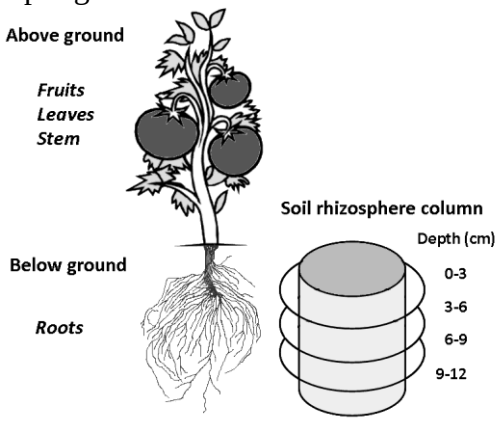
A growth experiment was carried out in a greenhouse at 28/20°C day/night temperature, with 11.5/14 hours (spring/summer) photoperiod, from March 26th to August 4th 2012; this period corresponds to the vegetative cycle of tomatoes (*L. esculentum* cv Cilao F1). The seedlings (about 10 cm) were placed in pots (5L each with 5kg of soil) containing a soil mixture (as described in section **2.2**) with a sand layer (4cm) at the bottom for drainage.

A total of 48 pots (6 pots for control test and each NP) were placed in a randomized block. After two weeks of adaption, the seedlings were spiked with Ag, CeO_2,Co, Fe_3O_4, Ni, SnO_2 and TiO_2 NP solutions (see section **2.1**) at 20 mg metal L^{-1} concentrations once per week, twice from the 13th week, to simulate a chronic dose of NPs supplied with irrigation. The nominal concentration added during the growing season was 100mg of NPs element kg^{-1} of soil. For the control test only water was supplied.

At the end of the experiment (130 days of growth), the soil of three tomato plants was sampled as follows: a Plexiglas® cylinder was inserted in the rhizosphere and a soil column of 12cm was sampled. The soil column was divided in 4 layers (each one 3cm deep). The deepest sample was sand (Fig. 2.2). Each tomato plant was separated into aerial part (stem and lives) and root, washed with deionised water and then prepared for further analysis. The fruits were also collected, washed, frozen at -80 °C and then lyophilized.

The rhizosphere soil samples were air dried, sieved (<2mm) and finely ground with an agate mill to determine the metal concentration as previously described in section **2.6.1.1**.

Figure 2.2 Soil and tomato sampling.



2.7.1.1. Vegetal tissue analysis

The influence of NP treatments on biomass production was evaluated at the end of the experiment collecting plant tissue and weighing the samples before and after drying at 60°C until achieving constant weight. The dry tissues (leaf, stems, root, fruits) of each plant were finely milled to facilitate acid digestion. Approximately a 0.4g sub-sample of plant tissues was treated with 8mL of concentrated Suprapur HNO_3 (Merck, Germany) plus 2mL of H_2O_2 (for electronic use, Carlo Erba, Italy) and digested in the microwave oven using the following program:

- 2 minutes at 250 Watt
- 2 minutes at 400 Watt
- 1 minute at 0 Watt (only ventilation to cool down)
- 3 minutes at 600 Watt

The content of nutrients (Ca, Mg, K, Na, P, S) and metal NPs (Ag, Ce, Co, Sn, Fe) in the leaves, stems, fruits and roots was quantified by ICP-OES. Blank and International Reference Materials (Olive leaves BCR-CRM 062) were analyzed to validate the method. In addition, standard solutions (0.5 mg L^{-1} Ag, Ce, Co, Sn) were analysed every 10 samples for quality control/quality assurance purposes.

Translocation Index (TI) was also calculated, which synthesises the capability of species to translocate nutrients and pollutants from roots to shoots (Paiva et al. 2002), according to the following equations:

TI = (DML)/(DMR+DMS+DML)*100 and

TI = (DMS)/(DMR+DMS+DML)*100

where, DMR, DML and DMS are the elements concentrations as a function of dry matters of roots, leaves and stem, respectively.

2.7.2. Experiment II: basil model

The second experiment was carried out growing *O. basilicum* plant in the greenhouse at 25-20°C day-night temperature, with a 14 hour photoperiod. The seedlings were placed in pots of 250 cm^3 filled with the soil mixture (see section **2.2** for the characteristics). A total of 48 pots (6 pots for control test and each NPs) were placed in a randomized block. After two weeks of adaption, the seedlings were spiked once per week with 50mL of Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 NP solutions (see section **2.1**) at 100mg metal L^{-1} concentrations, to simulate a chronic dose of NPs supplied with irrigation. For the control test only water was supplied. The treatment was repeated for 4 weeks and nominal concentration added during the experiment was 80mg of NPs element kg^{-1} of soil. Every week the plant growth was documented through leaf counting, whereas the physiological status was evaluated measuring the stomatal conductance at 48 hours after treatment. In addition, the photosynthetic efficiency was assessed after 48h from the 2^{nd} and 4^{th} treatment.

At the end of the experiment, 28 days after the first NP treatment, each plant was harvested, separated into aerial part (stem and lives) and root, washed with deionised water and then prepared for the following analysis: biomass produced (see section **2.7.1.1**), total element concentration (see section **2.7.1.1**), chlorophylls a and b, carotenoid and xanthophylls content and lipid peroxidation.

The soil samples were air dried and analysed for chemical characterization as described in section **2.6.1.1** and **2.6.1.3**. Due to the soil pH (7.5) the amount of accessible metals in soil samples was determined by soil extraction using 0.005M Diethylene-triamine-penta-acetic acid (DTPA) solution with a 1:2 ratio w/v according to Lindsay and Norvell (1978). After two hours of shaking, the soil suspension was centrifuged for 15 minutes at 1200 *g* and filtered through Whatman® 42; the concentration of elements in soil extracts was determined by ICP-OES.

2.7.2.1. Physiological parameters

Analysis of gaseous exchange and stomatal conductance are early indicators of plant stress, indeed both functions change rapidly in the presence of harmful factors and they can be measured with rapid and non-destructive techniques.

The stomatal conductance (mmol m^{-2} s^{-1}) was measured 48 hours after the NP treatment at 0, 7, 14, and 21 days, on six plants per treatment with the SC-1 Leaf Porometer (Decagon Devices, Inc., USA).

The leaf gas exchange (H_2O and CO_2 gas) was measured on attached leaf samples with an infrared portable CIRAS-2 (PP-System®, Hitchin, UK). This instrument consists of an infrared differential analyser (IRGA) connected to an automatic assimilation chamber (Parkinson's

Automatic Universal Leaf Cuvette, PAR 1000 mmol m^{-2} s^{-1}, 26°C, CO_2 13.63 mmol L^{-1} and 300cm^3 min^{-1} flow rate) and equipped with 18mm diameter, 2.5-cm^2 area cuvette inserts. Leaf transpiration rate (E, mmol m^{-2} s^{-1}), stomatal conductance (Gs, mmol m^{-2} s^{-1}) and net photosynthesis (Pn, µmol m^{-2} s^{-1}) were measured 48 hours after the NP treatment at day 14 and 21, on six plants per treatment.

In addition, at the end of the experiment leaf pigments and lipid peroxidation content were determined to evaluate possible NP impact on crop development and physiology.

Plant pigments were extracted from freeze-dried tissues according to Strickland and Parsons (1972). Briefly, 0.1g of leaf samples, from each treated plant, were milled in a mortar and 10mg of magnesium carbonate ($MgCO_3$) were added to neutralize solute acidity and to prevent the chlorophyll conversion in phaeophytin. Finally, 10mL of acetone was added to the milled material and then incubated for 12 hours in complete darkness. Subsequently, the samples were centrifuged at 10°C for 10 minutes at 10,000 rpm. Pigment content was then evaluated by measuring absorbance at the wavelengths maxima (470, 645 and 662 nm) for the solvent used in the extraction (pure acetone) (Moran 1982). An aliquot of the supernatant was collected (1cm cuvette) to perform the spectrophotometer analysis (DU 530, Beckman Coulter Inc., USA). The supernatant solutions were diluted by adding acetone, as necessary, to obtain a spectrophotometer reading in the range of 0.2 to 0.8 absorbance units at wavelengths of 645nm and 662nm.

The concentration of chlorophyll a (Chl a), chlorophyll b (Chl b) and the sum of leaf carotenoids and xanthophylls (c+x) were calculated using the following equations of Lichtenthaler and Buschmann (2001):

- Chl a (µg/mL) = [11,24*(abs 662)–2,04*(abs 645)]*dilution factor;
- Chl b (µg/mL) = [20,13*(abs 645)–4,19*(abs 662)]*dilution factor;
- c+x (µg/mL) = [(1000*(abs 470) – 1,90*(abs 662)–63,14*(abs 645)/214]*dilution factor.

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described by Hernandez and Almansa (2002). Fresh leaves (0.2g) were homogenized in 1mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15.000 *g* for 10 minutes. To 0.5mL of the aliquot of the supernatant, 1.5mL of 20% TCA containing 0.5% (w/v) TBA was added. The mixture was heated at 90°C for 20 minutes and then quickly cooled on ice. The contents were centrifuged at 10,000 *g* for 5 minutes and the absorbance was measured at 532nm. The value for non-specific absorption at 600nm was subtracted. The concentration of TBARS was calculated using a TBA acid calibration curve.

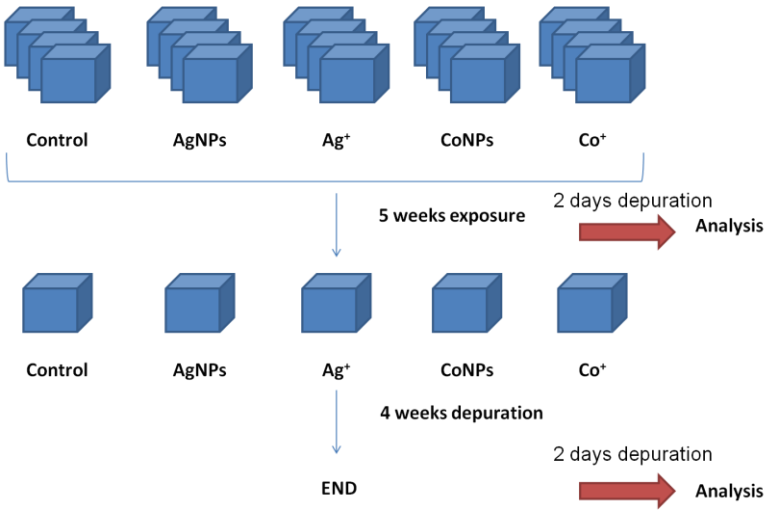
2.8. Interaction between earthworms and NPs

Earthworm-soil system was studied to assess whether NPs provided at chronic dose through the diet could exert metabolic stress or toxicity. For this purpose earthworms were exposed to the NPs by placing contaminated horse manure on the top of the soil to simulate sewage sludge disposal.

2.8.1. Experimental design

L. rubellus was exposed to Ag NPs, Ag^{+}, Co NPs and Co^{2+} through its diet for five weeks. Ten adult earthworms were kept in terrarium with 500g of soil mix (see section **2.2** for the soil characteristics) at 65% of WHC and fed once a week with ground horse manure (0.5g dry weight of manure per worm per week). Horse manure, from a non-medicated horse, was spiked 24 hours prior to feeding, with a water solution of NPs and ions (as nitrate) to reach the 65% of WHC. The concentration of both nanoparticle and ion solutions was 10mg of pollutant kg^{-1} dry horse manure for all substances. The experiment was carried out in quadruple. Every week the earthworms were counted and weighed to assess growth and survival. After five weeks of exposure to contaminated food, earthworms of three boxes of each treatment were transferred to Petri dishes for two days in order to empty their gut and then prepared for further analysis (Fig. 2.3). The earthworms of the fourth box were moved to another soil, having the same characteristics of the previous one, and fed for another month with unpolluted food (Fig. 2.3). After such period, earthworms were transferred into Petri dishes for two days in order to empty their gut, and then prepared for further analysis (Fig. 2.2).

Figure 2.3 Experimental design earthworm and NPs.



2.8.1.1. Chemical and biochemical soil analysis

At the end of the experiment soil samples were prepared for chemical and biological analysis as previously described in section **2.6.1.1** and **2.6.1.2**, respectively. Differently from the abovementioned method, in this experiment soil respiration was determined by measuring the CO_2 evolving from soil incubated under standard conditions. Briefly, 10g of soil at 50% of WHC was placed in 125mL glass bottles at 25°C, and the cumulative CO_2 accumulated in the headspace after 3-day incubation was determined by a gas chromatograph (Trace GC, Thermo Electron, USA) equipped with a thermal conductivity detector (TCD).

In addition, PLFAs were determined on soils and earthworms faeces according to the modified Bligh and Dyer method (White et al. 1979). Briefly, at the end of the first time of exposure, 5g of soil and about 0.5g of earthworm faeces were extracted with a single-phase mixture of chloroform/methanol/citrate buffer on a horizontal shaker (250 rpm) for 3 hours at room temperature. After centrifugation (3000*g*, 5 minutes) the supernatant was transferred to another glassware tube and the soil vortexed and re-extracted for another 3 hours with an additional volume of extractant. The combined supernatant was split into two phases by adding citrate buffer and chloroform and left overnight to obtain separation. The $CHCl_3$ layer was then transferred to a new tube and dried by using a rotavapor. Phospholipids were separated from neutral lipids and glycolipids by using silicic acid columns. Neutral lipids and glycolipids were eluted with chloroform and acetone separately. Phospholipids were obtained from methanol elution and dried by using the rotavapor. A mild alkaline methanolysis was used to convert phospholipids into Fatty Acid Methyl Esters (FAMEs) (Guckert et al. 1985). FAMEs were recovered with a *n*-hexane/chloroform (4:1, v/v) mixture, reduced to dryness by rotavapor and re-dissolved in 200 µL of *n*-hexane. FAMEs were detected on a gas chromatograph (Focus-GC, Thermo Scientific, USA) equipped with a flame ionization detector and a fused-silica capillary column Mega-10 (50m x 0.32mm I.D.; film thickness 0.25µm). The GC temperature progression was: initial isotherm at 115°C for 5 minutes, increase at a rate of 1.5°C per minute from 115 to 230°C, and final isotherm at 230°C for 2 minutes. Both injection port and detector were set up at 250°C, respectively and Helium at 1mL min^{-1} in a constant flow mode was used as carrier. The injected volume was 1µL in a splitless mode. Nonadecanoic acid methyl ester (19:0; cat no. N-5377) was used as an internal standard for quantification of FAMEs. The identification of the peaks was based on comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters mix cat no. 47080-U and Supelco 37 Component FAME mix cat no. 47885-U). The relative abundance of detected FAMEs

was expressed as mol %. The fatty acid nomenclature used was that described by Frostegård et al. (1993, 1996).

2.8.1.2. Earthworm tissue analysis

At the end of the first and second step of the experiment, some specimens were dried at 105°C, after 48 hours of depuration, and the metal concentration in the tissues was estimated by ICP-OES after digestion with HNO_3 and H_2O_2 (4:1 v/v) in the microwave oven with the program above described for the vegetable tissues (see section **2.7.1.1**)

Earthworms exposed for 5 weeks to the NPs were analysed for tissue fatty acid content by the following standard procedure described by Kennedy (1994). About 150mg of earthworm sub-samples were weighed in 10mL glass test tubes, 1mL of 4 N NaOH in 50% methanol was added and then the mixture was heated for 30 minutes at 100°C in a water bath. After cooling at room temperature, 2mL of 6 N HCl in methanol was added for methylation of dissolved fatty acids in a water bath at 80°C (10 minutes). Then 1mL hexane/methyl-tert-butyl ether (1:1, v/v) was added and lipids extracted by shaking for 10 minutes. The organic phase was transferred to a new test tube and the extraction was repeated. The combined organic phase was washed once with 0.25 N NaOH, and subsequently transferred to 2mL vials for analysis on the gas chromatograph as above described. The degree of unsaturation D was calculated according to (Kates 1986):

$D = \sum$ (% mono-unsaturated + 2 * % di-unsaturated + 3 * % tri-unsaturated + …)/100.

In addition, earthworms were analysed to evaluate the any changes in morphology or apoptotic frequency in their tissues. After the second step, for each treatments and box (Fig. 2.2), five purified earthworms were stored at 4°C in a test tube containing 4% paraformaldehyde in 0.1M phosphate buffered solution (pH 7.4). The earthworms were dehydrated in ascending concentration alcohols, and paraffin Paraplast (Bio-Optica, Italy) embedded. Groups of four serial sections (5µm thick) were either stained for histological observations by Alcian Blue (Serfőző & Elekes 2010) and Hematoxylin-eosin (Bio-Optica, Italy) (Gambardella et al. 2010). Apoptosis was assessed on the evidence of morphological characteristics, such as chromatin condensation with 4', 6-DiAmidino-2-PhenylIndole (DAPI) staining that is a fluorescent stain that binds strongly to A-T rich regions in DNA, as well as by a fluorescein-conjugated TUNEL test (terminal deoxy nucleotidyl transferase-mediated nick end labelling, Roche, Germany) (Ferrando et al. 2005). For fluorescence observations, nuclear DAPI counterstaining (1:1000, Molecular Probes, The Netherlands) was carried out. Negative control was performed by incubating sections with the Label Solution,

containing the nucleotide mixture without the transferase enzyme. Sections were examined under a Leica optical microscope (Leica, Germany) and visualized with a Leica software program using TIFF image formats.

2.9. Scanning electron microscopy ESEM-EDS

The observation of soil, vegetable and invertebrate tissues occurred under a Field Emission Gun Environmental Scanning Electron Microscope (FEG-ESEM, 200 QUANTA, FEI Company, The Netherlands) coupled with an X-ray microprobe for the elemental analyses (Energy Dispersive Spectroscopy- EDS by EDAX, USA).

The samples were investigated applying two methods as described below.

Samples were positioned on an aluminium stub using an adhesive carbon disc and dried at room temperature in a protected environment to avoid contamination. The presence of NPs in the soil or on the surface of vegetable tissues was identified with analyses performed by catching Back Side Electrons (BSE) in order to obtain information on the chemical nature of samples, rather than their morphology.

Plant and earthworm tissues were stored at 4°C in a test tube containing 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). These samples were processed for dehydration in ascending concentrations of alcohol solutions (70, 80, 95%) for 1 hour, then they were positioned on an aluminium stub using an adhesive carbon disc to analyze the external morphology. To verify the presence of NPs in samples' internal structure, once dehydration occurred, the specimen was embedded in paraffin and cut into transverse (10-12μm) or longitudinal sections. Sections were successively deparaffined, placed on an adhesive carbon disc and inserted in the chamber of the electron microscope (ESEM) for BSE analysis.

2.10. Statistical analysis

At least three replicates were included for each treatment in all experiments. The results are presented as means (± SD); significant differences were determined by one-way ANOVA, unless otherwise stated. Fisher's least significance difference (LSD) or Tukey's honestly significant difference (HSD) were performed as post-hoc tests. Statistical analyses were performed using the statistical package SPSS 15.0.1 (2006, IBM, Armonk, New York, USA).

Principal component analysis (PCA) is an unsupervised clustering method, which is a powerful tool for analysis of multivariate data, without requiring any knowledge of the dataset (Jambu 1991). PCA was used to transform a number of correlated variables into a smaller number of uncorrelated variables called principal components (Tabachnick & Fidell 2001).

PCA (Statistica 7.1 software, 2001, StatSoft, Tulsa, OK, USA) was performed on the standardised matrix of the chemical and physiological parameters of basil samples, including a total of 27 variables (leaf-stem-root fresh and dry weight, metal and macro nutrient content in each tissue, leaf transpiration rate, stomatal conductance, net photosynthesis, chlorophyll a and b).

In addition, PCA was carried out to investigate which fatty acids (FAs) were mostly affected by treatments. The data matrix for principal component analysis performed on FA profiles consisted of mol% of FAs with a concentration higher than 1.0%. The FAs that did not show significant differences among treatments, or $P<0.05$, as determined in preliminary analyses, were excluded from PCA to reduce the number of variables to fewer than the number of observations.

3. Results and discussion

3.1. Nanoparticles solubility

Ultrafiltration performed on the fresh NP suspensions showed that CeO_2, SnO_2 and TiO_2 NPs did not release ions or small complex while Ag, Co, Fe_3O_4 and Ni did. Indeed, in the filtrates the following levels were found: Ag NPs 3.46%; Co NPs 2.14%; Fe NPs 0.18%, Ni NPs 0.30%, expressed as a percentage of the nominal concentration.

3.2. Soil microbial biomass and nanoparticles

3.2.1. Experiment I: CeO_2, Fe_3O_4 and SnO_2 NPs impact on soil microbial biomass.

3.2.1.1. Physicochemical characterization of soil

Soil properties such as pH, cation exchange capacity, total organic carbon and total nitrogen remained stable throughout the experimental period (see Table 2.2). This is in agreement with the findings in soil polluted by heavy metals (Chander at al. 1995).

3.2.1.2. Biochemical characterization of soil

The biochemical parameters considered in the experiment are summarised in Table 3.1. After seven days of incubation, Cmic was 1187 and 431mg C kg^{-1} for M1 and M2 control soils respectively, while Nmic was 84 and 51.5mg N kg^{-1}; after two months, Cmic slightly declined in both horizons (1028 and 422mg C kg^{-1} for M1 and M2 respectively), whilst Nmic increased in the M1 soil (97.1mg kg^{-1}) and decreased in M2 (40.7mg kg^{-1}) (see Table 3.1). This decline over incubation time probably was due to the lack of organic substrate input (Chander and Brookes, 1991).

NP pollution did not significantly affect Cmic and Nmic. This evidence confirmed what Shah and Belozerova (2009) reported when soil was treated with different metal NPs, even at high rates. However, there are contradictory reports on the toxicity of metal and metal oxide NPs on soil microbial biomass (Dinesh et al. 2012); indeed, negative impact of metal oxide NPs on soil bacterial biomass have been also shown (Ge et al. 2011).

Turning to the details of the various treatments, in the samples spiked with CeO_2, Cmic declined proportionally to the dose in both incubation time and soil horizons. Similar behaviour can be observed for the M1 soil treated with magnetite. Conversely, in the same M2 soil after one week of exposure, Cmic increased as compared to the control and then decreased at 60 days. Tin dioxide

spiking promoted an increase in microbial C in both soils after seven days, but it decreased consistently after long exposure except at the higher dose in M1 soil.

These differences were not significant but this evidence suggested than NPs may interact with soil microbial biomass. Indeed, the C/N ratio of microbial biomass changed in NP contaminated soils over time. After one week of incubation at the lowest rate, in the soil polluted with Fe_3O_4 and SnO_2 the microbial C/N ratio increased up to 17 and 23, respectively; conversely, after 60 days, C/N, was around 10 for both unpolluted and polluted soils. Therefore, considering that fungi have a higher C/N ratio (5-15) than bacteria (3-6) (Paul & Clark 1996), the variation of the C/N ratio observed in the experiment was probably due to a change in bacterial biomass/fungal biomass ratio. As expected, the microbial C/N ratio of the M1 soil, the A1 horizon, was higher than in the A2 horizon of forest soil, probably because fungal biomass was higher on the surface than in deeper soil layers. Over 60 days, the microbial C/N ratio stabilized around 10 in both soils, as the result of the selection of microbial communities able to mineralize soil organic matter. The highest microbial C/N ratio values were found in the SnO_2 polluted soil, due to the dominance of fungal over bacterial biomass (Dilly et al., 2003). Indeed, tin oxide contamination has been found to stimulate growth of ectomycorrhizal mycelia in the short term (Wallander et al. 2003); in addition, low concentrations of toxic heavy metals, such as Cd and Pb (Stebbing 1982; Thompson & Couture 1991), may exert a hormetic effect. On the contrary, the low microbial C/N ratio of soil polluted with CeO_2-NPs was probably due to the predominance of bacterial over fungal biomass.

The metabolic quotient (qCO_2) significantly increased in the M1 soil treated with CeO_2-NPs ($P < 0.001$) after 7 days, while this enhanced in both SnO_2 and Fe_3O_4-NPs ($P < 0.001$) treatment after 60 days. The qCO_2 values after pollution with NPs were generally higher than in the NP-0 treatment and significant increases in these values in both soils were shown briefly after exposition to CeO_2 (Fig. 3.1). In M2 soil, significant ($P < 0.01$) increases of qCO_2 for SnO_2 and Fe_3O_4-NPs treatment were found at both incubation times, while in M1, the metabolic quotient significantly ($P < 0.001$) increased with SnO_2-NPs only after 60 days (Fig. 3.1). Increases in qCO_2 values have been attributed to a low efficiency of the utilization of substrate for growth when microorganisms are under stress (Giller et al. 1998, 2009). The qCO_2 showed values higher than two in NP polluted soils, probably indicating microbial stress. However, the qCO_2 value also depends on other factors than stress; for example, changes in the bacterial/fungal biomass can also affect this parameter (Wardle & Ghani 1995; Nannipieri et al. 2003). Furthermore, the rise of C mineralization rate can increase the C-labile pool (Jandl & Sollins 1997) as observed in samples polluted with SnO_2 and Fe_3O_4 NPs where labile C increased from 20 to 45% after exposure (see Table 3.1).

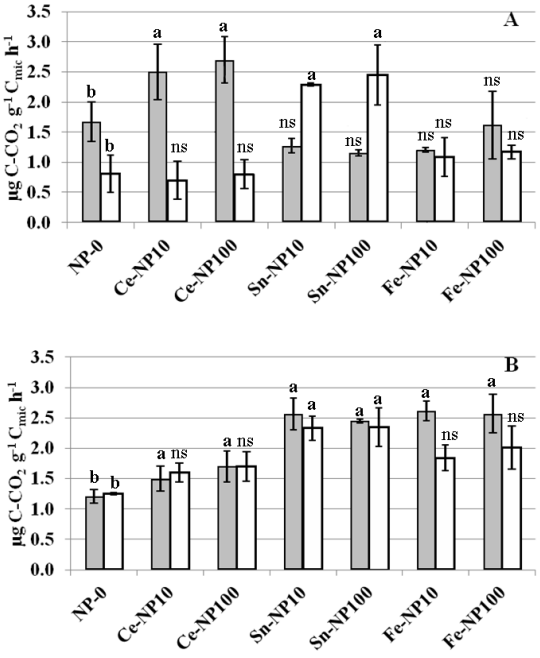
Table 3.1 Comparison between soil microbial biomass (Cmic and Nmic), labile pools (K_2SO_4- extractable C and N) amount and metal (Ce, Fe and Sn) $CHCl_3$-labile concentrations on two different soil samples (M1 and M2) at different concentrations of NPs (0, 10, 100 mg kg^{-1}) after 7 and 60 days of incubation. Standard deviation (SD) is reported in italic. Form Vittori et al. (2013).

		One week incubation						**Two months incubation**					
		Labile-C	**Labile-N**	**Cmic**	**Nmic**	**Uptake**	**C/N**	**Labile-C**	**Labile-N**	**Cmic**	**Nmic**	**Uptake**	**C/N**
		mg kg^{-1}				**µg kg^{-1}**		**mg kg^{-1}**				**µg kg^{-1}**	
M1	NP-0	321	84.5	1187	84.0	DL	14.1	302	126	1028	97.1	DL	10.6
	SD	*8*	*5.6*	*30*	*8.6*			*6*	*6*	*33*	*5.4*		
	CeO_2-NP1	215	104	808	58.3	3.10	13.8	162	110	665	93.2	DL	7.10
	SD	*15*	*6*	*24*	*5.6*	*0.10*		*9*	*5*	*29*	*1.8*		
	CeO_2-NP2	207	105	637	72.4	6.30	8.80	186	120	558	96.1	DL	5.80
	SD	*21*	*9*	*38*	*3.4*	*0.01*		*11*	*4*	*16*	*0.9*		
	Fe_3O_4-NP1	315	90.6	1070	64.5	102	16.6	496	158	1083	85.6	DL	12.7
	SD	*15*	*9.8*	*24*	*2.7*	*0*		*9*	*5*	*7*	*2.3*		
	Fe_3O_4-NP2	345	91.2	995	68.8	153	14.4	428	164	1013	74.5	DL	13.6
	SD	*12*	*6.5*	*38*	*5.5*	*0*		*8*	*3*	*7*	*7.2*		
	SnO_2- NP1	335	92.9	1474	63.8	DL	23.1	459	164	1025	93.5	DL	11.0
	SD	*15*	*6.8*	*24*	*7.6*			*2*	*4*	*10*	*2.1*		
	SnO_2- NP2	368	89.7	1253	74.2	DL	16.9	412	172	1316	92.1	DL	14.3
	SD	*12*	*7.4*	*38*	*8.4*			*6*	*3*	*7*	*1.2*		
M2	NP-0	116	53.8	431	51.5	DL	8.50	155	55.0	422	40.7	DL	10.4
	SD	*11*	*4.4*	*43*	*11.6*			*5*	*4.5*	*26*	*15.2*		
	CeO_2-NP1	119	45.3	295	38.3	69.6	7.70	83.9	46.7	355	22.4	41.9	15.8
	SD	*7*	*2.1*	*59*	*3.5*	*0.1*		*15.7*	*5.0*	*43*	*4.3*	*0.0*	
	CeO_2-NP2	149	47.7	225	40.5	89.5	5.60	112	56.0	176	16.1	43.6	10.9
	SD	*3*	*1.5*	*25*	*7.4*	*0.0*		*12*	*4.6*	*32*	*2.3*	*0.2*	
	Fe_3O_4-NP1	118	49.3	563	32.5	DL	17.3	156	43.6	389	29.8	DL	13.1
	SD	*14*	*5.6*	*24*	*7.6*			*3*	*4.1*	*10*	*2.3*		
	Fe_3O_4-NP2	109	46.3	551	38.7	DL	14.2	142	48.7	348	33.1	DL	10.5
	SD	*0*	*4.3*	*38*	*8.4*			*3*	*2.7*	*7*	*0.6*		
	SnO_2- NP1	144	48.6	521	40.8	DL	12.8	146	49.8	335	23.5	DL	14.3
	SD	*8*	*1.1*	*17*	*2.6*			*3*	*1.2*	*11*	*2.5*		
	SnO_2- NP2	105	42.4	556	41.5	DL	13.4	136	52.6	326	24.5	DL	13.3
	SD	*11*	*5.7*	*25*	*7.4*			*12*	*5.9*	*8*	*4.7*		

DL lower than detection limit; the values of DL were 0.01, 0.001, and 0.16 µg kg^{-1} for Ce, Fe and Sn, respectively.

Conversely, the K_2SO_4-extractable C and N remained stable over time in the control samples and in CeO_2 NP samples the C-labile pool slightly decreased at both doses and horizons.

Figure 3.1 Metabolic quotient (qCO_2) at different doses of NPs (0, 10, 100 mg kg^{-1}, respectively) after 7 days (grey columns) and 60 days (white columns) in M1 (figure A) and M2 (figure B). The lowercase letters (a and b) indicate a statistically significant differences (p<0.05) according to Student's *t* test between the treatments with NPs (a) and control test (b). Form Vittori et al. (2013).



3.2.1.3. Bioaccessibility of NPs in soil

The amount of bioaccessible NPs determined with solutions of differing ionic strengths are shown in Table 3.2. The concentration of elements due to NP dissolution in both soils decreased as follows: AR > EDTA > NH_4NO_3 > H_2O, since the different solutions have different extraction efficiency.

Indeed, the EDTA only extracted Fe in both unpolluted and magnetite polluted soils, whereas water alone extracted Sn in the M2 soil. The recovery of total elements after extraction with *aqua regia* was lower than the theoretical value. This confirmed that most of the analytical methods usually applied to assess the accessibility/availability of metals cannot identify free elements released from NPs (Gupta & Sinha, 2007).

All elements showed a high log partition coefficient Kp (>2.8), suggesting that they are characterized by low geochemical mobility in water (Cornelis et al. 2011).

Table 3.2 Concentrations of Ce, Fe, Sn found in reference soils M1 and M2, after different extraction: EDTA, NH_4NO_3, H_2O and aqua regia (AR) during the incubation. Also, the logarithm values of partition coefficients (Kp) are reported. Form Vittori et al. (2013).

		EDTA		NH_4NO_3		H_2O		AR		log (Kp)	
		7d	60d	7d	60d	7d	60d	7d	60d	7d	60d
		mg kg^{-1}		mg kg^{-1}		µg L^{-1}		mg kg^{-1}		L kg^{-1}	
M1	NP-0	DL	DL	0.20	0.04	3.90	4.30	20.1	23.4	3.70	3.70
	CeO_2-NP1	DL	DL	0.20	0.05	8.50	28.4	34.5	45.6	3.60	3.20
	CeO_2-NP2	DL	DL	0.20	0.05	60.8	89.5	59.3	52.3	3.00	2.80
	NP-0	179	162	DL	DL	1831	5659	46645	42593	4.40	3.90
	Fe_3O_4-NP1	214	166	DL	DL	2468	4256	44789	47465	4.30	4.00
	Fe_3O_4-NP2	245	156	DL	DL	3624	4267	45525	44432	4.10	4.00
	NP-0	DL	DL	DL	DL	DL	DL	1.60	1.70	DL	DL
	SnO_2-NP1	DL	DL	DL	DL	DL	DL	17.4	19.1	DL	DL
	SnO_2-NP2	DL	DL	DL	DL	DL	DL	50.4	58.9	DL	DL
M2	NP-0	DL	DL	0.40	0.20	4.1	3.90	21.2	23.3	3.70	3.80
	CeO_2-NP1	DL	DL	0.50	0.30	170	239	33.4	42.3	2.30	2.20
	CeO_2-NP2	DL	DL	0.40	0.30	863	601	89.7	90.1	2.00	2.20
	NP-0	372	423	0.20	0.80	2975	1207	29456	22763	4.00	4.30
	Fe_3O_4-NP1	375	409	0.20	0.90	3758	681	33261	24136	3.90	4.50
	Fe_3O_4-NP2	370	410	0.20	0.90	3081	876	37425	24512	4.10	4.40
	NP-0	DL	DL	DL	DL	DL	DL	0.9	0.8	DL	DL
	SnO_2-NP1	DL	DL	DL	DL	5.10	1.50	1.50	2.00	2.50	3.10
	SnO_2-NP2	DL	DL	DL	DL	5.80	2.90	2.90	3.00	2.70	3.00

DL lower than the detection limit; the values of DL for Ce, Fe, and Sn are 0.013, 0.001, 0.018 µg kg^{-1}, respectively

3.2.1.4. Determination of $CHCl_3$-labile (uptake) metal arising from NPs

Interesting changes in the amount of $CHCl_3$-labile metal were observed between soils and over incubation time. After seven days, both Ce and Fe were released after $CHCl_3$ fumigation of the CeO_2, and Fe_3O_4 treated M1 soils at both used doses whereas this did not happen for both SnO_2 treatments (Table 3.1). In the same soil the $CHCl_3$ labile fraction was not detectable after 60 days. The geogenic Fe was not stored in microbial cells according to Khan et al. (2009), while $CHCl_3$ labile Fe was detected after exposure to magnetite, probably because of the dissolution of magnetite with release of free iron (Bhilash et al. 2011). Conversely, after 60 days Fe content was undetectable probably due to the formation of aggregates and their interaction with soil particles, thus reducing their toxicity (Hassellöv et al. 2008). The M2 soil treated with CeO_2 NPs showed the presence of $CHCl_3$ labile fraction at both doses after 1 and 9 weeks (Table 3.1). Indeed, Ce can exist both as Ce(III) and Ce(IV) with the latter being more toxic (Oral et al. 2010) since it causes the oxidation of membrane components involved in the electron transport chain (Thill et al. 2006). The low solubility of Ce(IV) and its low reduction rate to Ce(III) may explain the slow dissolution rate of CeO_2-NPs in the environment (Deshpande et al. 2005); however, since the Ce(III) concentration in nanoparticles increases by decreasing NP size, Ce(III) can be solubilised (Lopez-Moreno et al. 2010; Roh et al. 2010) and thus free Ce(III) can be present in soil and taken up by microbial cells. The presence of Ce $CHCl_3$ labile pools may depend on the different composition of the microbial communities among the two soils with the presence of Ce-tolerant microorganisms in the M2 soil.

3.2.1.5. Characterization of NPs in soil

The FTIR spectra are characterized by a region composed of vibration bands at 530.9 and 472.5cm^{-1}, for CeO_2, at 622cm^{-1} for SnO_2 and at 571 and 440cm^{-1} for Fe_3O_4 (Fig. 3.2). These spectral regions were not activated in coarse sand fractions (125-500, 53-125 mm) of soil polluted with all NPs; as an example Fig. 3.3a and b only show CeO_2 data. The characteristic vibration bands of NPs were found in silt and clay fractions, as shown in Fig. 3.3c and d for CeO_2, respectively. The FTIR spectra of the clay fraction obtained in all treated soils showed that all NPs were localized in this fraction (Fig. 3.4). Electron microscopy confirmed the presence of NP aggregates in the clay fraction (Fig. 3.5). The SEM scan showed an irregular distribution of NPs in the clay fraction, because the presence of both NP agglomerates and NPs on the inorganic colloids was observed. The most present NP aggregates and clusters of different size had an average size of 500nm, thus the NPs could be associated to small size (2-53 and <2 mm) aggregates, which are rich in both labile organic C (Bol et al. 2009) and microbial biomass C (Van Gestel et al. 1996). Therefore, the

presence of NPs in the clay fraction suggests that NPs can affect most of microbial communities inhabiting soil.

Figure 3.2 FTIR spectra of a) CeO_2, b) SnO_2 and c) Fe_3O_4 NPs. Form Vittori et al. (2013).

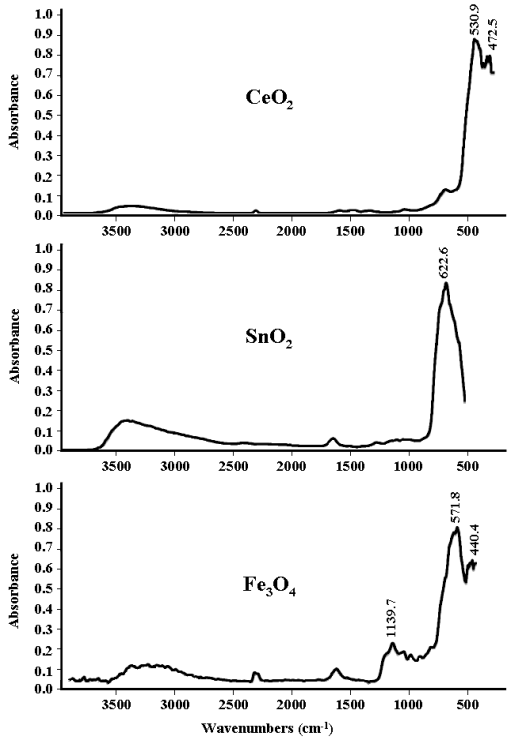


Figure 3.3 FTIR Spectra of 125-500 µm (a), 53-125 µm (b), 2-53 µm (c), <2 µm (d) soil fractions. Form Vittori et al. (2013).

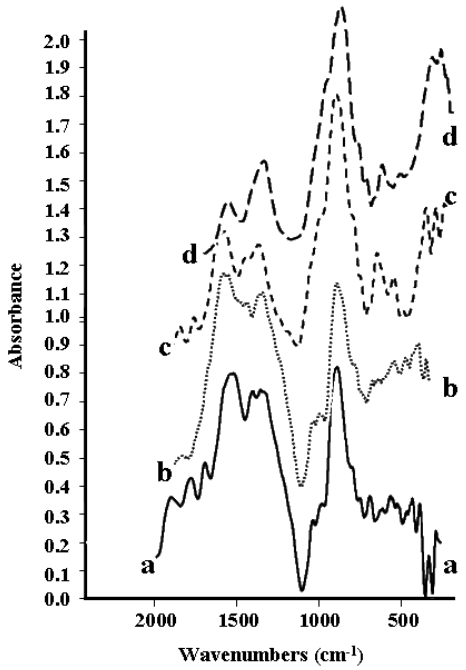


Figure 3.4 FTIR spectra of clay fractions of soil polluted with SnO_2 (a), CeO_2 (b) and Fe_3O_4 (c) and of unpolluted soil (d). Form Vittori et al. (2013).

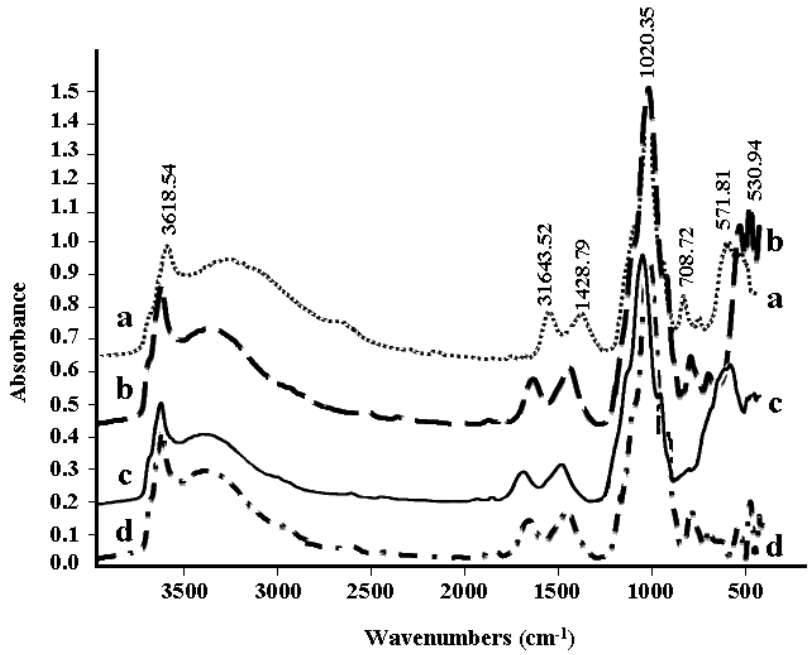
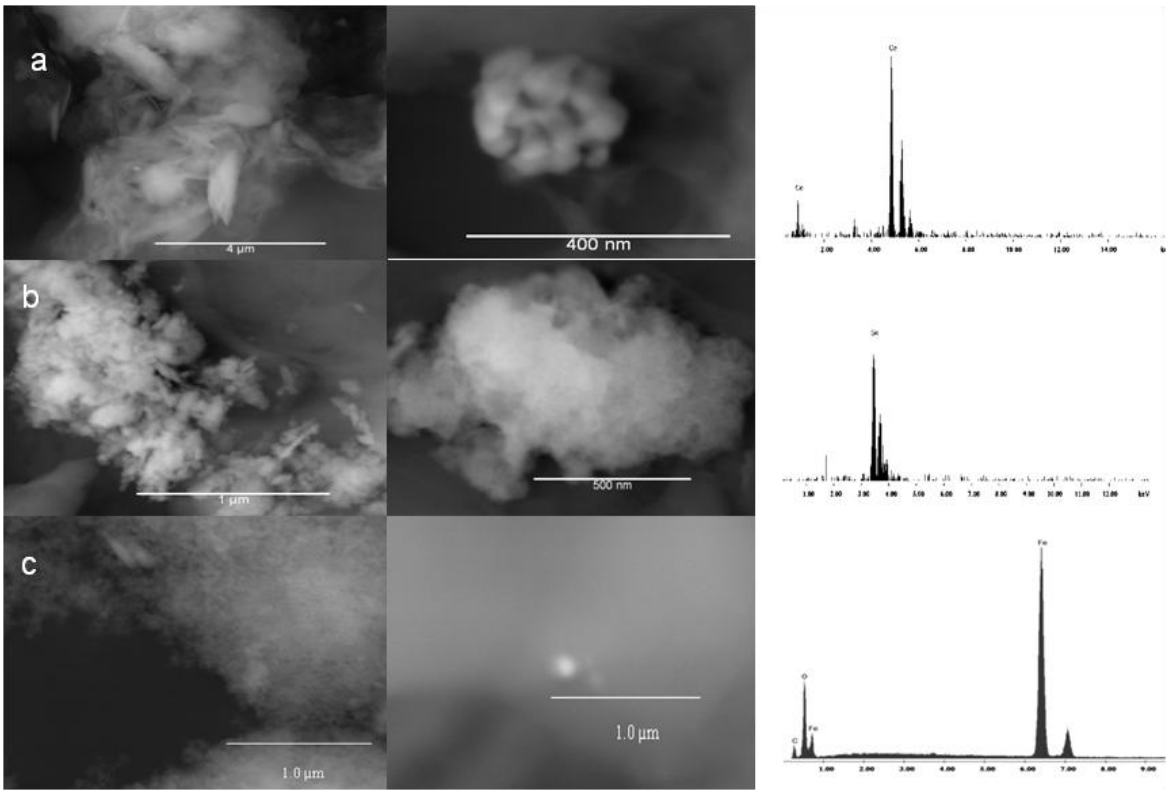


Figure 3.5 Aggregates of metal oxides-NPs in clay fractions of soil. ESEM images and X-ray spectra of CeO_2 (a), SnO_2 (b) and Fe_3O_4 (c)NPs aggregates. Form Vittori et al. (2013).



3.2.1.6. Microbial cultivable viable cell counts, DNA extraction and DGGE profile

The results of microbial cultivable viable cell count of soil M2 spiked with the highest dose of NPs are shown in Figure 3.6. Soil samples treated with SnO_2 NPs after 30 days of incubation showed a significant decrease (P<0.05), with respect to the control, of both bacteria and fungi counts of about 1 and 0.76 point of log, respectively. Notably, after 60 days, SnO_2 NPs treatment determined an increase of fungi counts (0.36 log). At the end of the incubation, the bacterial counts showed evident differences between control and Fe_3O_4 NPs (log 7.03 and log 6.86, respectively) and CeO_2 SnO_2 NPs (both log 6.37), with the highest and lowest cell count. Conversely, differences in fungi count are much lower and ANOVA analysis observed no significant differences between samples and control.

The DNA quantification showed an increase of DNA concentration of 64%, 18%, 58% in soil samples treated with CeO_2, Fe_3O_4, and SnO_2 NPs, respectively (Fig. 3.7). At 60 days, the DNA content decreased for CeO_2 and Fe_3O_4 NPs, conversely the difference between control and SnO_2 NPs remained high (43%). At the end of the incubation time there is also a decrement of DNA concentration in SnO_2 NPs samples (-9,9%). As mentioned above this compound can stimulate

growth of ectomycorrhizal mycelia in the short time (Wallander et al. 2003) probably due to an hormetic effect (Stebbing 1982; Thompson & Couture 1991).

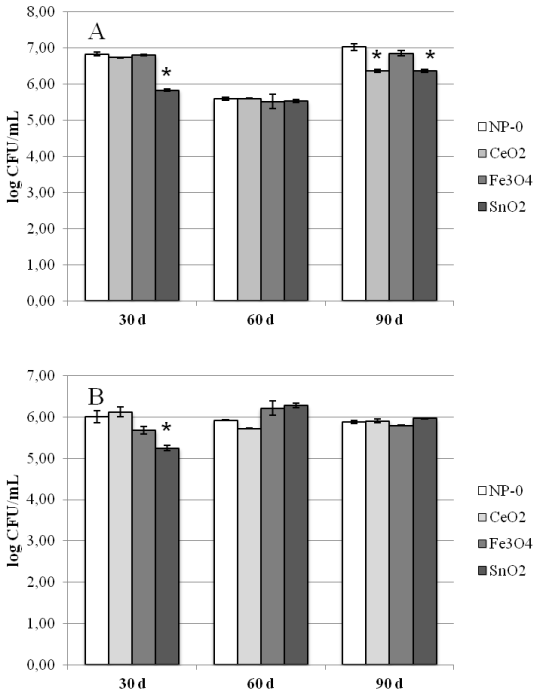


Figure 3.6 Cultivable biomass on TSA (A) and SDA plates (B) control (NP-0) and soil treated with CeO_2, SnO_2 and Fe_3O_4 NPs after 30, 60 and 90 days of incubation. significant differences with $P<0.01$ between NPs samples and the respective control are indicated with *.

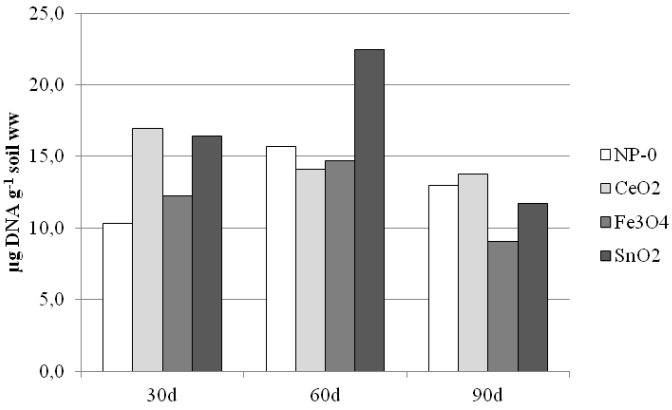
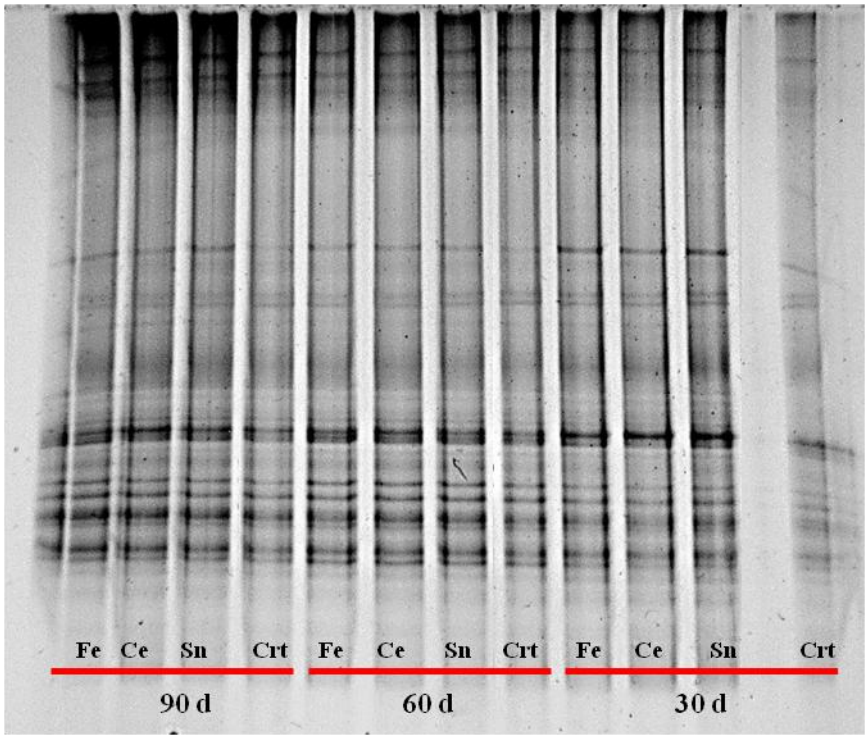


Figure 3.7 DNA quantification of control (NP-0) and soil treated with CeO_2, SnO_2 and Fe_3O_4 NPs after 30, 60 and 90 days of incubation (unique value).

In addition, the PCR-DGGE profile of control and NP treated soils (Fig. 3.8) did not change significantly upon incubation and or following CeO_2, Fe_3O_4 and SnO_2 NPs treatment.

This suggests that the NPs studied in this project—even if they were able to significantly modify the ecophysiological indicators—did not cause a shift in the bacterial community or a reduction of the soil complexity in the medium-term. As observed by Sciubba et al. (2014) the amendment with biosolids did not cause a shift in bacterial community in sandy loam soil. Notably, Nogueira et al. (2012) found that the structural diversity of the soil microbial community investigated by DGGE organic and inorganic ENMs has significantly affected the structural diversity of the soil bacterial community of OECD soil.

Figure 3.8 Representative DGGE profiles of soil amended with CeO_2, SnO_2 and Fe_3O_4 NPs and untreated controls (Crt) at the three different incubation times (30, 60 and 90 days) after 16S rDNA amplification with universal primers.



3.2.2. Experiment II: Ag NPs impact on soil microbial biomass.

3.2.2.1. Physicochemical characterization of soil

Soil properties such as pH (6.5±0.5), cation exchange capacity (16.5±0.7), total organic carbon (20.4±1.6) and total nitrogen (1.4±0.6) were not statistically affected by Ag NP pollution during the incubation period (three months), confirming what had already occurred in soil treated with other nanoparticles (Hänsch & Emmerling 2010; Ben-Moshe et al. 2013).

3.2.2.2. Biochemical characterization of soil

Table 3.3 shows the variation of biochemical parameters for the period of the experiment. As observed previously, in the control samples Cmic decreased by 50% of its value (from 467 to 226 mg Cmic kg^{-1}) after 60 days and the value remained stable until 90^{th} day; while, Nmic remained constant during the three months, being 31.3-39.2-35.2 mg Nmic kg^{-1} at day 30, 60 and 90 respectively.

Regarding the soil treated with Ag NPs, there was a decrease of Cmic and Nmic at both doses and exposure times ($P< 0.05$). The dose-dependent decrease is much more severe for the higher

dose at day 60, with an average value of 33mg Cmic kg^{-1} dry soil; after 90 days, this value increased to 56mg Cmic kg^{-1} dry soil, probably due to the proliferation of Ag tolerant microorganisms. The ANOVA test showed that the decrease of Cmic was statistically significant as compared to the control, unlike the Nmic, due to high variability of measurements.

Table 3.3 Comparison between soil microbial biomass (Cmic and Nmic), microbial C/N ratio, metabolic quotient (qCO_2), labile pools (K_2SO_4- extractable C and N) amount and Ag metal $CHCl_3$-labile concentrations on M3 soil at different concentrations of Ag NPs (0, 10, 100 mg kg^{-1}) after 30, 60 and 90 days of incubation.

		Cmic	**Nmic**	**C/N**	**qCO_2**	**Labile-C**	**Labile-N**
		mg kg^{-1}			**µg C-CO_2 h^{-1} g Cmic**	**mg kg^{-1}**	
30 d	NP-0	467,0	31,3	14,9	1,3	120,0	16,9
	Ag-NP1	216,0	24,1	9,7	2,1	76,0	9,5
	Ag-NP2	169,0	17,5	9,0	2,9	98,0	13,2
	ANOVA	**	ns		**	*	ns
60 d	NP-0	226,0	39,2	5,8	1,7	191,0	21,2
	Ag-NP1	113,0	6,3	17,9	3,4	51,0	9,6
	Ag-NP2	33,0	2,2	15,0	3,1	22,0	2,7
	ANOVA	**	*		**	*	ns
90 d	NP-0	215,0	35,2	6,1	1,8	214,0	17,6
	Ag-NP1	123,0	7,2	17,1	2,8	47,0	2,4
	Ag-NP2	56,4	3,5	16,1	3,3	18,0	1,2
	ANOVA	****	*		**	*	ns

ANOVA one –way test (Tukey's test $p<0.05$) was performed between Ag NPs doses and exposition times. ns = not significant, while significant values were indicated as * (<0.05), ** (<0.01), *** (<0.001).

The labile C- and N-pools increased by prolonging experiment times in the control while declining significantly ($P<0.05$) K_2SO_4 - extractable C, after exposure of soil to silver nanoparticles, but this did not occur for K_2SO_4 - extractable N.

The C/N microbial ratio in the control treatment decreased between 30 and 60 days by 60% while remaining stable at day 90. Conversely, C/N microbial ratio increased by 85 and 67% in NP1 and NP2 after 60 days and remained high (17 and 16 for NP1 and NP2, respectively) after 90 days of exposure, probably due to the severe decrease in the amount of bacteria. The soil exposure of Ag NPs increased the metabolic quotient (qCO_2) ($P<0.01$) and the variations were dose-dependent and significant compared to control (Table 3.3). Notably, Hänsch and Emmerling (2010) performed a medium-term experiment (4 months) spiking soil with an increasing dose of Ag NPs (3.2, 32, 320 µg Ag kg^{-1} soil) and observed no influence on microbial N and enzyme activities but found a significant decrease of microbial biomass with an increase of qCO_2, suggesting a lower efficiency of substrate use.

The increase in qCO_2 may come from microbial stress (Giller et al. 2009) due to pollution of Ag NPs in soil, but also to changes in the bacterial biomass/fungal biomass ratio (Wardle & Ghani, 1995; Nannipieri et al. 2003) as highlighted by the C/N ratio increase (Paul &Clark 1996).

3.2.2.3. Bioaccessibility and bioavailability of Ag NPs

The Ag NPs employed have PVP as a coating agent; it is made of water soluble non-ionic long chain polymers that coat the Ag NP surface and provide steric stabilization due to effects on ionic strength or cation valence (Whitley 2012). PVP coating on Ag NPs may decrease aggregation more than other coatings (e.g. sodium citrate, polysorbate) when it is exposed to high ionic strength electrolyte solutions (Huynh & Chen 2011). Therefore, it is reasonable to assume that the PVP coating enhanced mobility of these Ag NPs into soil micropores.

Table 3.4 shows the amount of Ag solubilised by extractants at different strength. The background level of Ag in the control soil was below the instrumental detection limit for NH_4NO_3 and EDTA (15 and 50µg kg^{-1}, respectively), whilst it was detectable in the water extract at 2µg kg^{-1}.

At the lowest dose, Ag was detectable only after 30 days and the concentration of this element due to NP dissolution decreased as follows: AR > NH_4NO_3 > EDTA > H_2O. Water alone extracted Ag at 60 and 90 days. NH_4NO_3 and the EDTA solutions are high saline extractants which may interfere with the instrument. In addition, the soil matrix complexity probably masked the low concentration of Ag in soil.

Table 3.4 Ag availability in soil measured after extraction with different solutions. The $CHCl_3$ labile Ag concentration, extracted with NH_4NO_3, is reported before and after fumigation

		NH_4NO_3		**H_2O**	**EDTA**	**AR**	**log Kp**
		µg kg^{-1}				**mg kg^{-1}**	**L kg^{-1}**
		unfumigated	fumigated				
30 d	NP-0	DL	DL	2	DL	0.15	1.9
	Ag-NP1	813	127	257	371	16.5	1.8
	Ag-NP2	1488	335	5395	9302	72.7	0.9
	ANOVA	DL	DL	**	DL	***	ns
60 d	NP-0	DL	DL	2	DL	0.16	1.9
	Ag-NP1	DL	DL	107	DL	15.8	2.2
	Ag-NP2	2607	2732	5325	4353	79.1	1.2
	ANOVA	DL	DL	**	DL	***	ns
90 d	NP-0	DL	DL	2	DL	0.15	1.9
	Ag-NP1	DL	DL	145	DL	14.6	2
	Ag-NP2	3102	3256	5423	5698	73.4	1.1
	ANOVA	DL	DL	**	DL	***	ns

AR is aqua regia extraction
DL lower than detection limit; the DL value extraction was 15 µg kg^{-1} for NH_4NO_3 extraction, 0.01 µg kg^{-1} for H_2O extraction and 50 µg kg^{-1} for EDTA extraction.
ANOVA one –way test (Tukey's test $p<0.05$) was performed between the SNP doses and exposition times. ns is not significant, while significant values were indicated as * (<0.05), ** (<0.01), *** (<0.001), respectively

Conversely, the Ag amount in NP2 soils can be ranked as EDTA>H_2O>NH_4NO_3. The average Ag amounts extracted with *aqua regia* were 16.5 and 75.4mg kg^{-1} dry soil for NP1 and NP2, respectively; these values were significant higher (P<0.001) than the control sample (0.15 mg kg^{-1} dry soil average of three times higher). The AR value detected in NP1 exceeds the theoretical dose (10mg kg^{-1} dry soil), while in NP2 the amount was lower than 100mg kg^{-1} at 30, 60 and 90 days, probably because of a problem in the soil homogenization.

The detection of Ag extracted by water showed a low instrumental background and thus the bioaccessibility of Ag can be calculated by partition coefficient (log Kp). The logarithm of partition coefficient was lower than 2.8 and this indicated that Ag NPs were soluble in soil samples. The amount of Ag solubilised by water increased with both dosage and exposure time, suggesting a high geochemical mobility of NPs (log Kp<2.8).

According to Khan et al. (2009) cells lysed with $CHCl_3$ can release trace elements and metals taken up by soil microorganisms. Silver content, extracted by NH_4NO_3 after $CHCl_3$ fumigation in soil exposed at NP1 was lower than DL from 60 to 90 days of exposure. The Ag stored in microbial biomass was detected only in NP2 after 60 and 90 days (125 to 152µg kg^{-1}, respectively), whereas after 30 days the Ag value in fumigated soil was lower than that detected in the non-fumigated sample.

Fumigation with $CHCl_3$ will probably alter PVP coating which would determine a decrease of Ag NP stability, forming insoluble complexes with anions, such as chloride (Sagee et al. 2012), or the cytosol compounds. Moreover, NH_4NO_3 is an high saline extractant which could disguise the determination of the low content of Ag in the NP1. A new interference-reducing approach that should be employed in order to obtain more sensitive measurement.

3.2.2.4. Microbial cultivable viable counts, DNA extraction and DGGE profile

The microbiological analysis confirmed the presence of stress conditions highlighted by the biochemical parameters and ecophysiological indexes.

Bacterial counts of Ag NPs samples decreased significantly (P<0.01) after 60 and 90 days of incubation compared to the control (Fig. 3.9). The decrease was particularly evident after 60 days of incubation, with a decrease of 3.5 log points as compared to counts at the beginning of the experiment and a decrease of 2.2 log points with respect to the control sample. The bacterial counts increased by 1.5 log after 90 days of incubation in the NP2 sample. The variation of counts of fungi obtained on SDA plates was much lower than those of bacterial counts. ANOVA analysis observed no significant differences in fungal counts among Ag NP samples and controls (P>0.05) (Fig. 3.9).

Although it is well established that cultivable microorganisms only represent a minor percentage of the microbiota inhabiting soils (Nannipieri et al. 2003), detecting the effect of anthropogenic pollutants, such as heavy metals, on the soil microbiota (Ellis et al. 2003) is particularly significant. Indeed, the DNA quantification showed decreased DNA concentration of 44%, 72%, 75% in soil samples treated with Ag NPs for 30, 60 and 90 days, respectively (Fig. 3.10).

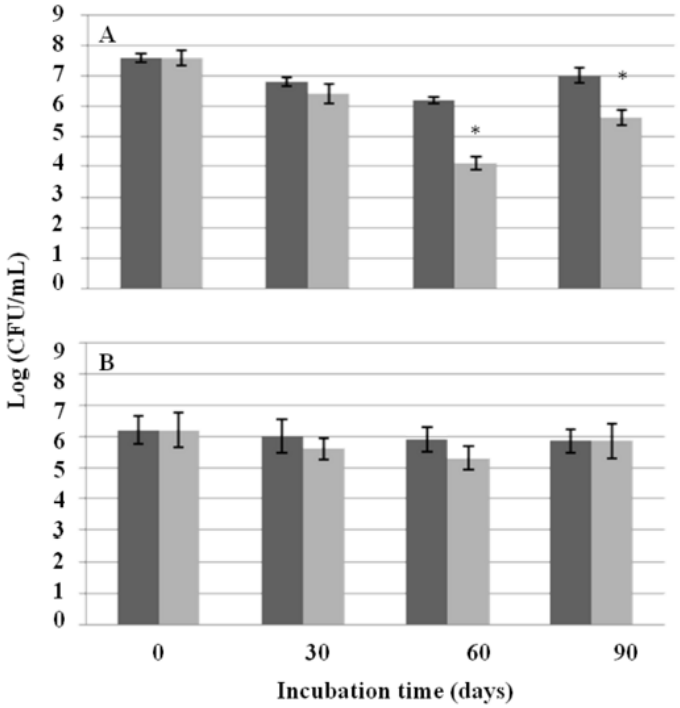


Figure 3.9 Cultivable biomass on TSA (A) and SDA plates (B) of control (dark gray) and the SNP (light gray) treated samples at the beginning of the incubation and after 30, 60 and 90 days of incubation. $P<0.01$ significant differences between Ag NPs samples and the respective control.

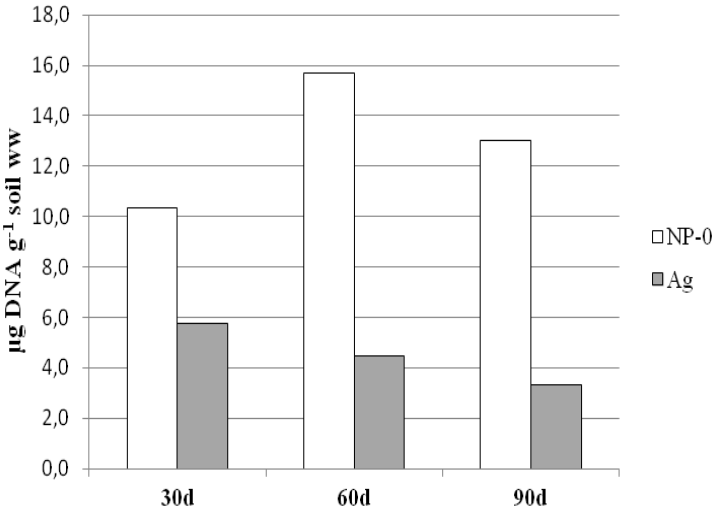
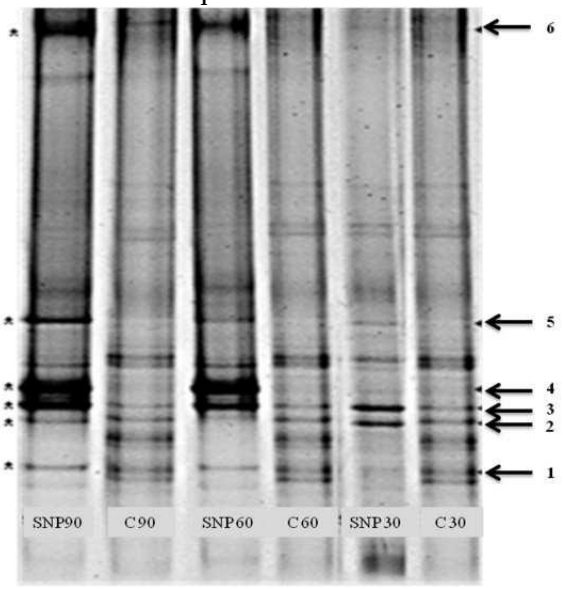


Figure 3.10 DNA quantification of soil treated with Ag NPs (unique value).

Considering the significant differences in bacterial counts and DNA quantification at the three incubation times, DGGE analyses of soil DNA after amplification with universal bacterial primer were carried out. PCR-DGGE (Fig. 3.11) showed a reduced complexity for all NP-treated samples compared to the corresponding controls; in addition, there was a reduction in the number of bands by prolonging the incubation time. The reduction of complexity, compared to the control, started after 30 days of incubation with Ag NPs. Two bands (2 and 3, see Fig. 3.11) showed a greater

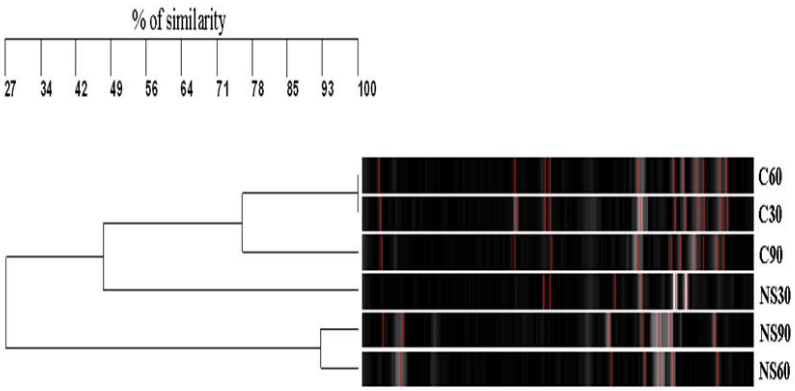
intensity in the profile obtained from the Ag NP samples at day 30 than that of the respective control, whereas some bands present in the control profile were absent in the pattern of the treated samples.

Figure 3.11 Representative DGGE profiles of soil amended with Ag NPs (SNP) and untreated controls (C) at the three different incubation times (30, 60 and 90 days) after 16S rDNA amplification with universal primers. Asterisks indicate the six main bands of the soil amended with Ag NPs at 90 days of incubation that were sequenced.



The control profiles (C30, C60 and C90) did not change significantly upon incubation. A greater intensity of band 3 was observed in the Ag NP samples at day 60 and 90 as compared to the profile at day 30. Moreover, bands 4 and 5 of 60th and 90th day patterns, which were almost undetectable in the control samples, showed an increased intensity in the Ag NP sample profile at 30 days. After 90 days of incubation, the DGGE profiles showed 6 dominant bands (numbered from 1 to 6 in Fig. 3.11), indicating the presence of Ag tolerant strains. These visual considerations are confirmed by analysis with SynGene software. The close similarity of the three control profiles indicated that no relevant changes were produced as the incubation proceeded in the absence of Ag NPs (Fig. 3.12).

Figure 3.12 SynGene software elaboration of DGGE profiles. Dendrogram shows the score similarity (%) among profiles (UPGMA clustering, Dice coefficient).



After 30 days of incubation, the Ag NP profile showed a shift in similarity compared to the control profile, indeed the similarity dropped to 27% after 60 and 90 days of incubation. Perturbations of the DGGE profile of arctic soil communities in the presence of silver nanoparticles (Kumar et al. 2011) and mixed metal nanoparticles (Kumar et al. 2012) have been reported, but the presence of putative resistant microorganisms is scarcely observed.

The six dominant bands present in the DGGE profile of Ag NP samples at 90 days were excised and sequenced (Table 3.5).

Table 3.5 Best-match identification of phylotypes (Seqmatch tool, from Ribosomal Database Project-II using the Basic Local Alignment Search Tool (BLAST) program) of excised DGGE bands amplified with universal bacterial primers.

Bands	**Closest match (NCBI accession number)**	**Percentage of Identity**
1	*Acidobacteriaceae* Bacterium Ellin5095 (AY234512.1)	98
	Acidobacteriaceae Bacterium Ellin311 (AF498693.1)	98
	Acidobacteriaceae Bacterium Ellin310 (AF498692.1)	98
2	*Luteibacter rhizovicinus* (S000323688)	100
3	*Dyella sp.*(AB461717.1)	99
4	*Dyella japonica* (AB681770.1)	100
5	*Edaphobacter modestus* (DQ528760, type strain)	100
6	*Dyella japonica* (AB681770.1)	99

The strains represented belonged to the Acidobacteriaceae (bands 1 and 5) and the Xanthomodaceae families (bands 2, 3, 4 and 6). *Acidobacteria* are widely distributed in soil, and are predominant in soil under broad-leaved forests, comprising 62% of the total sequences (Chan et al. 2008); they are involved in biogeochemical cycles of the rhizosphere soil (Lee et al. 2008). Although few culturable microorganisms belong to *Acidobacteria*, the diversity of this phylum by 16S rDNA sequences is nearly as great as the diversity within *Proteobacteria* (Kock et al. 2008). The rDNA sequence showing the highest similarity with band 1 (Bacterium Ellin5095) was obtained from DNA extracted from broad-leaved forest soil. Bacterium Ellin 210 and 311, which have the same degree of similarity with the rDNA of band 1, are two of the few culturable *Acidobacteria* described in literature (Sait et al. 2002). Although the ecological roles of *Acidobacteria* in soil are not well known, they are frequently present in bacterial communities responding to shift changes in soil properties (Ulrich & Becker 2006), as it occurred in this research. Not much is known about the *Edaphobacter* (band 5) genus of the *Acidobacteriaceae* family, since it has only been recently created (Koch et al. 2008); it comprises just two species (*Edaphobacter modestus* and *Edaphobacter aggregans*) isolated from forest and alpine soils. The

Dyella genus was created in 2005 (Xie & Yokota 2005) after the isolation of new strains from garden soil at the University of Tokyo. Phylogenetic analyses of 16S rRNA gene sequences of the isolated strains revealed a clear similarity to members of the family *Xanthomonadaceae*, but could not be inserted in any of the known genera. Bands 4 and 6 have the closest match to *Dyella japonica* strain isolated by Xie et al. (2005). All described *Dyella* spp. strains have been isolated from peculiar soils and environments, such as cliff soil and greenhouse soil, or activated sludge. Some of these strains are involved in the degradation of recalcitrant compounds (Li et al. 2009). Therefore, *Dyella* spp. strains appear to be adapted to harsh and peculiar habitats, in agreement with its presence in the Ag NP treated soils.

3.3. Plant and nanoparticles

3.3.1. Experiment I: Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 impact on tomato

3.3.1.1. Physicochemical characterization of soil

Soil pH (6.5±0.1), measured on bulk, was not statistically affected by NP pollution during the experimentation period.

The concentration of metal arising from NPs in treated soil after dissolution in *aqua regia* is shown in Table 3.6. The concentration in the surface layer (0-3cm) increased significantly (P<0.05) compared to the control, except for soil polluted with Fe_3O_4 and TiO_2 NPs. In the case of magnetite, there was an increase of Fe concentration in the 3-6cm soil layer; whereas in the case of the TiO_2 NPs treatment, the deepest soil layers were more enriched compared to the control. In general, the metal amount arising from NPs decreased with the depth, even if the lowest values were generally determined in the 3-6cm layer probably due to the presence of roots. Also the metal concentration in the rhizosphere increased significantly with respect to the control except for soil polluted with Fe_3O_4 and TiO_2 NPs, probably due to the high background level which can be found in the soil medium as compared to hydroponic solution.

Such greater amounts of elements arising from NPs in soil found in the rhizosphere soil and/or in the superficial layers (0-3cm, 3-6cm) of bulk soils as compared to deeper soil layers highlighted a low mobility of NPs in soil.

Several studies have been conducted in hydroponic solution (Rico et al. 2011), however the growth medium is important since surface-reactive particles such as clays, organic matter coated particles (Lee et al. 2012; Dimkpa et al. 2012; Du et al. 2011) can affect the behaviour of these

nanoparticles favouring their aggregation and thus decreasing the risk of toxicity (Dinesh et al. 2012).

Table 3.6 Concentration of elements derived from NPs in the soil rhizosphere and in the different layers of the soil column. SD is the Standard Deviation. The data were expressed as mg kg^{-1}, except for Fe (mg kg^{-1})

			Soil column (cm)							
	Rhizosphere		**0-3**		**3-6**		**6-9**		**9-12**	
Treatment		**SD**		**SD**		**SD**		**SD**		**SD**
Ag-NPs	26.1	3.2	36.9	0.2	9.9	0.09	0.9	0.04	DL	
Ag control	*DL*		*DL*		*DL*		*DL*		*DL*	
ANOVA	***		***		***		*		ns	
CeO_2-NPs	37.7	2.7	100.7	4.1	9.2	0.7	14.6	1.6	9.8	5.8
Ce Control	*5.2*	*0.7*	*11.0*	*2.5*	*6.8*	*1.5*	*9.8*	*0.5*	*12.5*	*0.4*
ANOVA	**		**		ns		ns		ns	
Co-NPs	45.6	2.9	64.6	0.1	8.8	0.3	6.8	0.1	7.6	0.2
Co control	*5.2*	*0.1*	*5.3*	*0.2*	*2.9*	*0.2*	*5.5*	*0.1*	*7.4*	*0.2*
ANOVA	**		**		**		ns		ns	
Fe_3O_4-NPs	5.6	3.3	11.5	0.4	10.4	0.2	17.1	0.3	16.5	0.2
Fe control	*5.6*	*0.8*	*12.1*	*0.3*	*5.9*	*0.2*	*12.2*	*0.2*	*16.1*	*0.4*
ANOVA	ns		ns		**		ns		ns	
Ni-NPs	71.1	5.0	32.6	0.3	8.1	0.2	23.4	0.3	24.3	0.9
Ni control	*12.2*	*1.6*	*24.1*	*0.4*	*11.5*	*0.2*	*21.3*	*0.2*	*26.8*	*0.8*
ANOVA	**		**		ns		ns		ns	
SnO_2-NPs	6.9	8.3	3.0	0.4	2.1	0.3	1.1	0.1	1.0	0.2
Sn control	*0.5*	*0.2*	*0.8*	*0.2*	*0.8*	*0.2*	*1.0*	*0.2*	*0.8*	*0.0*
ANOVA	**		*		*		ns		ns	
TiO_2-NPs	95.2	25.0	166.5	18.8	104.6	7.3	207.6	7.3	206.5	7.5
Ti control	*138.6*	*8.9*	*198.2*	*112.7*	*138.6*	*6.9*	*142.2*	*0.0*	*140.2*	*6.8*
ANOVA	ns		ns		ns		*		*	

DL lower than detection limit; the DL value was 0.03 mg kg^{-1} for Ag.
One-way ANOVA and Tukey's test (p<0.05) were used to determine statistical significance of differences between the treatment and the control means. P<0.001 ***, P< 0.01 **, P<0.05 * ns is not significant.

3.3.1.2. Plant growth

The plants exposed to NPs showed different vegetative growth (Table 3.7) especially in roots which exhibited a different morphology than those of control soil (Fig. 3.13).

Tomato plants exposed to Ag NPs showed a reduction of stem and root elongation with respect to the control, as observed by Lee et al. (2012) in *Sorghum bicolor*, but at the same time there was a significant increase of the stem dry weight (P<0.05). Oxidative stress and membrane damage are observed in nanophytotoxicity studies (Wang et al. 2011). An increase of lignifications has been assessed in transgenic tobacco were the overexpression of the peroxidise gene enhanced the generation of H_2O_2 (Kim et al. 2008). Conversely, in plants exposed to Co NPs the reduction of stem and root elongation is associated with a decrease of the aboveground dry biomass.

Table 3.7 Effect of NPs on dry matter of roots, stems and leaves of *Lycopersicon esculentum* plants grown in pots. Means followed by a different letter within a row are significantly different at $P<0.05$ according to the Duncan's multiple comparison test

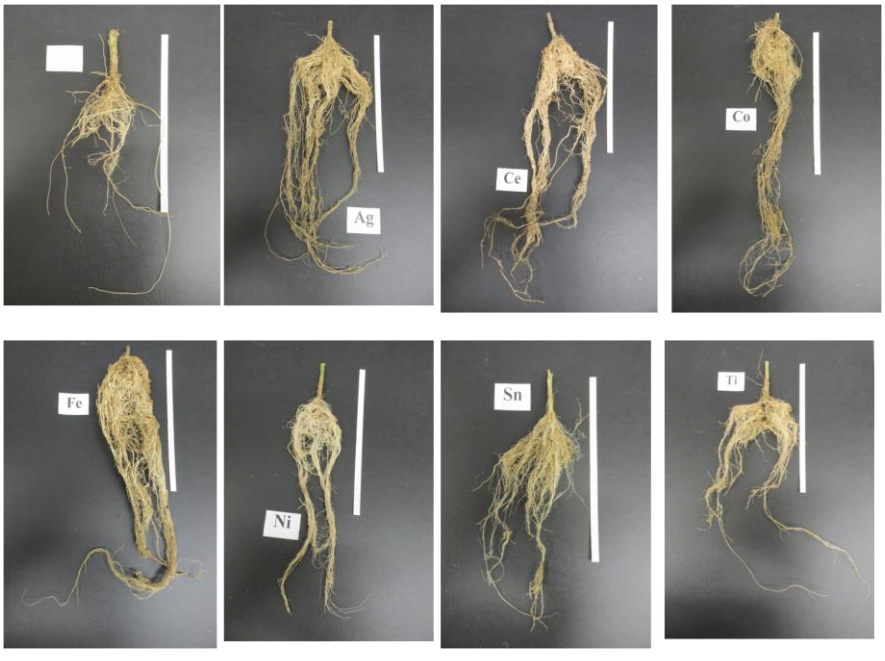
	Tomato morphological parameters									
	Dry matter (g dw per pot)						Height (cm)			
Treatment	Root		Stem		Leaves		Below ground		Aboveground	
Control	1.9	b	20.5	b	25.2	a	22	ab	98	a
Ag-NPs	1.6	b	26.2	a	24.2	a	19	b	82	b
CeO_2-NPs	2.2	ab	13.1	cd	15.7	c	23	ab	109	a
Co-NPs	1.5	b	10.3	d	18.3	b	15	b	84	b
Fe_3O_4-NPs	4.8	a	18.1	c	18.9	b	25	a	106	a
Ni-NPs	1.0	bc	26.1	a	12.1	d	15	b	93	ab
SnO_2-NPs	0.7	c	5.4	e	16.8	c	11	b	104	a
TiO_2-NPs	1.4	b	19.2	b	18.8	b	17	b	110	a

With regard to the effect of CeO_2-NPs on plants, in this experiment stem and root elongation were not affected, but aboveground dry biomass decreased.

Fe_3O_4 NPs promoted root growth for both elongation and dry matter parameters, however these results contradict what Lee et al. (2010) reported: inhibition of root elongation on *Arabidopsis thaliana* after exposure to Fe_3O_4 NPs in agar medium.

The exposure to Ni NPs determined a decrease of root and stem elongation associated to an increase of stem dry matter as Ag NP exposure did, but in addition it decreased the dry matter of leaves significantly.

Figure 3.13 Examples of tomato roots at the end of the experiment of Control, Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 NPs. Wight line is 15cm.



SnO_2 NP exposure reduced root elongation and the dry matter significantly of both aboveground and belowground biomass (P<0.05). As for TiO_2 NP treatment, reduction of leaf dry matter compared to the control was observed; these findings contrast with Song et al. (2013), who found that the biomass of plants exposed to TiO_2 NPs did not significantly vary among 1000-5000mg L^{-1} treatments.

Studies to support this data are very scant, most previous work focused on the early development stage assessing higher concentrations of toxicity than was studied in this experiment, which were also carried out in hydroponic system. The comparison of our results to bibliographical research showed that the fate and uptake of NPs varied in response to the environmental conditions. Further research is needed to examine the toxicity of NPs in real environmental settings.

3.3.1.3. Metal content in tomato tissues

Table 3.8 shows the concentration of elements arising from NPs. Generally, the concentration of these metals in the control tissues were lower than the instrumental detection minimum (DL, see values in Table 3.8). The largest amount of metal NPs was accumulated in tomato roots, except for Ni and TiO_2 NPs which showed no differences compared to the control.

Table 3.8 Comparison between the concentration of NPs elements of stem, leaves, root and fruit of tomato grown with or without (control) NPs SD is the Standard Deviation. The data are expressed as mg kg^{-1}

		Ag		Ce		Co		Fe		Ni		Sn		Ti	
			SD		SD		SD		SD		SD		SD		SD
Stem	Treatment	0.2	0.01	DL		0.4	0.001	38.4	0.5	0.9	0.1	DL		1.4	0.1
	Control	*DL*		*DL*		*DL*		*49.8*	*4.2*	*DL*		*DL*		*1.7*	*0.1*
	ANOVA	*				*		ns		*				ns	
Leaves	Treatment	1.1	0.05	DL		1.2	0.05	19.9	7.8	1.3	0.3	DL		3.3	0.8
	Control	*DL*		*DL*		*DL*		*20.5*	*8.6*	*1.1*	*0.5*	*DL*		*2.8*	*0.03*
	ANOVA	*				*		ns		ns				ns	
Root	Treatment	2.6	0.06	1.7	0.01	3.7		534.8	29.1	2.3	0.1	0.6	0.01	5.5	0.01
	Control	*DL*		*DL*		*0.2*		*383.5*	*12.3*	*3.2*	*0.3*	*DL*		*7.9*	*0.2*
	ANOVA	*		*		*		*		ns		*		ns	
Fruit	Treatment	0.3	0.01	DL		DL		116.8	7.9	0.8	0.1	DL		2.9	0.4
	Control	*DL*		*DL*		*DL*		*42.5*	*8.1*	*DL*		*DL*		*5.5*	*0.1*
	ANOVA	*						*		*				ns	

DL lower than detection limit; DL was of Ag (0.006 mg kg^{-1}), for Ce (0.01 mg kg^{-1}), for Co (0.0002 mg kg^{-1}), for Ni (0.01 mg kg^{-1}), and Sn (0.01 mg kg^{-1})

One-way ANOVA and Tukey's test (p<0.05) were used to determine statistical significance of the differences between treatment and the control means. P<0.001 ***, P< 0.01 **, P<0.05 * ns is not significant.

Ag, Co and Ni concentrations were higher than those of the tomato stem control. The concentration in leaves was significantly higher than the control only for Ag and Co (P<0.05). In addition, Ag, Fe and Ni were found in fruits at higher concentration than the control.

The translocation index (TI) showed that tomato plants had the ability to translocate Ag and Co from root to both stem and leaves (Table 3.9), whereas Ni accumulated in stems.

Table 3.9 Amount of NPs elements accumulated in stem, leaves and root are expressed as µg per pot (referred to grams of dry substance). Translocation Index (TI): values are expressed as the percentage of element of NPs from aboveground to below ground organs.

		Ag	Ce	Co	Fe	Ni	Sn	Ti
Stem	Treatment	5.2	DL	4.1	998.4	16.3	DL	26.9
	Control	*1.2*	*DL*	*DL*	*944.2*	*1.8*	*DL*	*34.9*
Leaves	Treatment	26.7	DL	22.0	240.6	24.6	DL	62.0
	Control	*1.5*	*DL*	*DL*	*517.8*	*27.8*	*DL*	*70.7*
Root	Treatment	4.2	3.7	5.6	2087.0	2.5	0.4	7.7
	Control	*0.1*	*DL*	*0.4*	*728.7*	*6.1*	*0.02*	*15.0*
				Translocation index (TI) %				
TI NP	Stem	14.4	DL	12.9	30.0	37.6	DL	27.8
	Leaves	74.0	DL	69.4	7.2	56.7	DL	64.2
TI Ctr	*Stem*	*DL*	*DL*	*DL*	*43.1*	*5.0*	*DL*	*28.9*
	Leaves	*DL*	*DL*	*DL*	*23.6*	*55.4*	*DL*	*58.6*

DL lower than detection limit; DL was of Ag (0.006 mg kg^{-1}), for Ce (0.01 mg kg^{-1}), for Co (0.0002 mg kg^{-1}), for Ni (0.01 mg kg^{-1}), and Sn (0.01 mg kg^{-1}).

CeO_2 and SnO_2 NPs did not translocate in the control nor in treated tomato. Sn concentration in soil and roots tissues was lower than expected, probably because of an underestimation of Sn concentration due to the incomplete dissolution of SnO_2 NPs in the acid mix used for the mineralization of soil and plants. As for CeO_2 NPs the literature is contradictory; in research conducted on maize plants, cerium was absent (Birbaum et al. 2010) or found at low concentrations in the shoot of plants grown in low organic matter soil (Zhao et al. 2012). Conversely, a study conducted on tomato plants grown in potting mix and treated with CeO_2 NPs (10-30nm) at increasing doses assessed the presence of cerium in the following order: root > stem > leaf > fruit (Wang et al. 2012). Translocation to the shoot is generally limited and depends on the NPs' primary diameter, as reported by Zhang et al. (2011).

No differences were found between control and TiO_2 NP samples. It can be observed that the translocation of Fe to leaves was lower than the control. Indeed, plants tend to limit the absorption or translocation to the aboveground organs of potentially toxic elements (if present in excess) under conditions of increased availability, thus lower values of translocation in soils contaminated with heavy metals compared to non-polluted areas can be found (Lübben 1993).

3.3.1.4. Nutrients content in tomato tissues

The average concentration of nutrients in tomato organs grown in soil polluted with NPs is reported in Table 3.10.

In general, abiotic and biotic stress affect the uptake of nutrients, metal immobilization in root cell walls, and metal accumulation in vacuoles, among others (Fernandes & Henriques 1991).

In this experiment, nutrient concentration in roots was higher for Ag, CeO_2, Co and Fe_3O_4 NP treatments with respect to the control especially for Ca and Mg. Conversely, Ni and Sn NP treatments determined a lower concentration, whereas no differences were found in TiO_2 NP treatments.

Ag and Ni NPs determined an increase compared to the control of Ca, Mg and Na contents in the stem, whereas SnO_2 NP treatment showed a lower concentration. According to Fernandes and Henriques (1991) and Wang et al. (2011) meal and metal oxide can damage the cellular membranes altering the plant capacity to absorb and transport some nutrients, thus modifying their nutritional value.

Finally, in all treatments, Ca and Mg content in leaves was higher than in the control.

Calcium content increased in all tissues, as it preserves the structural and functional integrity of plant membranes, stabilizes cell walls, regulates ion transport and selectivity and controls ion-exchange behaviour (Rengel 1992; Marschner 1995).

In addition, S content increased in leaves of plants treated with Ag, Co, Ni and Sn NPs, as observed by Trujillo-Reyes et al. (2014) in *Lactuca sativa* plants treated with Cu NPs. According to Grill et al. (1986-1987) plants respond to heavy metal stress by inducing SH-containing peptides such as phytochelatins (Gill et al. 1986,1987), which are capable of binding heavy metal ions via thiolate coordination. Phytochelatins can reduce free metal concentration in the cytosol by binding and transporting the metal to specific compartments, mainly the vacuole, prior to biotransformation into organic compounds or chemical reduction of the element (Salt et al. 1998). Indeed, Dago et al. (2014) observed an increment of synthesised phytochelatins ((γ-Glu-Cys)n-Gly, n=2–5) in both root and stem of *Hordeum vulgare* plants exposed to increasing concentrations of Hg, Cd and As ions.

The sum of nutrient concentration (Ca, K, Mg, Na, P and S) of stems treated with Ag and Ni NPs was higher ($P<0.05$) than in the control, whereas SnO_2 treatment showed a lower concentration ($P<0.05$) than to the control; no significant differences were found for the other treatments (Table 3.10).

The nutrient concentration in tomato fruit showed a high K content and a low Mg, P and S amount after irrigation with NPs compared to control (Table 3.10), probably due to the damage of cellular membranes which can alter the plant capacity to absorb and transport some nutrients (Fernandes & Henriques 1991; Wang et al. 2011).

Table 3.10 Comparison between concentration of macro elements of stem, leaves, root and fruit in tomato plants. One-way ANOVA and Tukey's test (p<0.05) were used to determine statistical significance of the differences between treatment and the control means; in bold treatments showing significant differences at P<0.05.

		Ca	K	Mg	Na	P	S
					g kg^{-1}		
Leaves	Control	19,5	8,7	3,2	2,3	2,4	11,1
	SD	*0,2*	*0,1*	*0,0*	*0,0*	*0,0*	*0,1*
	Ag-NPs	**28,6**	8,4	**5,0**	**3,5**	**3,9**	**16,8**
	SD	*1,9*	*0,7*	*0,3*	*0,2*	*0,3*	*0,9*
	CeO_2-NPs	**25,2**	8,8	**3,9**	1,9	2,4	12,3
	SD	*0,5*	*0,0*	*0,1*	*0,0*	*0,1*	*0,5*
	Co-NPs	**30,1**	8,7	**4,7**	**2,9**	2,2	**16,8**
	SD	*0,3*	*0,1*	*0,0*	*0,0*	*0,0*	*0,1*
	Fe_3O_4-NPs	**27,3**	8,5	**5,0**	**2,6**	2,3	**13,4**
	SD	*0,2*	*0,2*	*0,1*	*0,0*	*0,1*	*0,1*
	Ni-NPs	**24,9**	8,7	**4,2**	**2,5**	**2,6**	**13,2**
	SD	*0,3*	*0,1*	*0,0*	*0,0*	*0,0*	*0,2*
	SnO_2-NPs	**24,6**	8,5	**4,2**	2,2	**2,9**	**12,8**
	SD	*0,1*	*0,0*	*0,0*	*0,0*	*0,0*	*0,1*
	TiO_2-NPs	**26,2**	8,7	**4,2**	2,1	2,6	**13,3**
	SD	*0,2*	*0,1*	*0,1*	*0,0*	*0,0*	*0,3*
Stem	Control	11,8	8,5	2,4	3,3	1,4	2,9
	SD	*0,2*	*0,2*	*0,0*	*0,0*	*0,0*	*0,1*
	Ag-NPs	**17,3**	8,8	**3,3**	**4,9**	1,1	3,2
	SD	*0,1*	*0,1*	*0,1*	*0,1*	*0,0*	*0,1*
	CeO_2-NPs	10,5	**7,6**	2,3	2,9	1,2	**1,9**
	SD	*0,2*	*0,0*	*0,0*	*0,0*	*0,0*	*0,0*
	Co-NPs	12,0	7,8	**2,7**	3,4	**0,9**	**2,0**
	SD	*0,1*	*0,1*	*0,0*	*0,0*	*0,0*	*0,0*
	Fe_3O_4-NPs	12,0	8,4	2,5	**2,4**	**1,1**	**1,9**
	SD	*0,0*	*0,1*	*0,0*	*0,0*	*0,0*	*0,0*
	Ni-NPs	15,6	8,6	**3,8**	**4,5**	**1,1**	2,8
	SD	*0,3*	*0,1*	*0,1*	*0,1*	*0,0*	*0,0*
	SnO_2-NPs	**5,4**	**4,3**	1,1	1,7	0,8	1,2
	SD	*0,2*	*0,6*	*0,5*	*0,4*	*0,2*	*0,6*
	TiO_2-NPs	10,8	8,0	2,5	3,1	**1,6**	**1,9**
	SD	*0,1*	*0,1*	*0,0*	*0,0*	*0,0*	*0,0*
Root	Control	12,9	2,9	1,5	4,3	1,0	2,8
	SD	*0,3*	*0,1*	*0,1*	*0,4*	*0,1*	*0,1*
	Ag-NPs	**16,9**	**5,1**	**2,3**	**8,3**	**1,5**	**3,5**
	SD	*0,5*	*0,1*	*0,1*	*0,4*	*0,1*	*0,1*
	CeO_2-NPs	**16,9**	**3,6**	**2,0**	4,0	**1,5**	2,6
	SD	*0,1*	*0,0*	*0,0*	*0,0*	*0,0*	*0,0*
	Co-NPs	14,7	**6,1**	**3,3**	**6,7**	**1,5**	**3,6**
	SD	*0,1*	*0,0*	*0,1*	*0,0*	*0,0*	*0,0*
	Fe_3O_4-NPs	**21,9**	2,7	**2,0**	4,3	1,3	2,4
	SD	*0,3*	*0,0*	*0,0*	*0,1*	*0,0*	*0,1*
	Ni-NPs	12,1	**1,1**	1,0	**1,3**	0,7	1,6
	SD	*0,1*	*0,0*	*0,1*	*0,0*	*0,0*	*0,0*
	SnO_2-NPs	10,9	**1,0**	1,0	**1,1**	0,7	**1,4**
	SD	*0,3*	*0,0*	*0,1*	*0,0*	*0,0*	*0,0*
	TiO_2-NPs	13,4	3,1	1,7	5,4	1,3	2,6
	SD	*0,3*	*0,0*	*0,1*	*0,0*	*0,0*	*0,0*

Table 3.10 Continuation

		Ca	K	Mg	Na	P	S
					g kg^{-1}		
Fruit	Control	28,96	**1,86**	**4,74**	2,60	**3,11**	**12,09**
	SD	*0,26*	*0,09*	*0,04*	*0,01*	*0,00*	*0,18*
	Ag-NPs	24,42	2,04	4,54	2,55	2,98	11,16
	SD	*0,34*	*0,33*	*0,05*	*0,03*	*0,02*	*0,03*
	CeO_2-NPs	29,82	0,60	5,26	3,51	3,29	13,60
	SD	*0,15*	*0,01*	*0,01*	*0,01*	*0,01*	*0,02*
	Co-NPs	25,50	1,25	5,23	3,32	2,80	10,64
	SD	*0,19*	*0,07*	*0,02*	*0,03*	*0,02*	*0,02*
	Fe_3O_4-NPs	27,07	0,62	5,18	3,68	3,08	11,35
	SD	*0,14*	*0,00*	*0,02*	*0,02*	*0,00*	*0,07*
	Ni-NPs	29,34	1,76	5,05	3,46	2,99	14,07
	SD	*0,30*	*0,14*	*0,03*	*0,03*	*0,00*	*0,04*
	SnO_2-NPs	29,68	0,72	5,67	3,48	3,31	12,61
	SD	*0,10*	*0,16*	*0,08*	*0,00*	*0,03*	*0,14*
	TiO_2-NPs	24,95	3,44	4,55	3,13	2,75	11,56
	SD	*0,49*	*0,03*	*0,10*	*0,24*	*0,09*	*0,46*

3.3.1.5. ESEM analysis of tomato tissues

The ESEM images show the presence of nanoparticles within the root, but not in stem and leaves. Some examples of tomato roots exposed to NPs are shown in Fig. 3.14. Ag NPs were detected within root cells of tomatoes (Fig. 3.14) grouped as a large cluster (from 100 to 200nm) but also individually dispersed. Cluster formation from NPs was determined in tomato roots exposed to TiO_2 and SnO_2 NPs (Fig. 3.15, 3.16); the latter showed spherical clusters of different sizes whereas TiO_2-NPs were distributed parallel to the longitudinal section of roots probably associated with the absorption patterns of water and nutrients (Lee et al. 2010). Throughout the EDS spectra, nanoparticles were associated with soil compounds.

Fig. 3.14 a) b) c) d) ESEM images of Ag nanoparticles in tomato roots exposed at Ag NPs and e) f) EDS spectra of Ag NPs and natural nanoparticles

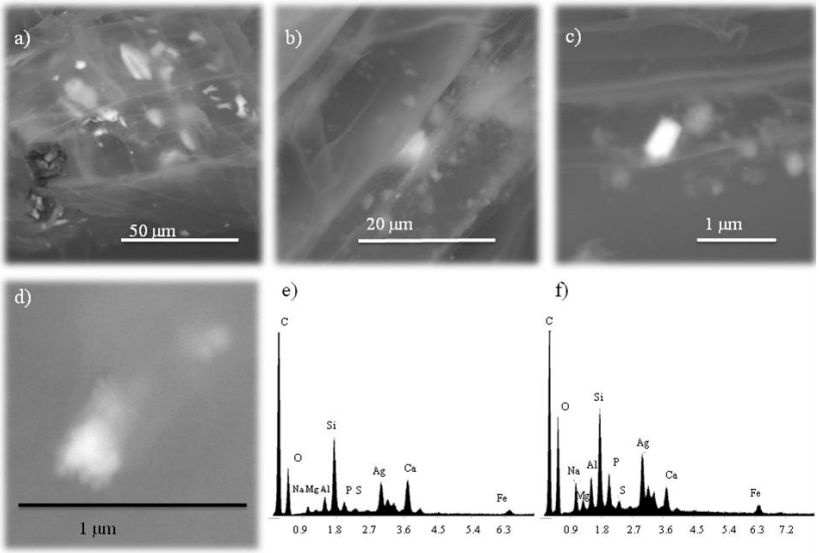


Fig. 3.15 a) b) c) ESEM images of TiO_2 NPs detected in root tissues of tomato exposed at TiO_2-NPs; d) EDS spectrum of TiO_2-NPs and natural nanoparticles

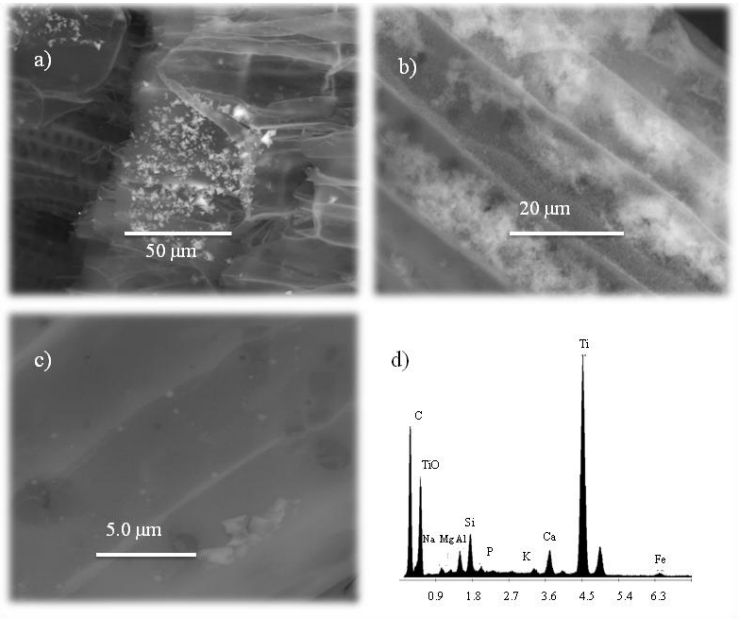
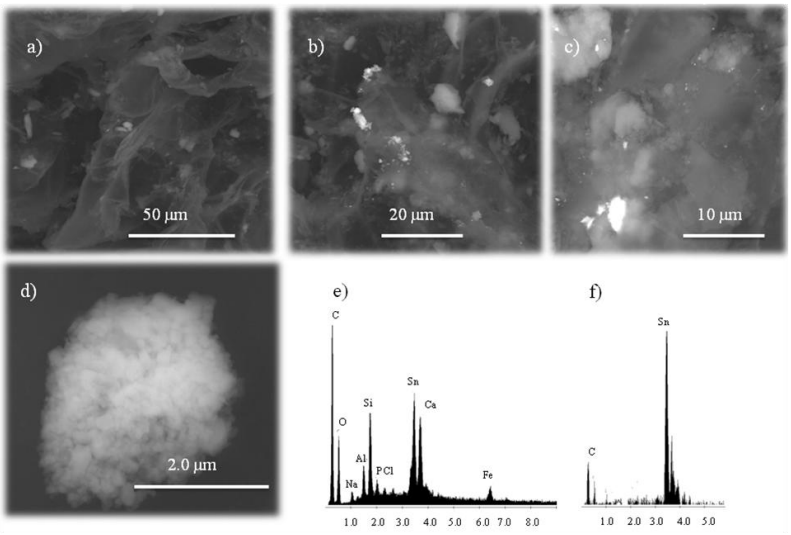


Fig. 3.16 a) b) c) d) ESEM images of SnO_2 nanoparticles detected in root tissues of tomato exposed at SnO_2-NPs; e) f) EDS spectra of SnO_2 NPs and natural nanoparticles



3.3.2. Experiment II: Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 impact on basil

3.3.2.1. Physicochemical characterization of soil

Soil pH (6.5±0.1), measured on the bulk, was not statistically affected by NP pollution during the experimentation period.

The concentration of metal arising from NPs in treated soil after dissolution in *aqua regia* is showed in Table 3.11. The metal concentration in the soil significantly increased over the control

except for soil polluted with Fe_3O_4 and TiO_2 NPs, probably due to the high background level which can be found in the soil medium with respect to hydroponic solution.

Table 3.11 Concentrations of Ag, Ce, Co, Fe, Ni, Sn and Ti found in basil soil, after different extraction: DTPA, H_2O and aqua regia (AR) at the end of the experiment. Also, the logarithm values of partition coefficients (Kp) are reported.

	DTPA	**H_2O**	**AR**	**log (Kp)**
	mg kg^{-1}	**µg L^{-1}**	**mg kg^{-1}**	**L kg^{-1}**
Control	DL	11.86	0.10	0.9
Ag-NPs	0.13	263.00	61.92	2.4
Control	0.45	DL	30.93	2.9
CeO_2-NPs	0.52	669.34	124.71	1.6
Control	0.16	25.32	7.81	2.5
Co	10.74	306.69	56.83	1.4
Control	19.20	3868	12743	3.5
Fe_3O_4-NPs	20.40	2867	12576	3.6
Control	0.49	23.43	34.72	3.2
Ni-NPs	1.78	77.30	106.87	3.1
Control	DL	DL	1.1	2.4
SnO_2-NPs	DL	97.90	3.97	1.6
Control	51.39	252.24	854.56	3.5
TiO_2-NPs	57.26	666.33	795.79	3.1

DL lower than detection limit; the values of DL in DTPA for Ag and Sn were 0.006, 0.02 mg kg^{-1} respectively, DL in H_2O for Ce and Sn were 0.03, 0.004 µg L^{-1}, respectively.

3.3.2.2. Bioaccessibility of NPs in soil

The concentration of elements due to NP dissolution decreased as follows: AR> DTPA > H_2O for Co, Fe_3O_4, Ni and TiO_2 NPs; whereas the recovery of metals in DTPA and H_2O is comparable for Ag and CeO_2 NPs; SnO_2 NPs are only detectable in AR and water (Table 3.11).

Fe_3O_4, Ni and TiO_2 NPs showed high values of log partition coefficient Kp (>2.8), suggesting that they are characterized by low geochemical mobility in water (Cornelis et al. 2011). Contrary to what was previously observed in bare soil, CeO_2 and SnO_2 NPs showed a partition coefficient of 1.6 probably due to the presence of plant roots which produce organic acids to counteract the metal elements (Taiz & Zeiger 1998).

3.3.2.3. Plant growth

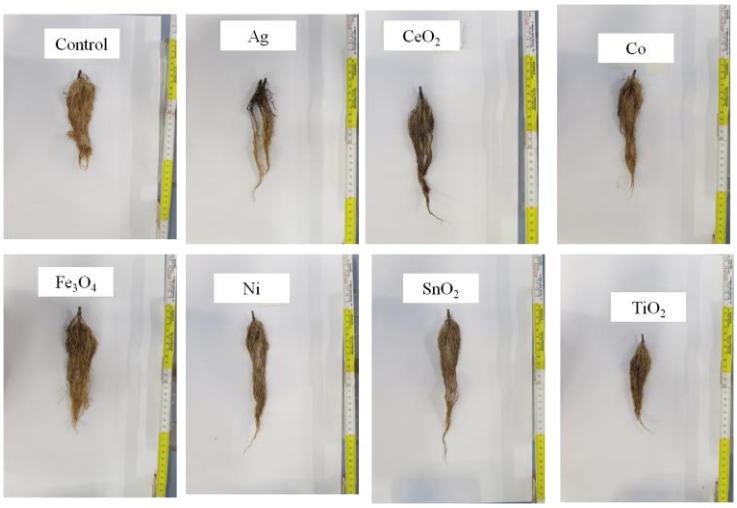
The plants exposed to NPs did not show significant differences in vegetative growth based on the leaf counts, as already observed by Wang et al. (2012) in tomato plants exposed to CeO_2 NPs.

Contrary to what was observed in tomato plants, basil exposed to Ag NPs showed a significant reduction of root and leaf dry matter, 30.4% and 14% respectively, as compared to the control (Table 3.12, Fig. 3.17). Such evidence was not surprising as similar results were obtained by Qian et al. (2013) who reported that fresh weight of *Arabidopsis thaliana* seedlings decreased significantly in Ag NP treatments compared to control. The variability of biological responses could be species-specific as observed by Lin and Xing (2008) or in our pilot test (comparison between strawberry and basil plants).

Table 3.12 Effect of NPs on dry matter of roots, stems and leaves of *O. basilicum* plants grown in pots. Means followed by a different letter are significantly different at $P<0.05$ according to the One-way ANOVA and Tukey's test.

	Dry matter (g dw per pot)					
Treatment	Root		Stem		Leaves	
Control	0.46	ab	0.31	a	1.00	a
Ag-NPs	0.32	b	0.30	a	0.86	b
CeO_2-NPs	0.45	ab	0.33	a	1.01	ab
Co-NPs	0.46	ab	0.33	a	1.03	ab
Fe_3O_4-NPs	0.47	a	0.33	a	1.02	ab
Ni-NPs	0.47	a	0.33	a	1.03	ab
SnO_2-NPs	0.45	ab	0.34	a	1.12	a
TiO_2-NPs	0.52	a	0.38	a	1.12	a

Figure 3.17 Examples of basil roots at the end of the experiment of Control, Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 NPs



Fe_3O_4, Ni and TiO_2 NPs determined higher root dry matter. Larue et al. (2012) observed an increase of root elongation in wheat seedlings treated with TiO_2 (14nm anatase and 22nm rutile) solution, they supposed that TiO_2 NPs would locally generate oxidative stress and enlargement of cell wall pores, which would in turn increase water flow and turgor into wheat roots and consequently enhance root elongation.

No significant difference was found in the stem dry weight.

3.3.2.4. Metal content in basil tissues and translocation index

Table 3.13 shows the metal content arising from NPs. A greater amount of metal-NPs was accumulated in basil roots, except for Fe_3O_4 and SnO_2 NPs which showed no differences compared to the control. Sn concentration in soil and roots tissues was lower than expected; probably, there was an underestimation of Sn concentration since, also in this case, SnO_2 NPs were not completely solubilised in the acid mix used for the mineralization of soil and plant.

Table 3.13 Comparison between the concentration of NPs elements of stem, leaves and root of basil grown with or without (Control) NPs.

		Ag µg kg^{-1}	Ce µg kg^{-}	Co µg kg^{-}	Fe mg kg^{-}	Ni µg kg^{-}	Sn µg kg^{-}	Ti mg kg^{-}
Leaves	Treatment	1388	1731	3334	131.7	3839	336.5	2.87
	Control	*194.6*	*DL*	*356.5*	*135.3*	1997	*304.8*	*2.74*
ANOVA		***	***	***	ns	***	ns	ns
Stem	Treatment	2081	DL	2829	39.04	652.6	494.8	1.08
	Control	*177.9*	*DL*	*296.2*	*45.27*	*411.4*	*497.1*	*1.33*
ANOVA		***	ns	***	ns	ns	ns	ns
Root	Treatment	5814	50858	71415	196.8	27340	167.6	8.17
	Control	*128.2*	*DL*	*1212*	*128.0*	*1882*	*143.3*	*3.74*
ANOVA		***	***	***	ns	***	ns	***

DL was the instrumental detection limit for Ce 0.2 mg kg^{-1}
One-way ANOVA and Tukey's test (p<0.05) were used to determine statistical significance of the differences between treatment and the control means. P<0.001 ***, P< 0.01 **, P<0.05 * ns is not significant

Ag, Co and Ni concentrations in the basil stem were higher than the control as observed previously in tomato plants. As the concentration of the elements was assessed by ICP-OES, we cannot distinguish between ions and NPs; however these NPs seems to be the most mobile probably due to rapid aging in the soil medium. The concentration in leaves was significantly higher compared to the control for Ag, CeO_2, Co and Ni (P<0.05). Differently than the previous experiment, CeO_2 NPs were smaller (15-30nm), which confirms the results of Zhang et al. (2011), who suggested that the translocation to the shoot was limited and depended on the NPs primary diameter.

In Table 3.14, metal concentration in basil per pot and translocation indexes are shown. As expected, the amount of metal arising from NPs was higher in the treated plants than in the control, but the translocation index showed a good capacity of plant roots to stop most of the ENPs (Lin & Xing 2008) in an experiment using relatively brief exposure.

Table 3.14 Amount of NPs elements accumulated in basil stem, leaves and root are expressed as µg per pot (referred to grams of dry substance). Translocation Index (TI): values are expressed as the percentage of element of NPs from aboveground to below ground organs.

		Ag	Ce	Co	Fe	Ni	Sn	Ti
Leaves	Treatment	1261,1	2080,3	3465,2	123688,0	2556,6	344,8	3109,4
	Control	*217,4*	*260,6*	*361,3*	*137883,4*	*1975,7*	*301,0*	*2844,2*
Stem	Treatment	564,8	97,0	1009,3	12528,0	238,4	150,8	386,7
	Control	*57,0*	*90,1*	*97,3*	*15651,7*	*137,5*	*152,4*	*435,9*
Roots	Treatment	1954,1	22079,0	35763,4	88932,6	13270,8	50,6	4102,1
	Control	*60,4*	*110,9*	*565,3*	*59510,1*	*870,4*	*66,0*	*1742,0*
		Translocation index (TI) %						
		Ag	Ce	Co	Fe	Ni	Sn	Ti
Leaves	NPs	29,9	8,4	9,1	55,0	15,6	62,9	40,9
	Control	*65,0*	*56,2*	*35,1*	*64,4*	*66,2*	*57,3*	*56,3*
Stem	NPs	17,5	0,4	2,7	5,6	1,5	26,5	5,1
	Control	*16,9*	*19,3*	*9,4*	*7,0*	*4,7*	*30,5*	*8,5*
Roots	NPs	52,5	91,2	88,2	39,5	82,9	10,5	54,0
	Control	*18,0*	*24,5*	*55,5*	*28,5*	*29,2*	*12,2*	*35,2*

3.3.2.5. Nutrient content in basil tissues

The average concentration of nutrients in basil organs grown in soil polluted with NPs is reported in Table 3.15.

The main differences were observed in the nutrient concentration in roots. Indeed, Ca was significantly higher in all treatments as compared to the control (P<0.001). As mentioned above, Ca is an important element for the structural and functional integrity of plant membranes.

In vitro tests conducted by Romeis et al. (2000) showed that transgenic tobacco cells subjected to membrane damage activate the calcium-dependent protein kinase and increase the content of calcium in the cytosol as a plant defensive response. Our hypothesis is that the Ca accumulation in different tissues is a response to the membrane damage generated directly or indirectly by NPs.

Ag treatments determined a decrease of Mg and S content in basil roots as compared to the control, while both control and Ag treatment showed the lowest concentration of P (P<0.01). The variation of Ca content persisted in the leaves of plants that were treated with CeO_2, Fe_3O_4 and Ni, where NPs showed the highest values, while Ag NPs exhibited the lowest (P<0.05). Similarly Trujillo-Reyes et al. (2014) observed a variation of the content of nutrients in *Lactuca sativa* plants treated with Cu NPs: an increase of S and Ca in roots, an increase of S and a decrease of Mn, P, Ca and Mg in leaves. As already observed, previous works (Fernandes & Henriques,1991, Wang et al.

2011), the plant capability to absorb and transport some nutrients, thus modifying their nutritional value, can be damaged by metal and metal oxide.

No significant differences were found in stem nutrient concentrations.

Table 3.15 Comparison between the concentration of macro elements of stem, leaves and root in basil plants. Means followed by a different letter are significantly different at $P<0.05$ according to the One-way ANOVA and Tukey's test.

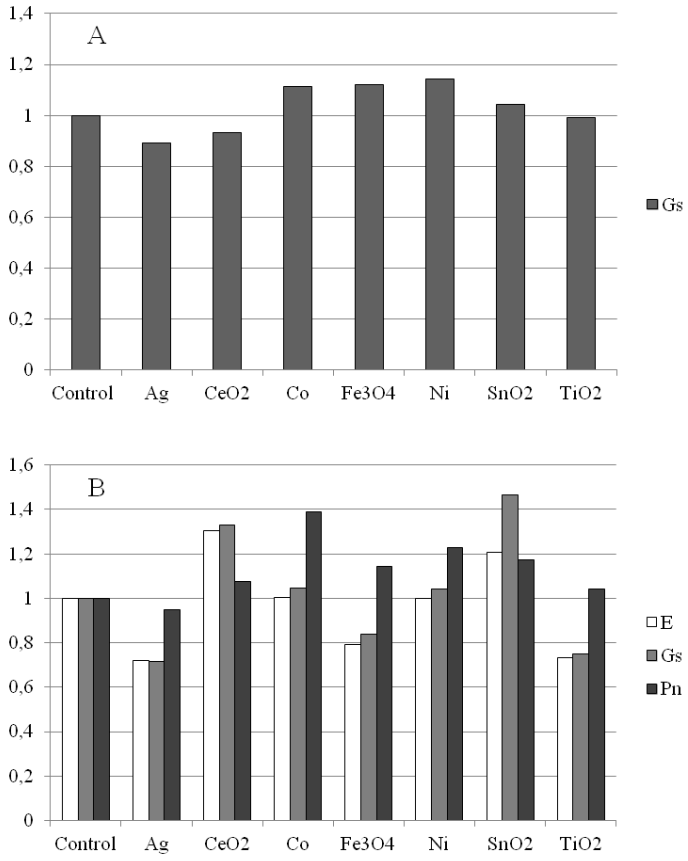
		Ca		K		Mg		Na		P		S	
		g kg^{-1}											
Leaves	Control	18,19	ab	2,66	a	4,72	a	0,52	ab	3,07	a	1,90	b
	Ag-NPs	15,68	b	2,16	a	4,97	a	0,64	ab	3,05	a	2,66	a
	CeO_2-NPs	18,52	a	1,97	a	4,56	a	0,78	a	3,04	a	1,82	b
	Co-NPs	17,38	ab	2,73	a	4,25	a	0,54	ab	3,06	a	1,78	b
	Fe_3O_4-NPs	18,73	a	2,29	a	4,82	a	0,56	ab	3,17	a	1,91	b
	Ni-NPs	18,60	a	2,90	a	4,85	a	0,44	b	3,12	a	1,90	b
	SnO_2-NPs	17,99	ab	2,58	a	4,38	a	0,47	ab	3,28	a	1,81	b
	TiO_2-NPs	18,42	ab	3,45	a	4,69	a	0,46	b	3,06	a	1,78	b
ANOVA			*		ns		ns		*		ns		***
Stem	Control	10,00	a	7,63	a	2,07	a	1,38	a	1,17	ab	0,92	a
	Ag-NPs	6,48	a	6,73	a	1,99	a	3,43	a	1,20	ab	1,14	a
	CeO_2-NPs	9,23	a	7,27	a	2,15	a	2,31	a	1,23	ab	1,00	a
	Co-NPs	8,15	a	6,93	a	1,78	a	1,78	a	1,05	ab	0,84	a
	Fe_3O_4-NPs	8,67	a	6,32	a	2,09	a	1,53	a	1,08	ab	0,80	a
	Ni-NPs	8,13	a	7,68	a	2,22	a	1,60	a	1,11	ab	0,80	a
	SnO_2-NPs	10,17	a	8,22	a	2,18	a	1,93	a	1,60	a	1,40	a
	TiO_2-NPs	8,87	a	7,12	a	1,92	a	1,26	a	0,99	b	0,69	a
ANOVA			ns		ns		ns		ns		.		ns
Root	Control	6,99	c	4,79	a	6,99	a	7,84	a	1,47	b	5,63	a
	Ag-NPs	9,17	ab	6,65	a	4,29	b	7,10	a	1,49	b	3,21	b
	CeO_2-NPs	9,13	ab	6,39	a	6,98	a	8,14	a	1,53	ab	5,51	a
	Co-NPs	8,49	bc	4,05	a	8,21	a	6,98	a	1,88	ab	6,57	a
	Fe_3O_4-NPs	9,61	ab	6,82	a	8,45	a	9,65	a	1,83	ab	6,70	a
	Ni-NPs	9,13	ab	5,98	a	7,71	a	8,18	a	1,75	ab	6,14	a
	SnO_2-NPs	8,65	bc	4,53	a	7,03	a	7,05	a	1,72	ab	5,45	a
	TiO_2-NPs	11,02	a	6,32	a	8,26	a	7,96	a	1,99	a	5,99	a
ANOVA			***		ns		***		ns		**		***

One-way ANOVA and Tukey's test ($p<0.05$) were used to determine statistical significance of the differences between treatment and the control means. $P<0.001$ ***, $P< 0.01$ **, $P<0.05$ * ns is not significant

3.3.2.6. Physiological parameters

Figure 3.18 shows leaf gas exchange measured with both Leaf Porometer and CIRAS-2. Due to the intrinsic heterogeneity of the stomatal conductance (Weyers & Lawson 1997) to reduce the high variability of the measurements, the values were normalized to the control. No significant differences were found but both instruments highlighted a reduction of the leaf gas exchange in the plants treated with Ag NPs.

Fig. 3.18 Stomatal conductance (ST) and leaf gas exchange (E=evaporation; Gs= stomatal conductance; Pn= net photosynthesis) measured with Leaf Porometer (A) and CIRAS-2 (B).



Plant pigment concentrations are summarised in Table 3.16. No significant differences were found except for chlorophyll b which in plants treated with Ni and SnO_2 showed a lower concentration compared to the control. Notably, Song et al. (2013) determined a decrease of both chlorophyll a and b in tomato plants treated with Ag NPs at 100-1000 mg L^{-1}, even if a comparable silver uptake was recorded in leaves, 0.9mg kg^{-1} and 1.3mg kg^{-1} in tomato plants and in basil, respectively; this difference may be due to the growth of tomato plants in Hoagland's solution in Song's study; indeed differences in species and medium might account for our differing results.

Lipid peroxidation is shown in Table 3.17. TBARS is a classical marker of oxidative stress, but the accumulation of metal arising from the NPs in basil plants did not show any significant difference compared to the control. Similarly to what described by Larue et al. (2012), in wheat exposed to TiO_2 NPs did not impact photosynthesis and did not induce any global oxidative stress in leaves.

Table 3.16 Comparison between the concentration of chlorophyll a (Chl a), chlorophyll b (Chl b), the sum of leaf carotenoids and xanthophylls (c+x) and relative ratio. Means followed by a different letter are significantly different at $P<0.05$ according to the One-way ANOVA and Fisher's test. ns is not significant

	Chl a	**Chl b**		**x+c**	**a/b**	**(a+b)/(x+c)**
				mg g^{-1} ww		
Control	0,827	0,359	a	0,280	2,31	4,23
Ag-NPs	0,869	0,364	a	0,277	2,39	4,45
CeO_2-NPs	0,825	0,352	a	0,279	2,34	4,21
Co-NPs	0,843	0,373	a	0,275	2,26	4,43
Fe_3O_4-NPs	0,795	0,350	a	0,270	2,27	4,24
Ni-NPs	0,783	0,323	b	0,265	2,43	4,17
SnO_2-NPs	0,697	0,298	b	0,237	2,34	4,21
TiO_2-NPs	0,853	0,371	a	0,281	2,30	4,36
ANOVA	ns	*		ns	ns	ns

Table 3.17 Lipid peroxidation evaluated with TBARS in basil leaves. Means followed by a different letter are significantly different at $P<0.05$ according to the One-way ANOVA and Fisher's test. ns is not significant

	TBARS		
	µg g^{-1}	SD	ANOVA
Control	2.040	0.441	ns
Ag-NPs	2.129	0.791	ns
CeO_2-NPs	2.120	0.203	ns
Co-NPs	1.548	0.138	ns
Fe_3O_4-NPs	1.932	0.302	ns
Ni-NPs	1.691	0.253	ns
SnO_2-NPs	2.094	0.946	ns
TiO_2-NPs	1.879	0.389	ns

In plants exposed to Ag, Co, Fe_3O_4, Ni and TiO_2 NPs the decrement of gas exchange is decoupled to the Pnet. Our hypothesis is that tissues involved in photosynthesis are not damaged by NPs (as plant pigment and lipid peroxidation showed); conversely NPs could alter the root capacity to absorb water and nutrients and decrease the evapotranspiration (Asli et al. 2009).

3.3.2.7. PCA analysis

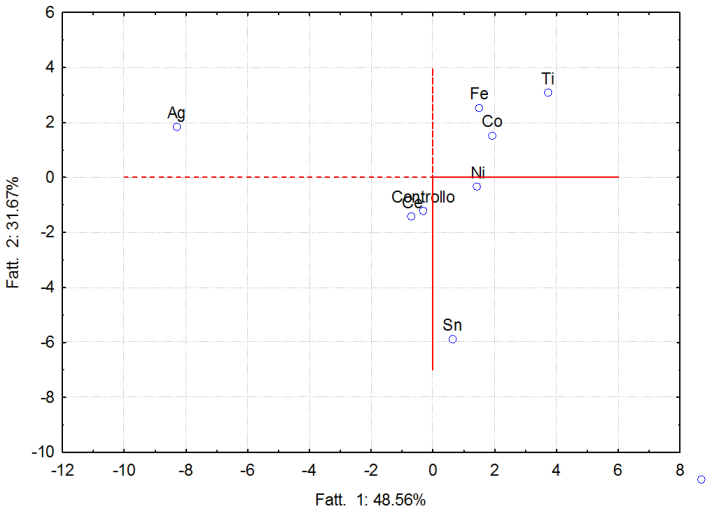
The intercorrelations between the various chemical and physiological parameters of basil were determined by PCA and presented in Fig. 3.19. The criteria to define the number of principal components (PCs) explaining most of the total variance among a certain number of variables is the Kaiser criterion, based on the concept that a PC with an eigenvalue < 1.0 has no legitimacy for the description of total variance (Kaiser 1960). Out of the seven extracted PCs, the last three did not

satisfy the Kaiser criterion; the first and second PCs were chosen because they cumulatively explained 80,22% of the total variance (Table 3.18). The first PC accounted for 48.56% of the total variance and showed high loadings (> 0.75) for the biomass production (PFF, PSF, PSFU and PSR); NP metal content in leaves (AGF and FEF), roots (AGR and SNR) and stem (AGFU); macro elements in leaves (CAF and SF), roots (MGR and SR) and stem (NAFU).

Table 3.18 Factor loadings of the eleven variables used in the principal component analysis on the first two principal components (PC1, PC2).

Variable*	PC1	PC2
PFF	0.752	0.130
PSF	0.877	-0.324
PFFU	0.698	0.138
PSFU	0.754	0.191
PSR	0.983	-0.005
AGF	-0.924	0.247
FEF	-0.931	0.172
AGFU	-0.928	0.238
CEFU	0.104	-0.919
FEFU	-0.365	-0.815
SNFU	-0.286	-0.750
TIFU	-0.200	-0.935
AGR	-0.923	0.243
SNR	-0.869	-0.270
CAF	0.828	-0.156
SF	-0.937	0.243
KFU	0.193	-0.858
NAFU	-0.925	-0.018
PFU	-0.247	-0.910
SFU	-0.502	-0.777
MGR	0.965	0.145
PR	0.742	0.371
SR	0.923	0.082
EVAP	0.175	-0.800
GS	0.211	-0.891
CLA	-0.303	0.797
CLB	-0.103	0.806
% of variance	48.56	34.66

Fig. 3.19 Principal component analysis based on chemical and physiological parameters of basil. The scatter plot reports the projection of eight treatments on the first two components PC1 and PC2. accounting for 80.22% of total variability.



*PFF= wet weight leave; PSF= dry weight leaves; PFFU= wet weight stem; PSFU= dry weight stem; PSR= dry weight root; AGF= Ag leaves; FEF=Fe leaves; AGFU= Ag stem; CEFU= Ce stem; FEFU= Fe stem; SNFU= Sn stem; TIFU= Ti stem; AGR= Ag root; SNR= Sn root; CAF= Ca leaves; SF= S leaves; KFU= K stem; NAFU= Na stem; PFU= P stem; SFU= S stem; MGR= Mg root; PR= P root; SR= S root; EVAP= evaporation; GS= stomatal conductance; CLA= chlorophyll a; *CLB= chlorophyll b.

The positive branch of the first PC indicated a correlation between the biomass production (PFF, PSF, PSFU and PSR) and the content of macro elements in leaves (CAF) and root (MGR and SR).

The negative branch of the first PC indicated that the Ag NP treatment (AGF, AGFU and AGR) influenced the content of macro elements in leaves (SF) and stem (NAFU).

The second PC accounted for 31.66% of the total variance and showed high loadings (> 0.75) for NP metal content in stem (CEFU, FEFU, SNFU and TIFU), macro elements in stem (KFU, PFU, SFU) and physiological parameters (EVAP, GS, CLA, CLB).

The negative branch of the second PC indicated a correlation between NP metal content in stem (CEFU, FEFU, SNFU and TIFU) and macro elements in stem (KFU, PFU) with physiological parameters (EVAP, GS).

In the positive branch of the second PC only plant pigments (CLA, CLB) were loaded.

A partial separation among NP treatments in the scatterplot was achieved by combining the first and the second PCs. As reported in Figure 3.19, the combination of control, CeO_2, Co, Fe_3O_4, Ni TiO_2 were closely aggregated in the northeast quadrant; while Ag and SnO_2 are separated at west and south, respectively.

PCA confirmed the negative effect of Ag NPs on plant growth and highlighted the influence on element transport such as higher content of Fe and S in leaf and Na in stem, a lower content of Mg and S in root. SnO_2 treatment influenced the translocation of elements to a higher degree of Ce, Fe, Ti, K and P in the stem.

3.4. Earthworm and nanoparticles

3.4.1. 1st part

3.4.1.1. Chemical and biochemical properties of soil

The manure used to fed earthworms increased TOC content in all treatments whereas no differences were found for TN: consequently, the C/N ratio at the end of the experiment tripled compared to its initial value (Table 3.19).

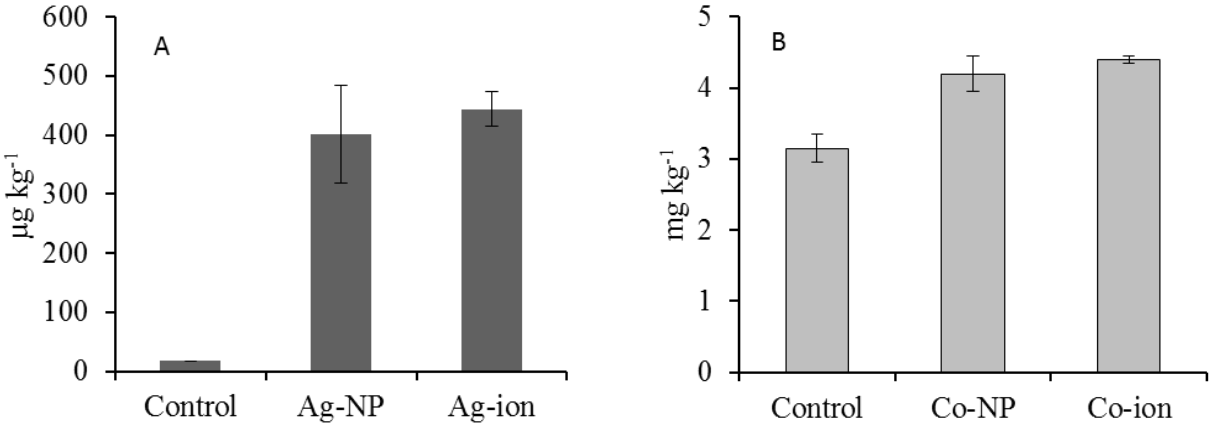
Only the Co^{2+} treatments increased the extractable C and decreased Cmic compared to the control; while on the contrary, Nmic did not show significant differences among treatments. Also the ratio Cmic/Nmic and soil respiration rate did not significantly change among treatments, whereas the qCO_2 was highest in the soils where earthworms were fed with both Co-NP and Co^{2+} contaminated food (Table 3.19), probably due to microbial stress (Giller et al. 1998, 2009) and changes in the composition of microbial communities inhabiting soil (Wardle & Ghani, 1995; Nannipieri et al. 2003).

Table 3.19 Chemical and biochemical properties of soil after 4 weeks of exposure. One-way ANOVA and Fisher's test ($p<0.05$) were used to determine statistical significance of the differences between treatment and the control means. $P<0.05$ significant differences between NPs and ions treatment and the control are reported in bold

Treatment	TOC %	TN %	TOC/TN	Extr C mg kg^{-1}	Cmic mg kg^{-1}	Nmic mg kg^{-1}	C/N	CO_2 mg CO_2-C kg^{-1} d^{-1}	qCO_2 mg CO_2-C kg^{-1}Cmic h^{-1}
Control	21.1	0.85	24.8	705.2	1291.8	106.0	13.3	44.9	1.45
Ag-NPs	20.4	0.81	25.2	700.9	1229.3	106.6	14.3	41.5	1.40
Ag^{+}	16.4	0.63	26.4	715.7	1257.9	72.2	25.1	42.1	1.39
Co-NPs	16.7	0.71	23.6	734.3	1177.3	94.1	12.8	45.3	1.61
Co^{2+}	16.8	0.71	23.9	797.3	975.8	52.2	21.7	48.9	2.08
ANOVA	**3.9**	**0.16**	**2.8**	**67.9**	**172.6**	73.1	19.1	8.9	**0.13**

Ag and Co concentrations significantly increased in soil polluted with both NPs and ion forms compared to control (Fig. 3.20).

Figure 3.20 Ag and Co concentration in soil after 5 weeks of exposure. Results are mean ± standard deviation of three treatments.



Soil PLFAs were significantly affected by treatments and by the form through which pollutants were supplied (Table 3.20). Generally, all treatments increased the content of bacterial FAs, whereas fungal content was increased only by Ag NPs and Co^{2+}, thus leading to a decrease in the bacteria/fungi ratio.

Soil treated with Co^{2+} and Co NPs showed slightly higher values of total and G- bacterial PLFAs as compared to the control soil; moreover, a lower G+/G- bacterial ratio occurred in Co^{2+} treatment.

Changes in the ratio of Gram-negative to Gram-positive bacteria have been related to the quality of organic matter in the soil. A higher proportion of G- bacteria is usually interpreted as a shift from oligotrophic to more copiotrophic conditions in the soil (Yao et al. 2000).

Table 3.20 PLFAs (% mol) in soils. One-way ANOVA and Fisher's test ($p<0.05$) were used to determine statistical significance of the differences between treatment and the control means. $P<0.05$ significant differences between NPs and ions treatment and the control are reported in bold

FAs	Control	Ag-NPs	Ag^{+}	Co-NPs	Co^{2+}	ANOVA
C14:0	3.5 ± 0.1	3.4 ± 0.1	3.57 ± 0.26	3.59 ± 0.14	3.27 ± 0.09	**0.26**
C15:0i	5.0 ± 0.1	4.9 ± 0.1	5.21 ± 0.27	5.11 ± 0.19	4.83 ± 0.16	**0.32**
C15:0a	3.8 ± 0.1	3.8 ± 0.1	3.97 ± 0.19	3.85 ± 0.19	3.60 ± 0.14	**0.26**
C15:0	1.6 ± 0.0	1.5 ± 0.0	1.63 ± 0.10	1.60 ± 0.08	1.47 ± 0.05	**0.12**
C16:0i	3.2 ± 0.1	3.2 ± 0.1	3.21 ± 0.14	3.19 ± 0.07	3.03 ± 0.08	0.19
C16:0	25.0 ± 0.8	23.9 ± 0.2	24.71 ± 1.63	24.31 ± 1.01	22.86 ± 0.44	**1.72**
C17:0i	5.9 ± 0.4	6.0 ± 0.4	5.99 ± 0.33	5.77 ± 0.09	5.91 ± 0.11	0.57
C16:1w5	6.5 ± 0.4	6.7 ± 0.5	6.55 ± 0.62	6.19 ± 0.48	6.36 ± 0.51	0.90
C17:0	2.2 ± 0.1	2.2 ± 0.1	2.19 ± 0.21	2.11 ± 0.23	2.27 ± 0.26	0.35
C17:0cy	2.0 ± 0.1	2.3 ± 0.2	2.45 ± 0.06	2.71 ± 0.30	2.57 ± 0.07	**0.31**
C18:0	6.1 ± 1.2	5.0 ± 0.5	5.44 ± 0.31	5.41 ± 0.20	5.69 ± 0.28	1.14
C18:1w9	14.3 ± 0.4	14.5 ± 0.1	14.03 ± 0.85	14.08 ± 0.72	14.86 ± 0.73	1.15
C18:1w7	6.0 ± 0.4	7.0 ± 0.7	6.47 ± 0.87	6.94 ± 0.81	7.79 ± 0.28	**1.21**
C18:2w6.9	9.8 ± 0.4	10.2 ± 0.3	9.40 ± 0.81	9.65 ± 0.78	10.70 ± 0.05	**0.99**
C19:0cy	3.0 ± 0.1	2.9 ± 0.2	2.96 ± 0.15	2.89 ± 0.15	2.90 ± 0.10	0.26
C20:0	2.3 ± 0.5	2.5 ± 0.4	2.21 ± 0.27	2.60 ± 0.07	1.88 ± 0.09	**0.58**
Bacteria	32.7 ± 1.1	33.8 ± 0.6	34.09 ± 0.69	34.17 ± 0.70	34.38 ± 0.44	**1.32**
Fungi	9.8 ± 0.4	10.2 ± 0.3	9.40 ± 0.81	9.65 ± 0.78	10.70 ± 0.05	**0.99**
BacteriaG+	17.9 ± 0.7	17.9 ± 0.6	18.39 ± 0.29	17.93 ± 0.51	17.37 ± 0.32	**0.88**
BacteriaG-	11.0 ± 0.3	12.2 ± 0.7	11.88 ± 0.76	12.54 ± 0.86	13.26 ± 0.30	**1.16**
Bacteria/fungi	3.4 ± 0.1	3.3 ± 0.1	3.65 ± 0.31	3.56 ± 0.30	3.21 ± 0.06	**0.37**
G+/G-	1.6 ± 0.0	1.5 ± 0.1	1.55 ± 0.12	1.44 ± 0.14	1.31 ± 0.05	**0.18**

Also PLFAs identified in earthworm faeces after their depuration (2 days) showed significant differences among treatments (Table 3.21). Most significantly, earthworms fed with contaminated food showed higher amounts of bacterial PLFAs in their faeces than those fed with uncontaminated food. The faeces of worms fed with Ag and Co NPs showed higher G- bacterial PLFAs compared to the other treatments; moreover, lower G+/G- bacterial ratio occurred in Ag and Co NPs.

The bacterial/fungal PLFA ratio in faeces of earthworm was affected only by Ag ion and NPs (Table 3.21).

Works to support this data are very limited, and few studies have been conducted about the impact of ENMs on soil microbial community composition using PLFA. Notably, Shah and Belozerova (2009) did not observe differences in the FA profiles in soil exposed to Si, Pd, Au and Cu NPs (at 0.013% or 0.066% w/w rate) in 15 days of incubation. Conversely, Kumar et al. (2012) assessed striking differences between control and arctic soil treated with Ag, Cu and Si NPs (all at 0.022%, w/w; 176 days of incubation): Gram-positive signature FA (15:1 ISO, Cavigelli et al. 1995) in treated soil was found to be above a 1% cut-off; while a marker for Gram-negative bacteria (23:0 3OH, Cavigelli et al. 1995) showed reductions to below 1% of the total peak area, suggesting that these types with a thinner cell wall and the general inability to form spores may be more susceptible to NP-mediated toxicity. In our study we did not detect a significant increase of Gram-

positive bacteria, probably due to the presence of earthworms that reduce soil microbial biomass and are able to immobilise nutrients (Cole at al. 2002).

Table 3.21 PLFAs (% mol) in faeces. One-way ANOVA and Fisher's test (p<0.05) were used to determine statistical significance of the differences between treatment and the control means. P<0.05 significant differences between NPs and ions treatment and the control are reported in bold

FAs	Control	Ag-NPs	Ag^+	Co-NPs	Co^{2+}	ANOVA
C14:0	2.5 ± 0.0	2.3 ± 0.1	2.5 ± 0.5	2.6 ± 0.0	3.3 ± 0.6	1.00
C15:0i	2.9 ± 0.3	2.8 ± 0.1	2.6 ± 0.4	2.7 ± 0.1	3.5 ± 0.0	0.68
C15:0a	4.1 ± 0.4	3.6 ± 0.1	3.6 ± 0.5	3.6 ± 0.2	3.8 ± 0.2	0.91
C15:0	2.2 ± 0.2	8.5 ± 0.5	14.5 ± 1.6	4.2 ± 0.2	11.8 ± 1.1	2.49
C16:0i	1.6 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.4 ± 0.3	1.1 ± 0.1	0.46
C16:0	27.0 ± 0.4	26.4 ± 0.3	25.4 ± 0.4	28.7 ± 0.0	25.2 ± 0.0	0.88
C17:0i	8.9 ± 1.3	10.0 ± 1.0	7.9 ± 0.3	9.2 ± 0.4	9.5 ± 0.1	2.12
C16:1w5	5.3 ± 0.5	6.2 ± 0.2	6.0 ± 0.1	6.8 ± 0.1	6.4 ± 0.6	1.05
C17:0	1.3 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	1.3 ± 0.0	1.2 ± 0.0	0.12
C17:0cy	5.2 ± 1.4	8.1 ± 1.3	6.2 ± 0.4	6.3 ± 1.8	4.6 ± 0.5	3.19
C18:0	10.5 ± 3.4	5.9 ± 0.5	5.6 ± 0.3	5.8 ± 0.1	4.6 ± 0.6	4.09
C18:1w9	11.2 ± 0.2	7.8 ± 0.4	9.0 ± 0.0	8.6 ± 0.2	8.6 ± 0.5	0.94
C18:1w7	9.5 ± 1.1	9.3 ± 0.4	8.2 ± 0.6	11.4 ± 0.0	9.4 ± 0.6	1.75
C18:2w6.9	5.1 ± 0.5	4.9 ± 0.1	4.7 ± 0.0	4.9 ± 0.3	5.2 ± 0.5	0.97
C19:0cy	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.2	0.32
C20:0	0.7 ± 0.1	0.6± 0.0	0.6 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	0.16
C22:0	0.8 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.31
Bacteria	36.6 ± 2.4	45.2 ± 0.4	45.5 ± 1.3	40.8 ± 0.9	45.5 ± 0.7	3.54
Fungi	5.1 ± 0.5	4.9 ± 0.1	4.7 ± 0.0	4.9 ± 0.3	5.2 ± 0.5	0.97
BacteriaG+	17.7 ± 2.3	17.6 ± 0.8	15.2 ± 1.4	17.1 ± 1.2	18.0 ± 0.1	3.59
BacteriaG-	15.2 ± 0.1	17.9 ± 0.9	14.7 ± 1.1	18.0 ± 1.8	14.3 ± 0.2	2.75
Bacteria/Fungi	7.1 ± 0.2	9.2 ± 0.4	9.5 ± 0.3	8.2 ± 0.7	8.7 ± 1.0	1.65
G+/G-	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.0	0.36

3.4.1.2. Chemical and biochemical properties of earthworms

At the end of the exposure, earthworms fed with Ag contaminated food showed higher Ag content than those fed with uncontaminated food, being 649.3 ± 13.5 and 487.3 ± 37.0µg Ag kg^{-1} for Ag NPs and Ag^+ treatment respectively. As regards earthworms fed with Co contaminated food, higher values of Co were found in both Co treatments compared to earthworms fed with uncontaminated food (Table 3.22), but Co^{2+} showed a very high value probably due to the fact that cobalt is an essential element that is homeostatically controlled in organisms, including earthworms (Neuhauser et al. 1984).

A slight increase of Ca and P was detected in earthworms tissues, while for the other major elements no differences were found as a function of varied exposure. The Ca/P ratio slightly increased with exposure of earthworms to Ag and Co NPs, 0.67±0.01 0.66±0.02 respectively, while no significant differences occurred in the ratio which ranged from 0.59 to 0.66 (Table 3.23).

Table 3.22 Ag and Co concentration (μg kg^{-1}) in earthworm tissues after 5 weeks of exposure. Results are mean ± standard deviation of three treatments.

Treatments	Ag		Co	
Control	<DL	c	2914.8 ± 184.5	b
Ag-NPs	649.3 ± 13.5	a	2737.1 ± 111.3	b
Ag^{+}	487.3 ± 37.0	b	2923.2 ± 16.7	b
Co-NPs	<DL	c	4272.9 ± 457.7	b
Co^{2+}	<DL	c	12157.3 ± 724.7	a
Significance	***		***	

DL detection limit; DL was 26.9 and 75.8 (μg kg^{-1}) for Ag and Co. respectively. Within the column. different letters indicate significant differences (P<0.05) among treatments.

Table 3.23 Major elements concentration (g kg^{-1}) in earthworm tissues after 5 weeks of exposure. Results are mean ± standard deviation of three treatments. Ca/P ratio was also reported.

Treatments	Ca	K	Mg	Na	P	S	Ca/P
			g kg^{-1}				
Control	4.1	5.8	0.8	9.1	6.9	6.9	0.59
SD	*0.3*	*0.7*	*0.1*	*0.2*	*0.2*	*0.2*	*0.01*
Ag-NPs	4.6	6.7	0.9	9.2	6.9	6.9	0.67
SD	*0.3*	*1.1*	*0.2*	*0.6*	*0.1*	*0.1*	*0.01*
Ag^{+}	4.5	6.4	0.9	8.9	7.1	7.1	0.63
SD	*0.2*	*0.9*	*0.4*	*0.2*	*0.1*	*0.1*	*0.02*
Co-NPs	4.7	6.3	0.9	9.4	7.1	7.1	0.66
SD	*0.5*	*1.3*	*0.1*	*0.5*	*0.1*	*0.1*	*0.02*
Co^{2+}	4.4	7.3	0.9	8.8	7.2	7.2	0.61
SD	*0.1*	*0.4*	*0.0*	*0.1*	*0.2*	*0.2*	*0.02*

As the FAs in the earthworm's body depend on species (Albro et al. 1992; Paoletti et al. 2003) and diet (Hansen & Czochanska 1975; Sampedro et al. 2006), FAs in body or gut of earthworms have been used as indexes of response to environmental stress (Crockett et al. 2001). Therefore in this research, FA content in body tissues of *L. rubellus* was used as marker to evaluate the impact of nanoparticles on earthworms.

A total of 28 FAs were identified and quantified in earthworm tissues and 18 of them showed significant differences among treatments (Table 3.24). The most abundant FAs (in average >10 % mol) were 20:5w3, 20:3w3, 18:1w9c followed by 18:0 and 18:2w6,9c, whereas the remaining FAs had a % mol less than 5. Poli-unsaturated FAs (PUFAs) were the most abundant and ranged from 34.5 to 43.4 mol%, in the control and Co^{2+} treatment, respectively. PUFAs decreased according to the following sequence: Control>Ag NPs>Co NPs>Ag^{+}>Co^{2+} (Table 3.25). Also mono-unsaturated FAs showed a similar pattern to PUFAs with higher values in the control and the lowest ones in Co^{2+} treatment. On the contrary, saturated FAs showed an opposite trend with the lowest values in the control and the highest in Co^{2+} treatment. Consequently the ratio SAFA/MUFA was the lowest in the control, followed by Ag NPs, Ag^{+} and Co NPs, and finally by Co^{2+} treatment, which exhibited a higher uptake of metal elements (12,2 mg Co kg^{-1} dw, see Table 3.22). The degree of

unsaturation showed significant differences between the control and both ion treatments, showing the highest value in the former (Table 3.25).

Table 3.24 FAs (% mol) in earthworm tissues. One-way ANOVA and Fisher's test (p<0.05) were used to determine statistical significance of the differences between treatment and the control means. P<0.05 significant differences between NPs and ions treatment and the control are reported in bold

FAs	Control	Ag-NPs	Ag^{+}	Co-NPs	Co^{2+}	ANOVA
C12:0	2.9 ± 0.1	3.2 ± 0.1	4.2 ± 0.7	3.4 ± 0.0	5.1 ± 0.1	**0.3**
C13:0	0.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.1
C14:0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.8 ± 0.2	1.3 ± 0.3	**0.2**
C14:1w5	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.1
C15:0	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1
C15:0i	2.6 ± 0.1	2.1 ± 0.3	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	**0.2**
C16:0	0.4 ± 0.1	0.7 ± 0.0	2.1 ± 0.2	1.8 ± 0.1	3.1 ± 0.6	**0.3**
C16:0i	1.1 ± 0.3	1.2 ± 0.2	0.7 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	**0.2**
C16:1w7	3.1 ± 0.4	3.3 ± 0.5	3.2 ± 0.7	2.5 ± 0.2	2.7 ± 0.1	0.5
C16:1w9	2.4 ± 0.1	1.9 ± 0.0	1.8 ± 0.1	1.7 ± 0.0	1.6 ± 0.0	**0.1**
C17:0	1.9 ± 0.3	3.4 ± 0.0	4.1 ± 0.0	3.7 ± 0.3	4.7 ± 0.4	**0.3**
C17:0cy	2.2 ± 0.6	1.9 ± 0.8	3.1 ± 0.9	3.0 ± 1.5	3.2 ± 0.5	1.0
C18:0	9.8 ± 0.2	11.4 ± 0.0	12.3 ± 0.3	11.9 ± 0.1	13.1 ± 0.2	**0.2**
C18:1w7	0.8 ± 0.2	1.4 ± 0.2	1.1 ± 0.0	0.8 ± 0.5	1.0 ± 0.0	0.3
C18:1w9c	13.1 ± 0.5	11.7 ± 0.2	11.0 ± 0.4	10.5 ± 0.1	10.3 ± 0.1	**0.3**
C18:1w9t	0.8 ± 0.2	1.1 ± 0.1	0.5 ± 0.0	1.1 ± 0.3	0.3 ± 0.0	**0.2**
C18:2w6.9c	9.0 ± 0.3	8.2 ± 0.1	8.2 ± 0.2	8.1 ± 0.0	7.4 ± 0.7	**0.4**
C18:3w3	2.0 ± 0.6	2.5 ± 0.2	2.7 ± 0.2	2.6 ± 0.1	3.2 ± 0.6	0.4
C18:w6.9t	1.4 ± 0.2	2.1 ± 0.1	1.9 ± 0.7	2.7 ± 0.2	2.0 ± 0.4	**0.4**
C20:0	0.6 ± 0.5	1.0 ± 0.2	1.3 ± 0.6	1.5 ± 0.5	1.5 ± 0.7	**0.5**
C20:1w9c	9.3 ± 0.4	8.8 ± 0.2	9.1 ±0.1	9.2 ± 0.4	9.5 ± 0.2	0.3
C20:2w6	2.0 ± 0.0	2.0 ± 0.2	1.8 ± 0.1	1.6 ± 0.1	0.9 ± 0.5	**0.3**
C20:3w3	13.4 ± 0.4	12.2 ± 0.2	10.1 ± 0.6	11.5 ± 0.3	8.8 ± 0.7	**0.5**
C20:5w3	15.6 ± 0.1	14.8 ± 0.0	13.8 ± 0.4	14.6 ± 0.1	12.1 ± 0.9	**0.5**
C22:0	0.9 ± 0.0	0.9 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.7 ± 0.3	**0.2**
C23:0	0.3 ± 0.0	0.6 ± 0.2	0.3 ± 0.3	0.3 ± 0.3	0.6 ± 0.2	0.2
C24:0	1.0 ± 0.5	1.2 ± 0.6	1.3 ± 0.3	1.3 ± 0.0	1.5 ± 0.3	0.4
C24:1w9	1.1 ± 0.2	0.6 ± 0.2	0.8 ± 0.3	0.6 ± 0.0	0.7 ± 0.2	**0.2**

Table 3.25 Molar percentage of total saturated. mono- and poli-unsaturated fatty acids. One-way ANOVA and Fisher's test (p<0.05) were used to determine statistical significance of the differences between treatment and the control means. P<0.05 significant differences between NPs and ions treatment and the control are reported in bold

Type of FAs	Control	Ag-NPs	Ag^{+}	Co-NPs	Co^{2+}	ANOVA
SAFA	19.4 ± 0.3	23.6 ± 0.4	27.8 ± 2.4	26.4 ± 1.0	33.0 ± 2.6	**1.8**
MUFA	31.2 ± 0.8	29.4 ± 0.1	28.0 ± 0.1	26.9 ± 0.8	26.6 ± 0.5	**0.6**
PUFA	43.4 ± 0.2	41.7 ± 0.3	38.4 ± 1.8	41.0 ± 0.3	34.5 ± 1.8	**1.2**
SAFA/MUFA	0.6 ± 0.0	0.8 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	**0.1**
Unsaturation degree	1.8 ± 0.0	1.7 ± 0.0	1.6 ± 0.1	1.7 ± 0.0	1.4 ± 0.1	**0.04**

SAFA. saturated fatty acids; MUFA. mono-unsaturated fatty acids; PUFA. poli-unsaturated fatty acids

Changes in the degree of unsaturation usually indicate a response of soil organisms to environmental stress and have consequences on membrane fluidity. Indeed, saturated FAs can pack

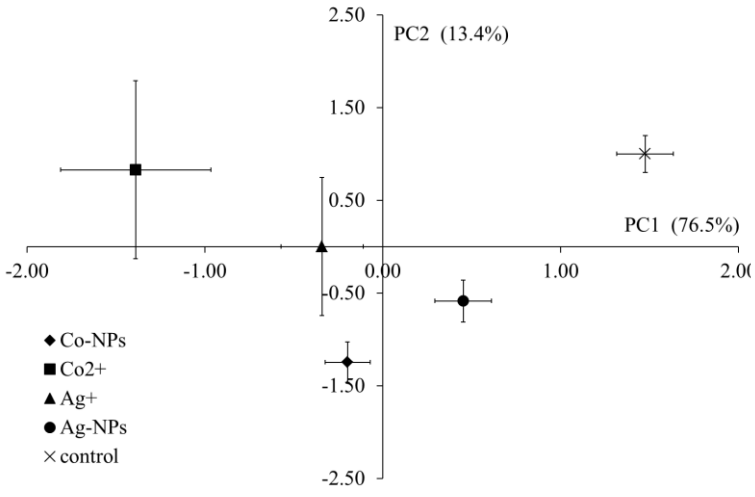
together better than the unsaturated ones and, therefore, make membrane more viscous and less permeable (Collins et al. 1990). Moreover, a low degree of unsaturation decreases the susceptibility of FAs to free radicals (García et al. 2005). The observed reduction of the degree of unsaturation in earthworm tissues agrees with other similar research carried out on soil microorganisms (Čertik et al. 2005; Frostegård et al. 1993; Howlett & Avery 1997; Markowicz et al. 2010; Paraszkiewicz et al. 2009; Yang et al. 2009) and could be ascribed to a defensive mechanism that reduces the ability of heavy metals to generate oxidative stress on membrane lipids (Howlett & Avery 1997; Yang et al. 2009).

Principal component analysis carried out on earthworm FA tissues extracted two principal components that explained almost 90% of variance (Table 3.26). Along PC1 (76.5% of explained variance) treatments were separated from each other and from the control; but with Ag^{+} treatment very close to the Co-NPs treatment; however, within each metal, ion and NP treatments were separated. PC1 showed the highest loading scores for 12:0, 16:0, 17:0, 18:0 FAs in the negative branch and 16:1w9, 18:1w9, 18:2w6,9c, 20:3w3 and 20:5w3 FAs in the positive branch, showing a clear separation between saturated and unsaturated FAs. PC2, which accounted for 13.4% of variance, had the highest loading score for 18:2w6,9t and 22:0 FAs in the negative and positive branch, respectively. Moreover, PC2 seemed to separate the two ion treatments from the two NP treatments, with the first ones closer to the control (Fig. 3.21), probably due to a different mechanism of NP delivery to cells (Tsyusko et al. 2012; Hayashi et al. 2013).

Table 3.26 Explained variances and factor loadings for each variable on the unrotated principal components (PC) from 24 original soil chemical and biochemical variables

Variable	PC1	PC2
C12:0	-0.890	0.343
C15:0i	0.747	0.476
C16:0	-0.962	0.151
C16:1w9	0.907	0.378
C17:0	-0.965	-0.127
C18:0	-0.972	-0.123
C18:1w9c	0.921	0.295
C18:2w6.9c	0.910	-0.035
C18:2w6.9t	-0.355	-0.841
C20:2w6	0.832	-0.322
C20:3w3	0.938	-0.100
C20:5w3	0.938	-0.310
C22:0	0.442	0.853
% of variance	76.5	13.5

Figure 3.21 Principal component analysis carried out on earthworm tissues FAs. The overall explained variance by the two components was 89.9%.

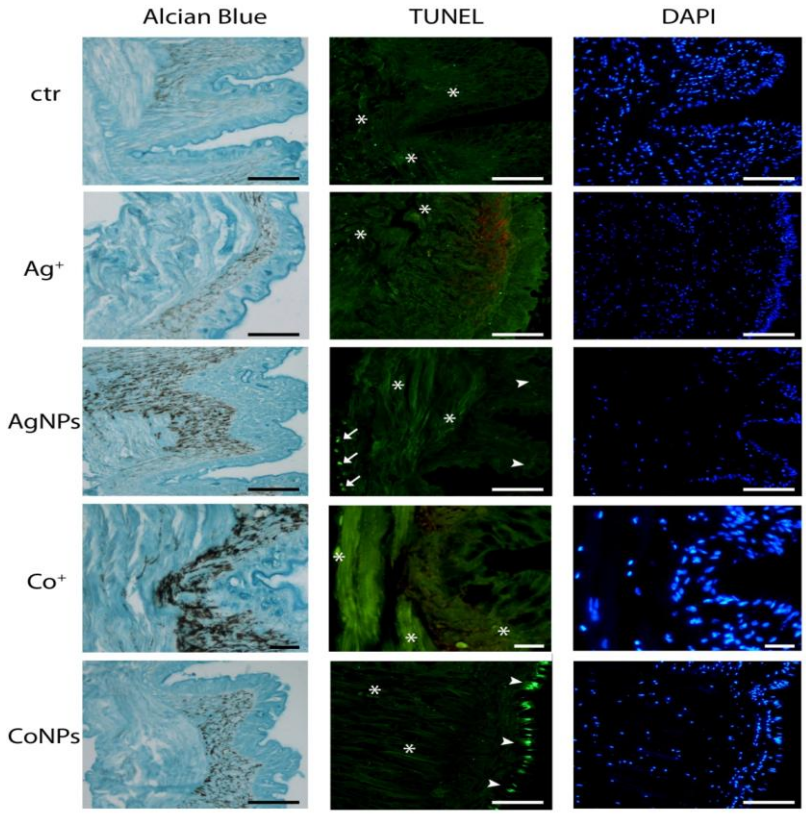


3.4.2. 2nd Part: evaluation of metal depuration and healthiness of *L. rubellus*

Interestingly, earthworms fed with Ag NP contaminated food showed a comparable value of Ag concentration at the end of the exposure and at the end of the depuration; while no Ag was detected in earthworms fed with Ag^{+}. Conversely, Co concentration remained stable in worms fed with Co^{2+}, as observed by Coutris et al. (2012).

The histological and immunohistochemical analyses performed after four weeks of depuration of earthworms in new unpolluted soil are shown in Fig. 3.22.

Figure 3.22 Histomorphology of *L. rubellus* cuticle stained with Alcian Blue and immunohistochemical detection of apoptosis in *L. rubellus* cuticle during the experiment. ctr = control. Note TUNEL-positive nuclei in the musculature in all experiments (asterisks). Cuticle is immunopositive to TUNEL only after treatment with Ag NPs and Co NPs (arrowheads). while TUNEL-positive nuclei between musculature and intestine were only observed after Ag NPs-treatment (arrows). White and black bars are 100 µm.



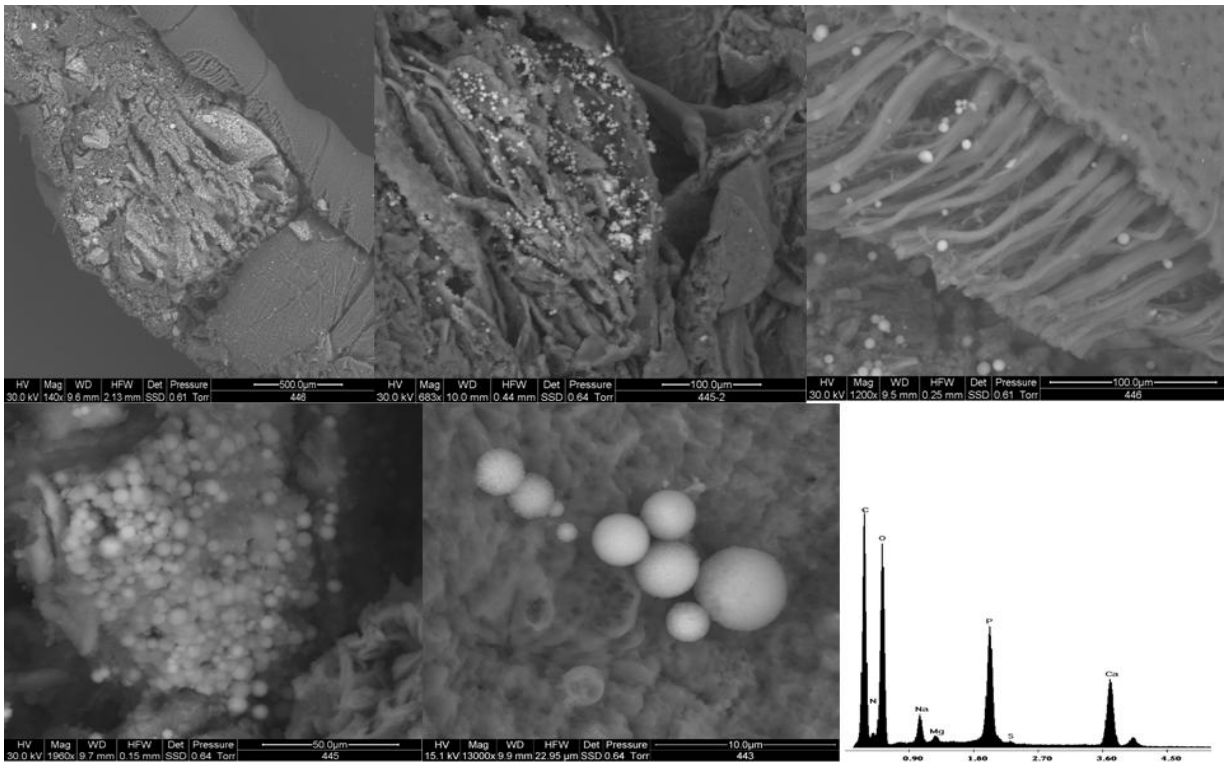
Hematoxylin-eosin staining did not show changes in cuticule morphology or in the circular and longitudinal musculature. Moreover, Alcian Blue staining showed that cuticule function (production of acidic mucins) did not change after exposure to ions, therefore further immunohistochemical analysis was conducted.

The apoptotic frequency in earthworms tissues was analysed by TUNEL test, while DAPI test, which detects DNA and highlights the presence of cell nuclei, was performed as a control to avoid false positive error in TUNEL test interpretation.

Few TUNEL-positive elements were detected in the control samples: they were almost exclusively located in muscle tissue. A similar distribution was observed in all treated samples and no differences in the distribution was observed between control and ion-exposed samples. Besides, Ag-NP and Co-NP treated samples also showed numerous TUNEL-positive nuclei in the cuticule. In addition, exposure to Ag-NPs evidenced TUNEL-positive nuclei along the side between musculature and gut. Notably, Lapied et al. (2011) found an increase of apoptotic frequency in *Lumbricus terrestris* after exposure to TiO_2 NPs in cuticule and in intestinal tissues: the tissues that were most exposed to NPs (dermal contact and ingestion of contaminated feed).

The ESEM-EDS observations verified the presence of the Co and Ag NPs and some particles of the soil with few NPs entrapped internally in the tissues of earthworm gut (Fig. 3.23). The Ag and Co content in purged earthworms tissues was higher than that of control in Ag NP and Co^{2+} treatments for Ag and Co elements, respectively (Table. 3.27).

Figure 3.23 ESEM images of the Ca-P spherules found in the earthworms tissues.



By ESEM investigation, the presence of calcium-phosphate spherules similar to hydroxypatite (Fig. 3.23) were found between the mouth and clitellum of earthworms which had been exposed to Ag, Co NPs and Co^{2+}. A significant increase of Ca and P content was detected in earthworm bodies

after exposure as reflected by increased of Ca/P ratio (Table. 3.27), while no significant change in other major elements between different treatments and the control were observed (data non shown).

Table 3.27 Ag and Co concentration (μg kg^{-1}) in earthworm tissues at second step after 4 weeks of depuration. Results are mean ± standard deviation of three treatments. Ca/P ratio was also reported.

Treatments	**Ag**		**Co**		**Ca/P**
Control	<DL	b	3146 ± 324.6	b	0.65
Ag-NPs	469.5± 61.1	a	3172.1 ± 111.3	b	0.80
Ag^{+}	<DL	b	3185.2 ± 278.7	b	0.72
Co-NPs	<DL	b	3899.9 ± 377.7	b	0.72
Co^{2+}	<DL	b	10514.6 ± 112.7	a	0.74
Significance	***		***		***

The identification of calcium-phosphate precipitates in the musculature is quite interesting. Indeed, calcification—namely precipitation of calcium-phopshate crystals—is the response to short-term or long-term inflammation in human cells. Presence of "foreign bodies" can induce inflammation and some cells can die by apoptosis and release apoptotic bodies or bodies still not well-defined enough to release matrix vesicles. These small membrane-bound microparticles have the capacity to concentrate calcium and phosphate to allow crystal nucleation and thus act as the first nidus for mineralization (Reynolds et al. 2004). The calcium-phosphate spherules identified probably are a sort of calcification that is the exit of an inflammatory reaction. What is surprising is the Ca/P ratio close to 1.67 of the hydroxypatite and the similarity of morphology, size and composition that is identified in the human body in some forms of cancer (Gatti et al. 2008; Gatti & Montanari 2008).

4. Conclusions

4.1. Soil microbial biomass and NPs

In the first experiment the impact of CeO_2, Fe_3O_4 and SnO_2 NPs on soil microbial biomass has been evaluated.

The engineered metal oxides-NPs did not influence significantly microbial biomass C and N but affected the C/N microbial ratio and increased the metabolic quotient (qCO_2); this aspect is probably due to microbial stress and changes in the bacterial biomass/fungal biomass ratio as shown by viable count.

The determination of metals dissolved from NPs by chemical methods employing EDTA, NH_4NO_3 *aqua regia* extraction, was less suitable in assessing the NPs hazard of NPs in soil than extraction by water and use of the soil partition coefficient (log Kp). Water extraction showed that elements of NPs had low solubility.

Both FTIR and ESEM analysis indicated that NPs were associated to small aggregates rich in labile organic C, microbial biomass and clays. This suggests that NPs can interact with most of microbial communities inhabiting soil, therefore future research should investigate the effects on soil microbial diversity and the relationships between changes in microbial composition and microbial functionality.

The DGGE analysis underlined that the metal oxide investigated, even if able to significantly modify the ecophysiological indicators, did not induce any shift in bacterial community or reduction of soil complexity in the medium-term. However, the decrease of microbial efficiency of substrate use could be relevant in a long term perspective. Further experiments should be carried out with more than two sampling times in order to better identify a response trend. In the second experiment, the previous experimental design was applied to evaluate the impact of a polyvinylpyrrolidone-coated Ag NPs, which are used for the incorporation into consumer products. The Ag concentration employed are higher than the predicted values, but are in line with those in biosolids. The results showed that Ag NPs had a dose-dependent antibacterial effect on soil microbial biomass; with a decrease in microbial biomass C and viable bacterial counts due to the antimicrobial action of Ag nanoparticles.

Data obtained in this study provided evidence that the bacterial community of forest soils is highly influenced by the presence of Ag NPs, both quantitatively and qualitatively, with the selection of tolerant strains.

Future research should be focused on the activities of Ag NPs resistant bacterial strains so as to evaluate their contributions to the global soil functionality.

4.2. Plant and NPs

The uptake, bioaccumulation, biotransformation and risk of nanoparticles for food crops are not still well understood.

In the first experiment the uptake and translocation of Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 NPs in tomato plants grown in soil polluted though water irrigation, simulating a chronic exposure, was assessed.

The metal oxides- and metal- based engineered nanoparticles affected differently the morphological parameters, the uptake and the translocation of elements from NPs in various tomato organs. The dry mass of roots was enhanced by Fe_3O_4 exposure and depressed by SnO_2, Co and Ni treatments. Low mobility of NPs was found in bulk and rhizosphere soils, except for TiO_2 NPs which was leached. The rhizosphere soil was enriched by NPs, which were detected in root tissues using ESEM-EDS analysis. Ag, TiO_2 and SnO_2 NPs were detected within root cells of tomato grouped as a large cluster. The absorption of water and nutrients by plants probably determine the root and rhizosphere enrichment of NPs.

The determination of metal concentration in tomato organs by ICP-OES showed that the tomato plants exposed at Ag, Co and Ni NPs had metal concentration higher than the control; in addition, the long term exposure to relative low concentrations of NPs can determine a disorder of macro nutrients absorption. Notably, plants treated with Ag NPs showed Ag contamination in the fruits, but the translocation mechanism should be further investigated.

In the second test, the impact of Ag, CeO_2, Co, Fe_3O_4, Ni, SnO_2 and TiO_2 NPs was assessed in basil plants through chemical and physiological parameters. Conversely to what observed in tomato, Ag NPs treated basil showed a significant reduction of root and leaf dry matter with respect to the control. Discrepancy in biological responses could be specie-specific. However, a similar pattern was found for the metal concentration arising from NPs: the larger amount of metal-NPs was accumulated in basil roots and the concentration in leaves was significantly higher compared to the control for Ag, CeO_2, Co and Ni.

Notably, also in the relative short exposure there was an accumulation of Ca in roots, suggesting that the metabolic alteration in plants could be aimed at counteracting the membrane damage generated directly or indirectly by NPs.

The physiological parameters (gas exchange and plant pigments concentrations) did not show significant differences; probably tissues involved in the photosynthesis are not damaged by NPs.

Nevertheless, further studies are required to evaluate the impact of these NPs over several generation and their fate in food chain.

4.3. Earthworms and NPs

The final part of the thesis aimed at determining whether Ag and Co NPs could damage soil microbial biomass and earthworm functionality more than their ionic form.

Ag and Co added to soil as NPs, or ion through food for *L. rubellus*, affected both the earthworm and soil microbial biomass C. More precisely, soil microbial biomass and membrane fluidity of the earthworm decreased. Noteworthy, Co^{2+} determined a reduction in soil microbial biomass and the *L. rubellus* membrane fluidity. It is suggested that the physiological alteration in earthworms could be aimed at counteracting the risk of oxidative stress likely induced by the exposure to ions also released by the NPs.

The investigation of the effect of Ag and Co NPs and Ag and Co ions exposure after one month of depuration, checked through the cell apoptosis of *L. rubellus* cuticule, and the presence of NPs in the earthworm body, showed no differences between controls and ion-treated specimens. In the specimens exposed to NPs, TUNEL-positive nuclei were almost exclusively detected in the cuticule, besides being present between the musculature and the intestine after exposure to Ag NPs. Changes in distribution of TUNEL-positive nuclei after exposure to Ag NPs showed an alteration of cell renewal, probably due to the persisting exposure to Ag. The presence of NPs inside the earthworms was hardly identified but the ESEM-EDS analysis identified the presence of Ca/P spherules (calcification) in the tract between the mouth and the clitellum.

Further long-term experiments are needed to assess the dissolution and the toxicokinetics of NPs.

In conclusion, some remarks can be made about NPs toxicity in soil:

Generally toxicity is influenced by NP core elements: CeO_2, Fe_3O_4 and SnO_2 NPs determine a lower impact on bacterial community with respect Ag NPs.

The impact of NPs on organisms is specie-specific: the exposure at Ag NPs in tomato plant determine an increase of root dry matter while in basil the same parameter decreases.

Experiments conducted in media closer to real conditions showed a decrease in toxicity with respect to *in vitro* test or hydroponic tests.

With regard to the methodological aspects, only very advanced and expensive techniques, such as bulk extended X-ray absorption fine structure spectroscopy and XANES, could really achieve a proper *in situ* characterization of NPs in soil.

However, a multidisciplinary approach, involving physical, chemical and biological skills could pave the way to draw the right conclusions and accomplish a deeper comprehension of the effects of NPs on soil and soil inhabitants.

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List of papers

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2. L. Vittori Antisari, **S. Carbone**, A. Gatti, A. Fabrizi, G. Vianello (2012). "Toxicological effects of engineered nanoparticles of earthworms (*Lumbricus rubellus*) in short exposure". EQA, vol. 8, p. 51-60, DOI: 10.6092/issn.2281-4485/3750
3. L. Vittori Antisari, **S. Carbone**, A. Fabrizi, A. Gatti, G. Vianello (2011). "Response of soil microbial biomass to CeO_2 nanoparticles". EQA, vol 7, p. 1-16, DOI: 10.6092/issn.2281-4485/3829
4. C. Gambardella, L. Gallus, A. Gatti, M. Faimali, **S. Carbone**, L. Vittori Antisari, C. Falugi, S. Ferrando "Toxicity and transfer of metal oxide nanoparticles from microalgae to sea urchin larvae". Chemistry and Ecology. *In press:* doi:10.1080/02757540.2013.873031
5. C. Gambardella, T. Mesarič, T. Milivojević, K. Sepčić, L. Gallus, **S. Carbone**, S. Ferrando, M. Faimali "Effects of selected metal oxide nanoparticles on Artemia salina larvae: evaluation of mortality, and behavioural and biochemical responses". Environmental Monitoring and Assessment. *In press:* doi: 10.1007/s10661-014-3695-8
6. L. Vittori Antisari, **S. Carbone**, F. Gaggia, L. Baffoni, D. Di Gioia, G. Vianello, P. Nannipieri "Silver uptake by soil microbiota, and loss of microbial biomass and bacterial diversity in a sandy clay loam soil polluted with silver engineered nanoparticles"
7. L. Vittori Antisari, **S. Carbone**, A. Gatti, G. Vianello, P. Nannipieri3 "Uptake and translocation of metals and nutrients in tomato grown in soil polluted with metal oxides- (CeO_2, Fe_3O_4, SnO_2, TiO_2) or metal- (Ag, Co, Ni) engineered nanoparticles"
8. L. Vittori Antisari, **S. Carbone**, G. Vianello, V. A. Laudicina, L. Badalucco, A. Gatti, S. Ferrando, C. Gambardella, C. Falugi "Earthworm model for a new nanoecotoxicity test: lipid metabolism disorders and cell apoptosis of cuticle in *Lumbricus rubellus*"
9. **S. Carbone**, T. Hertel-Aas, E.J. Joner, D.H Oughton. "Bioavailability of Ce and Sn nanoparticles evaluated by dietary uptake in the earthworm *Eisenia fetida* and sequential extraction of soil and feed"

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