Effects of Engineered Nanoparticles on Crops, their Symbionts, and Soil Microbial Communities

Effecten van synthetische nanodeeltjes op gewassen, hun symbionten en microbiële bodemgemeenschappen

Janine Moll

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Effecten van synthetische nanodeeltjes op gewassen, hun symbionten en microbiële bodemgemeenschappen

(met een samenvatting in het Nederlands)

Proefschrift

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Janine Moll

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Promotor: Prof. dr. M.G.A. van der Heijden

Copromotor: Dr. F. Widmer

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Chapter I

General Introduction

1. Definition of nanoparticles

Nanoparticles (NPs) are one of the promising key technologies of the 21st century. NPs can consist of any material; it is only their size that defines them. According to the recommendations of the European Commission (2011), NPs are particles that are smaller than 100 nm in at least one dimension. Therefore carbon nanotubes (CNTs), which are in the micrometer range in length but have a diameter in the nano size, are also classified as NPs. Furthermore, the European Commission defines the term nanomaterial (NM) as materials containing 50% or more NPs (ratio between number of particles with size between 1-100 nm and larger sized particles). Aggregated or agglomerated NPs are also included in this definition because single particles might be released from these aggregates and agglomerates (European Commission, 2011).

What makes NPs special compared to larger particles (primary particles>100 nm) of the same material is their high surface area to volume ratio. Because of this enhanced surface area of NPs, more atoms are close to the surface and can increase chemical reactivity, change surface free energy, change atomic structure, and size-quantization effects can occur (Wigginton et al., 2007). Therefore NPs can exhibit different physical and chemical properties than the same volume of larger particles (>100 nm) of the same material (Gleiter, 2000; Heiligtag and Niederberger, 2013). For example, optical changes occur due to size effects. Gold NPs in suspension are red, whereas larger particles appear golden.

2. Different nanoparticles and their applications

2.1 Natural nanoparticles

NPs are not a new invention. They also exist naturally (Handy et al., 2008; Hochella et al., 2015). Some examples of natural NPs are fractions of particles of incomplete combustion, particles released into the atmosphere by volcanoes, aerosols of sea spray, and fractions of soil colloids and clay minerals (Figure 1, Hochella et al., 2008; Heiligtag and Niederberger,

2013). In addition, because their size is <100 nm, viruses and proteins are considered to be biological NPs (Heiligtag and Niederberger, 2013). It is estimated that biogeochemical processes on the earth's surface produce many thousands of terra grams of inorganic and organic natural NPs (Hochella et al., 2015).

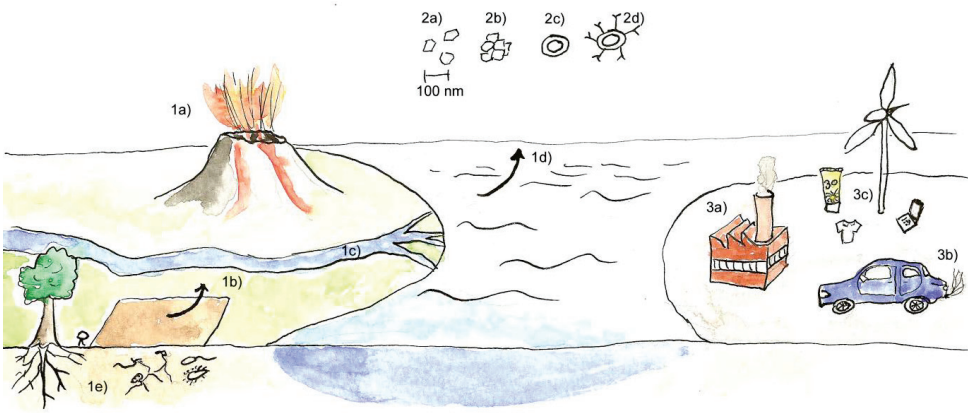

Figure 1: Overview of natural sources of NPs (1), different kinds of NPs (2) and sources of engineered NPs (3). Natural NPs can be formed by 1a) volcano eruptions, 1b) wind erosion of soils and weathering of rocks, 1c) colloids in surface waters, 1d) particles in sea spray and 1e) NPs can be formed by biological processes. NPs can either consist of 2a) single particles, 2b) agglomerates of NPs, 2c) a core with a coating, or 2d) a core with coating and functional groups attached. Part 3 shows sources of engineered NPs. NPs can be released to the environment as a result of 3a) fabrication of NPs, 3b) incomplete combustion, and 3c) due to usage and discarding of NP containing products, such as sun protection creams, clothes, electronic devices, and composite materials.

2.2 Engineered nanoparticles

Humans were already using NPs as pigments, for instance silver NPs in glazed ceramics, in the 9th century (Heiligtag and Niederberger, 2013). However, it was only possible to see NPs

after the development of the electron microscope, and only in the last decades has science started to investigate NPs systematically and discover their huge potential for developing new materials (Heiligtag and Niederberger, 2013). There are many different types of engineered NPs, for example metal(-oxide) NPs, carbon based NPs and semi-conducting NPs. In the case of metal based NPs, they can either be soluble, e.g. Ag NPs, or insoluble as TiO_2 NPs. NPs can be produced from a single material, or they can be designed as a core with a coating and functional groups attached (Figure 1).

There are numerous applications for engineered NPs. For example, they are used in electronic devices and computer storage devices, as composite materials, as well as in batteries, paints, cosmetics, food additives, clothes, and many other products (Piccinno et al., 2012; Heiligtag and Niederberger, 2013). The ten most produced NPs in 2012 were TiO_2, ZnO, SiO_2, FeO_x, AlO_x, CeO_x, CNTs, Fullerenes, Ag and quantum dots (Piccinno et al., 2012). Three of the most produced NPs, i.e. TiO_2 NPs, carbon nanotubes (CNTs) and CeO_2 NPs, are described in detail in sections 2.3 to 2.5.

2.3 Titanium dioxide (TiO_2) nanoparticles

Titanium is an abundant element in soils, with concentrations ranging between 0.1 and 10 % (Scheffer F. et al., 2002). Titanium dioxide comprises parts of different minerals, e.g. Biotite, Amphibole, Ilmenite, Titanite and Titanomagnetite, which occur for instance in metamorphic or volcanic rocks. Three forms of TiO_2 exist, each with a different crystal structure depending on the connections of TiO_6 octahedrals, i.e. anatase, brookite and rutile (Landmann et al., 2012). Rutile orginates from rocks and is the most abundant form of TiO_2. During weathering processes, TiO_2 is not bound to clay but mainly precipitates in form of anatase (Scheffer F. et al., 2002). Brookite is metastable and therefore rare (Landmann et al., 2012).

During the last years, TiO_2 NPs have been industrially produced for use as pigments, photo-catalysts, or in sunscreen products. TiO_2 NPs are photo-catalysts meaning that light induced

molecular transformations can take place on the surface of the catalyst (Linsebigler et al., 1995). Anatase shows a higher photo-catalytic activity than rutile (Luttrell et al., 2014). Due to the photo-catalytic activity of some TiO_2 NPs, reactive oxygen species (ROS, e.g. $HO^{\bullet}$, $O2^{\bullet-}$ and H_2O_2) are produced by the interaction of UV radiation and NPs (Harbour et al., 1985; Uchino et al., 2002). These ROS can undergo further reactions with other compounds. For example, it has been reported that TiO_2 NPs irradiated with UV light can photo-split water into oxygen and hydrogen (Fujishima, 1972; Fujishima and Zhang, 2006) and can act as photo-catalyst to decompose compounds such as cyanide (Frank and Bard, 1977). The photo-catalytic properties of TiO_2 are also useful in solar cells (O'Regan et al., 2002) and in air conditioners for oxidizing contaminants (Obee and Brown, 1995). One specific example of a photo-catalyst is the TiO_2 NP P25 (a mixture of 79% anatase and 21% rutile).

TiO_2 in its non-nano form has a high refractive index and therefore is an effective white pigment with a high covering power. During the grinding of pigments, a fraction of NPs are formed. As white pigments, TiO_2 NPs are found in ceramics, plastic, textiles, and food. In the food industry, E171 (food grade pigment TiO_2 NPs) is used as a pigment in milk, ice cream, mozzarella and chewing gum (Sánchez and Gutierrez-Lopez, 2015). It is estimated that people consume 1 mg titanium kg^{-1} body weight per day (Weir et al., 2012). Yang et al. (2014) reported that it is more likely that E171, rather than P25, is entering the environment because it is used so frequently in food. TiO_2 NPs are mainly produced as pigments, while as photo-catalysts they play a minor role (Piccinno et al., 2012; Keller et al., 2013).

TiO_2 NPs are also used in cosmetics and sunscreen products due to their ability to adsorb and scatter UV light (Wiechers et al., 2013). Because titanium particles >100 nm scatter light, sunscreens containing these larger particles result in a whitish layer on the skin. Decreasing the size to 15 to 50 nm reduces the light scattering but maintains UV protection (Wiechers et al., 2013). Therefore, primarily TiO_2 NPs are used in sunscreens. As cytotoxic ROS can be produced by TiO_2 NPs, TiO_2 NPs in sunscreens are coated, for example, with SiO_2. This

coating reduces ROS production by the TiO_2 core (Smijs and Pavel, 2011). Skin is a good barrier for particles, and several studies have shown that TiO_2 NPs remain on the surface and do not penetrate into the living skin (Dussert et al., 1997; Mavon et al., 2007). Regarding toxicity, TiO_2 NPs in food products and sunscreens have been declared to be non-toxic by the U.S. Food and Drug Administration (Nohynek and Dufour, 2012; FDA, 2014). However, inhaled TiO_2 NPs have been reported to cause chronic inflammation in the pulmonary system (Ferin et al., 1992; Borm and Kreyling, 2004).

2.4 Carbon nanotubes

Carbon nanotubes (CNTs) are tubes consisting of graphene and can either be single walled (SW) or multiwalled (MW) with several layers of graphene. CNTs can be longer than the nano-scale, but their diameter is <100 nm. It is not entirely clear if both SWCNT and MWCNTs occur naturally (MacKenzie et al., 2008), but natural CNTs have been found in a coal petroleum mix (Velasco-Santos et al., 2003). With the current detection methods, it is difficult to analyze SWCNTs in samples and test whether they occur naturally (MacKenzie et al., 2008). CNTs can be formed pyrogenically or as combustion by-products and are part of soot (Nowack and Bucheli, 2007). CNTs are used in a variety of applications, such as rechargeable batteries, composite materials, and thin-film electronics (De Volder et al., 2013). CNTs have a high tensile strength and thermal conductivity and can conduct currents up to 10^9 A cm^{-2} (De Volder et al., 2013). There are health risk associated with the inhalation of CNTs, as several studies have shown that they can cause inflammation in the pulmonary system in mice and rats (Lam et al., 2004; Muller et al., 2005).

2.5 Cerium oxide nanoparticles

Cerium (Ce) is a rare earth metal and one of the most insoluble oxides under ambient conditions (Von der Kammer et al., 2012). Cerium is occurring in the earth's crust in concentrations of 66 µg g^{-1} dry soil in average (Tyler, 2004). In general, between 1 and 270 mg kg^{-1} CeO_2 occurs in European top soils (Von der Kammer et al., 2012). In top soils of

Swedish forests, between 11 and 68 µg of elemental cerium g $soil^{-1}$ has been found (Tyler, 2004). Cerium oxide (CeO_2) in its nano form is used in polishing, catalysts, ceramics, electronics, diesel fuel additives, solid oxide fuel cells, paints, environmental remediation, and biomedicine (Andreescu et al., 2014). CeO_2 NPs have two oxidation states (Ce^{3+} and Ce^{4+}), and the presence of both states in the NP powder contributes to their reactivity (Sun et al., 2012; Andreescu et al., 2014). Similar to TiO_2 NPs, ROS can also be produced on the surface of these particles. Additionally, CeO_2 NPs are also able to reduce ROS, e.g. H_2O_2, to water and oxygen (Andreescu et al., 2014). For biomedical applications, CeO_2 NPs were reported to have the potential to protect cells from damage by radiation (Tarnuzzer et al., 2005). CeO_2 NPs protected healthy human cells from radiation while tumor cells were not protected (Tarnuzzer et al., 2005). Furthermore, CeO_2 NPs have been reported to protect spinal cord neurons from oxidative injuries (Das et al., 2007). As with other NPs, inhalation of CeO_2 NPs can cause health problems, as. several studies have shown that CeO_2 NPs lead to inflammation and cytotoxicity by oxidative stress (Cho et al., 2010; Srinivas et al., 2011).

3. Release of nanoparticles to the environment

Production and use of NPs and NP containing materials often results in their unintentional release into the environment (Figures 1 and 2). Because quantification and characterization of NPs in soils is challenging (Handy et al., 2008; Von der Kammer et al., 2012), estimates of environmental concentrations are based on information about production rates and scientific studies which have quantified NPs and investigated how they change in the environment over time (Gottschalk et al., 2013; Keller et al., 2013; Sun et al., 2014; Wang et al., 2016). Estimated concentrations of NPs in the environment vary depending on the material and the environmental compartment, e.g., air, water and soil. TiO_2 is, in addition to SiO_2, the most produced NP worldwide (78000 t y^{-1} in 2010), and therefore high environmental concentrations are expected compared to NPs produced in lower quantities (Keller et al., 2013). The global input of NPs into soil is estimated to be 38’200, 500 and 1’400 t per year, for TiO_2 NPs, CNT and CeO_2 respectively (Keller et al., 2013). For Europe, the estimated

input of TiO_2 NP is 0.13 and 1'200 µg kg^{-1} soil y^{-1} for natural and sludge treated soils, respectively, and the estimated CNT input is 5.1 and 999 ng kg^{-1} y^{-1} (Figure 2, Sun et al., 2014).

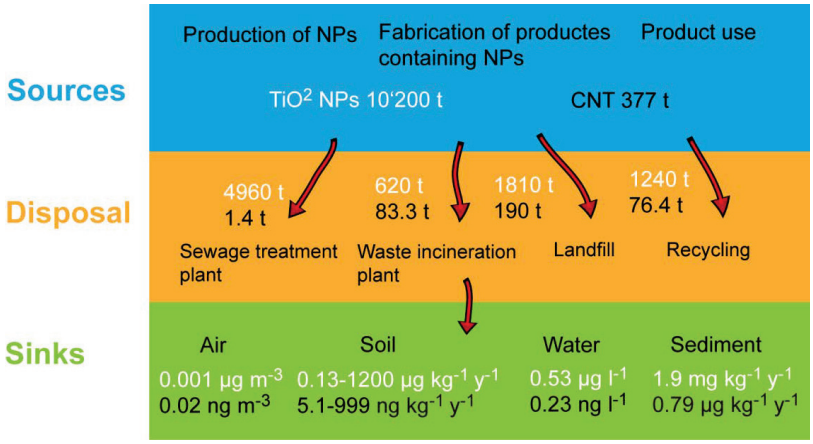

Figure 2: Production, disposals and sinks for TiO_2 NPs (white) and CNTs (black) for Europe for the year 2012 based on the study of Sun et al. (2014). The numbers in the sources section indicate the amount of NPs produced in 2012 (TiO_2 in white, CNT in black), and their material flow to different disposal locations are indicated in the disposal section. Numbers in the sink section indicate modes of estimated environmental concentrations of NPs (TiO_2 in white, CNT in black) for the air and surface water, as well as yearly concentration increases in sediments and soils. For soils, a concentration range is shown depending on whether or not sewage sludge is applied.

These expected environmental concentrations are based on modeled calculations. Measuring the concentration of NPs in environmental samples can be challenging depending on the matrix, (air, water, and soil) and the elementary composition of the NP (Handy et al., 2012; Von der Kammer et al., 2012). In air, NPs can be detected by their size, but concentrations need to be above the detection threshold. In water, the particle size can be

detected by dynamic light scattering or electron microscopy, and concentrations can be assessed if the background concentration of the element of the investigated NPs is low. However, NPs tend to agglomerate in water, and therefore diameters of agglomerates are often measured by dynamic light scattering instead of single particle sizes (Von der Kammer et al., 2012). Depending on the material, it is not possible to distinguish natural NPs from the manufactured ones. Soil is a very complex matrix, and thus the detection of NPs is even more challenging than in liquids. Soils are very heterogeneous and possess different chemical and physical properties, which influence the fate of NPs in soils. Depending on the type, NPs can leach from soils and be transferred to freshwater or be bound to soil particles (heteroagglomeration) and thus accumulate over time (Fang et al., 2009; Nowack et al., 2012; Cornelis et al., 2014). Currently, for TiO_2 and CeO_2 NPs, only indirect detection and quantification by determining total elemental titanium or cerium concentration is available. Analyzing and characterizing TiO_2, CeO_2 NPs and CNTs in soils is difficult. Additionally, more specific analytical methods are needed to be able to detect and quantify NPs in environmental samples at environmental concentrations in order to support ecotoxicological tests and assess the risk of engineered NPs (Hassellöv et al., 2008; Von der Kammer et al., 2012).

4. Ecotoxicological concerns over nanoparticles

NPs are released into the environment (see section 3) by the usage and discarding of NP-containing products, and therefore it is important to test whether they influence the environment. Earlier studies have observed that effects of NP on plants in hydroponic systems depended on the NP type, as well as on the plant species investigated (Foltete et al., 2011; Larue et al., 2012a; Fan et al., 2014). For example, TiO_2 NPs affected wheat root elongation when exposed to 100 mg l^{-1} for one week, while rape seed under the same conditions was unaffected (Larue et al., 2012c). NP type also plays a role in how strongly plants are affected in soils, as shown for Ag NPs, MWCNTs and Cu NPs on zucchini biomass (Stampoulis et al., 2009). Fan et al. (2014) observed that not only plants are

affected in hydroponic systems, but also the interaction between plants and microbes. Pea growth and nitrogen fixation by symbiotic rhizobia was assessed and revealed a delayed nitrogen fixation in the presence of TiO_2 NPs (Fan et al., 2014). Results of hydroponic systems are not directly comparable with soil systems because of the highly different environment (particles in liquid vs. solid environments). A few studies have conducted NP exposure experiments in soil systems, and contrasting effects of NPs were found, with the results again depending on plant species, soil type and NP type (Du et al., 2011; Priester et al., 2012; Feng et al., 2013; Burke et al., 2014). Additionally, it has been shown that community structures of microorganisms (Ge et al., 2011; Ge et al., 2012; Frenk et al., 2013; Shrestha et al., 2013; Burke et al., 2014) and interactions of microorganisms with plants can be altered by NPs in soils (Priester et al., 2012; Feng et al., 2013).

A wide range of studies investigating the ecotoxicity of NPs have been conducted in aquatic ecosystems, but few studies have focused on soils. This may be because it is easier to conduct chemical analytics of NPs in aquatic systems than in soil systems (Handy et al., 2012). In order to study the impact of NP on terrestrial plants, several studies have used hydroponic and liquid cultures as model systems. The matrices, i.e., growth media, are less complex than soil, and investigations on NP concentrations and agglomeration are possible. NPs are often better suspended in aqueous media at extreme pH levels and at low ionic strength. However, good growth conditions for plants and soil microorganisms are at pH around 7, and ionic strength is increased because plants and microorganisms need nutrients. Under these conditions, NPs tend to agglomerate and sediment (Allouni et al., 2009). With help of stabilizers, such as natural organic matter (NOM), NP agglomeration and sedimentation decreases in growth media at pH around 7 (Chowdhury et al., 2012). However, such additives need to be included in control treatments during experiments to exclude the possibility that these additives affect the organisms (Handy et al., 2012).

To be able to estimate the risk of NPs in soils, more studies in soil systems are needed that assess different species and NPs in the same system under the same conditions to make studies comparable. For this, standardized tests need to be developed for determining the ecotoxicity of NPs. OECD provides several tests for assessing the ecotoxicity of numerous chemicals. However, NPs have different specificities than other chemicals, and tests might need to be adjusted for this (Kühnel and Nickel, 2014; OECD, 2014). Currently, no standard testing system exists for assessing potential effects of NPs on plants, soil microorganisms, and their interaction.

5. Nanoparticles in agricultural ecosystems

Nanoparticles are released into soils (including agricultural soils, see section 3) and have been reported to affect plants and soil microorganisms under certain conditions (Section 4). As mentioned in Section 3, application of sludge as fertilizer can further increase the concentration of engineered NPs in agricultural soils (Sun et al., 2014). Humans depend on agricultural ecosystems for food production, and therefore it is important to assess whether engineered NPs released into agricultural ecosystems affect soil health as well as crop quality and quantity.

An additional source of introduced NPs into soils is the application of NP-containing agrochemicals. Agrochemicals can affect the environment, including soil life, negatively, (Johnsen et al., 2001; Howe et al., 2004; Rattner, 2009; Guilherme et al., 2012). Applying lesser amounts of agrochemicals with an increased efficiency could potentially reduce the impact on the environment and would have economic advantages. To increase the efficacy of agrochemicals, nano-based agrochemicals are in consideration to be used. Because of their enhanced reactivity, they could be applied in lesser amounts, or biodegradable polymers could be used as nano-carriers of active compounds (Kah and Hofmann, 2014; Mastronardi et al., 2015; Servin et al., 2015; Subramanian et al., 2015). By using agrochemicals encapsulated in NPs, the release of active compounds can be time controlled

(Nair et al., 2010; Khot et al., 2012; Servin et al., 2015). For example, nanomaterials, e.g. nano-clay, can retain nutrients and serve as a long term reservoir for nutrients (Servin et al., 2015). Subsequently, the nutrient flux to the plants can be synchronized with plant uptake, and nutrient leaching can be reduced. As another example, some NPs, e.g., TiO_2, Ag and ZnO NPs, have been reported to suppress plant diseases due to their antimicrobial activity (Servin et al., 2015), and bactericidal effects have been reported for these NPs (Fabrega et al., 2009; Jiang et al., 2009; Simon-Deckers et al., 2009; Li et al., 2011; Bandyopadhyay et al., 2012; Ge et al., 2012; Rodrigues et al., 2012; Rousk et al., 2012; Lin et al., 2014). In publications and patents for NP- containing agrochemicals, the following NPs are used: lipid/polymer nano emulsions, TiO_2, Al, CuO, ZnO, CNTs, Ag, SiO_2 and sulfur (Gogos et al., 2012), but rare earth element oxides such as CeO_2 can also be found in patents for fertilizer production (Servin et al., 2015). Until now, there have only been a few plant protection products and fertilizers on the market that include NPs in their formulation (Mastronardi et al., 2015). However, because of the increasing number of patents (Gogos et al., 2012; Mastronardi et al., 2015), more of these NP-based products may soon appear on the market. The application of NP based agrochemicals would increase the environmental concentrations of engineered NPs and thus could also cause adverse effects on the environment. Therefore, suitable experimental systems and model-organisms are needed to test whether engineered NPs affect agricultural ecosystems.

6. Potential model-organisms for testing NPs in agricultural ecosystems

Most studies investigating potential effects of NPs in agricultural systems have focused either on plants or microorganisms. However, plant-microorganism interactions provide important ecosystem functions, e.g. nitrogen fixation (van der Heijden et al., 2015a). Therefore, it is important to consider potential effects of NPs on the symbiosis between plants and microorganisms when assessing the ecotoxicity of NPs. Legumes are interesting as potential model plants because they interact symbiotically with both fungi and bacteria. Additionally,

crops important for human nutrition, such as wheat, should be considered for testing whether NPs affect plants. One example is red clover (*Trifolium pratense*) which forms a symbiosis with rhizobia and arbuscular mycorrhizal fungi. Red clover, is indigenous to Europe, the Near East, North Africa and central Asia (Boller et al., 2010) and widespread in grasslands. It is protein-rich during the growing season and is therefore important for animal nutrition (Boller et al., 2010) and often cultivated in grasslands in Switzerland.

Red clover forms a symbiosis with *Rhizobium trifolii*. Rhizobia are a group of beneficial soil organisms that form a symbiosis with legumes (Fabacea) and are able to biologically fix nitrogen. For example, *R. trifolii* associated with red clover can fix more than 300 kg N ha^{-1} y^{-1} (Carlsson and Huss-Danell, 2003), making clover also useful as a green manure. Signaling between the two partners is important for forming the symbiosis and is thoroughly reviewed in Oldroyd et al (2013), Heidstra et al. (1996) and D'Haeze et al. (2002). Briefly, legumes secret a specific flavonid that attracts rhizobia and activates their NodD gene (Goethals et al., 1992; Maj et al., 2010). Transcription of the nod gene then begins and Nod factors (lipo-oligosaccharides) are produced (Stokkermans et al., 1995). This nod factor then triggers the root hairs to deform and build infection threads where the bacteria penetrate the root cortex (Stokkermans et al., 1995; Gehring et al., 1997). Division of the root cortex and pericycle cells leads to the formation of nodules (Heidstra and Bisseling, 1996). At the same time, rhizobia differentiate into bacteroids, and in this form they are able to fix nitrogen (Gardiol et al., 1987). Legumes provide amino acids and dicarboxylic acids to the rhizobia in exchange for ammonium and aspartate (Lodwig et al., 2003). For assessing nitrogen fixation in plants, the isotopic ratio of ^{15}N and ^{14}N can be measured (Unkovich et al., 2008). Atmospheric nitrogen (N_2) contains a constant fraction of ^{15}N of 0.3663%. Soil mineral nitrogen contains a fraction of ^{15}N isotopes which is commonly 0.001-0.007% above the ratio of ^{15}N in air and differs among soils (Unkovich et al., 2008). Plants utilizing only soil nitrogen have the ^{15}N signature of that soil, but for legumes with nitrogen fixation, this ^{15}N signature is diluted and is in the range between that of the soil and the air (Unkovich et al., 2008). By adding additional

^{15}N to soils, this difference can be increased (Unkovich et al., 2008).This makes it possible to assess the amount of biologically fixed nitrogen in plants.

More than 80% of all terrestrial plants, including red clover and wheat, form a symbiosis with soil fungi called arbuscular mycorrhizal fungi (AMF) (Wang and Qiu, 2006; van der Heijden et al., 2015b) AMF belong to the phylum Glomeromycota (Schüssler et al., 2001). AMF germinate from spores, and their mycelial growth is stimulated by root exudates like strigolactones. Hyphae penetrate plant roots and form characteristic structures in the root cortex, i.e. arbuscules and vesicles. Arbuscules are important for nutrient exchange, and vesicles act as storage organs. Plants supply AMF with carbon compounds in exchange for phosphorus, nitrogen and micronutrients like Zn and Cu (Smith and Read, 2008) . Besides the nutritional benefits for the host plant, AMF can also alleviate biotic and abiotic stresses like plant pathogens, heavy metals, drought and high salt concentrations (Azcón-Aguilar and Barea, 1996; Augé, 2001; Hildebrandt et al., 2007; Estrada et al., 2013). Furthermore, they can have a positive effect on soil structure and water retention (Augé, 2001; Rillig, 2004). Thus, AMF provide ecosystem services, which highlights their importance for terrestrial and agro-ecosystems.

Not only symbiotic microorganisms, but also microorganisms in general, should be assessed for NP-induced changes in their community. Soil microorganisms provide important ecosystem functions such as nutrient cycling, regulation of microclimate and local hydrological processes (Altieri, 1999; Doran and Zeiss, 2000). However, besides the beneficial aspects, soil microorganisms can also be pathogens (Weller, 1988). Microorganisms can be assessed in soil by their community structure (Hartmann and Widmer, 2006). Disturbances of the soil like the presence of heavy metals (Frostegård et al., 1996), agricultural farming practices (Hartmann et al., 2015), physical soil disturbance (tillage) (Feng et al., 2003) and NPs have been reported to change microbial community structure (Ge et al., 2012; Frenk et al., 2013; Judy et al., 2015).

In this thesis, ecotoxicological testing systems are developed to assess whether engineered NPs affect red clover, wheat, *R. trifolii*, AMF and soil microorganisms. Additionally, these testing systems are compared and evaluated for their suitability for ecotoxicological testing of engineered NPs.

7. Project NANOMICROPS

This thesis is part of the project NANOMICROPS (Effects of NANOparticles on beneficial soil MIcrobes and CROPS) of the Swiss National Science Foundation project "Opportunities and risks of nanoparticles" (NRP 64). The goal was to assess whether agriculturally relevant NPs affect beneficial soil microorganisms and crops, and what the fate of these NPs is. To achieve this, two PhD projects were performed. One focused on the chemical analytics of nanoparticles in environmental samples (Gogos, 2015) and the other one, this PhD thesis, investigates the ecotoxicology of NPs in model agricultural systems. We assessed TiO_2 NPs, CNTs and CeO_2 NPs because of the release of these NPs to soils and other ecosystem compartments due to their high production and widespread use, in addition to their relevance in agriculture (e.g. application together with agrochemicals). As mentioned in section 3, detection, quantification and characterization of NPs in environmental samples is challenging. Therefore, we developed testing systems for NPs in which both ecotoxicological testing as well as characterization of the NPs could take place. We started the tests with a TiO_2 NP exposure experiment of *R. trifolii* in liquid cultures and hydroponic systems (Chapter II), followed by the investigation of effects of different NPs on red clover grown in soil (chapter III), effects of TiO_2 NPs on wheat and soil microbial communities (Chapter IV), and effects of different NPs (TiO_2, CeO_2 and CNT) on soil microbial communities (Chapter V). In all three testing systems we used the same TiO_2 NPs (P25) and red clover variety to facilitate comparisons across experiments. In the soil experiment, we also used an additional plant species, i.e. wheat, as well as additional NPs (CeO_2 NPs and CNTs). The goal of these experiments was to assess if (1) agriculturally relevant bacteria (*R. trifolii*) are affected by NPs, (2) if red clover and wheat are affected by NPs, (3) if community structure of

microorganisms is altered by NPs, (4) if symbioses between the tested plant species and AMF and *R. trifolii* (red clover only) are affected and (5) if the results obtained in hydroponic systems is comparable to soil systems. Finally, a general discussion about testing systems for NPs on plants and soil microorganisms is presented (Chapter VI, Figure 3).

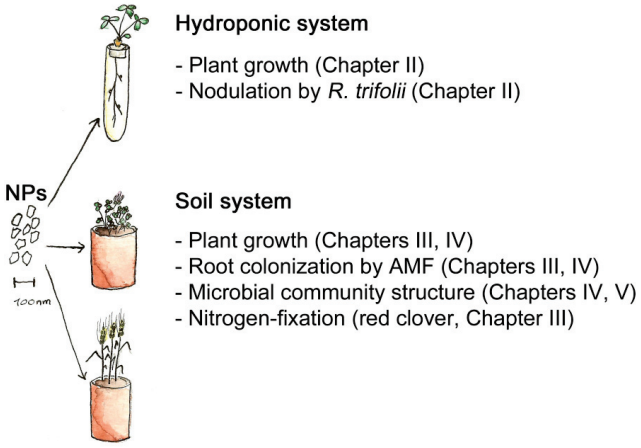

Figure 3: Overview of experiments performed in this thesis. Experiments with hydroponic and soil systems using red clover and wheat have been performed. The measured endpoints and the chapters where the experiments are presented are indicated.

Chapter II

Effects of titanium dioxide nanoparticles on red clover and its rhizobial symbiont

Janine Moll[1,2], Annette Okupnik[1,3], Alexander Gogos[1,4], Katja Knauer[5], Thomas D. Bucheli[1], Marcel G.A. van der Heijden[1,2,6], Franco Widmer[1]

[1] Institute for Sustainability Sciences ISS, Agroscope, Zurich, Switzerland

[2] Plant-Microbe-Interactions, Department of Biology, Utrecht University, Utrecht, the Netherlands

[3] Department of Ecotoxicological Impact Research and Ecotoxicology, Technische Universität Berlin, Berlin, Germany

[4] Departement Process Engineering, eawag, Duebendorf, Switzerland

[5] Federal Office for Agriculture FOAG, Berne, Switzerland

[6] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland

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1. Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) are in consideration to be used in plant protection products. Before these products can be placed on the market, ecotoxicological tests have to be performed. In this study, the nitrogen fixing bacterium *Rhizobium trifolii* and red clover were exposed to two TiO_2 NPs, i.e., P25, E171 and a non-nanomaterial TiO_2. Growth of both organisms individually and their symbiotic root nodulation were investigated in liquid and hydroponic systems. While 23 and 18 mg l^{-1} of E171 and non-nanomaterial TiO_2 decreased the growth rate of *R. trifolii* by 43 and 23% respectively, P25 did not cause effects. Shoot length of red clover decreased between 41 and 62% for all tested TiO_2 NPs. In 21% of the TiO_2 NP treated plants, no nodules were found. At high concentrations certain TiO_2 NPs impaired *R. trifolii* as well as red clover growth and their symbiosis in the hydroponic systems.

Keywords

Nanoparticle, plant, microorganisms, ecosystem services, agriculture

2. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) are manufactured worldwide at an estimated quantity up to 88'000 t y^{-1}, making them one of the most used NPs (Keller et al., 2013). TiO_2 NPs are used for instance in cosmetics, plastics and paint (Robichaud et al., 2009; Gottschalk et al., 2010; Piccinno et al., 2012). Also in food TiO_2 particles are used for white coloring and are labeled in Europe as E171 independent of a certain particle size (European Food Safety Authority, 2004). These applications of TiO_2 NPs have resulted in considerable releases into the environment. Due to the larger quantities applied food grade TiO_2 NP pigments (e.g. E171) have a higher probability to enter the environment than photocatalysts (e.g., P25) (Weir et al., 2012; Yang et al., 2014). In Europe it has been estimated that TiO_2 NP inputs into soils may reach 0.13 µg kg^{-1} y^{-1} and, if sewage sludge is applied, may be as high as 1200 µg kg^{-1} y^{-1} (Sun et al., 2014). Because of their photo-protective and photocatalytic properties, TiO_2 NPs are also considered for use in plant protection formulations to modify the lifetime of active ingredients (Gogos et al., 2012; Khot et al., 2012). Future application of plant protection formulations could result in estimated additional TiO_2 NP input into soils ranging from 3 to more than 5000 µg kg^{-1} y^{-1} (Gogos et al., 2012; Khot et al., 2012; Kah et al., 2013). Therefore, it is important to determine possible effects of TiO_2 NPs on plants, soil organisms and ecosystem functions as basis for an environmental risk assessment.

Legumes and their nitrogen fixing bacterial symbionts are important providers of nitrogen in agricultural systems, representing a central ecosystem service (Herridge et al., 2008). To perform nitrogen-fixation a complex sequence of signaling between rhizobia and plants takes place, which results in morphological alterations of root hairs and nodule formation (Heidstra and Bisseling, 1996). An important legume is red clover (*Trifolium pratense*), which is used as fodder crop and green manure due to its symbiosis with the nitrogen fixing bacterium *Rhizobium trifolii*. Up to 373 kg nitrogen ha^{-1} y^{-1} can be fixed by the symbionts *R. trifolii* and *T. pratense* (Carlsson and Huss-Danell, 2003). The importance of legumes for agricultural

systems is expected to increase in the future because legumes increase nitrogen availability in soil and reduce the reliance on mineral nitrogen (N) fertilization (Carlsson and Huss-Danell, 2003). Effects of TiO_2 NPs on nitrogen-fixation have been reported for other legume-rhizobia models such as pea (Fan et al., 2014) and barrel clover (Chen et al., 2015). For these reasons, it is important to investigate whether TiO_2 NPs have adverse effects also on other symbiotic legume-rhizobia interactions such as e.g., red clover and *R. trifolii*

Hydroponic systems are suitable to assess plant development under highly controlled conditions. In particular, exposure to NPs can more easily be controlled and effective NP concentrations and particle size can be determined over time, which is of importance when assessing effects on plant performance (Handy et al., 2012). However, many NPs tend to aggregate and sediment in growth media depending on, e.g., NP concentration, pH, ionic strength, humic acid and protein content of the medium (Allouni et al., 2009; Chowdhury et al., 2012). Therefore it is important to determine the actual exposure concentration and the NP quality during exposure (Harris et al., 2014). Various studies have reported experiments with TiO_2 NPs in hydroponic systems, which have revealed contrasting effects on plant growth and biomass production and nitrogen fixation (Castiglione et al., 2011; Larue et al., 2012a; Jacob et al., 2013; Fan et al., 2014; Frazier et al., 2014). These effects may depend on plant species as well as NP types, concentrations, and qualities. To the best of our knowledge, effects of TiO_2 NPs on the important fodder crop red clover and its symbiosis with *R. trifolii* have not been assessed yet.

In this study, we used a liquid culture system to assess growth of *R. trifolii* exposed to different TiO_2 NPs. We then developed a small scale hydroponic system to assess the impact of TiO_2 NPs on red clover and root nodulation by *R. trifolii*. E171 (100% anantase) was chosen because food grade TiO_2 NPs have the highest probability to get released to the environment (Weir et al., 2012; Yang et al., 2014). As a non-nano material (European Commission, 2011) we chose an anatase particle with average particle size larger than 100

nm (non-nanomaterlal (NNM) TiO_2). To assess whether a fraction of rutile crystal structure changes potential effects, we also included P25 (20% rutile, 80% anatase) in our assessment. TiO_2 NPs can be dissolved at low pH (pH<3). However, at pH between 3 and 8 no ions were detected as shown for P25 (Aller et al., 1990; Schmidt and Vogelsberger, 2006). We chose $ZnSO_4$ as a positive control because it has been reported to affect plant growth (Ebbs and Kochian, 1997; Kaya et al., 2000). We aimed to determine whether (1) TiO_2 NP concentrations and qualities changed over the duration of the experiment, and whether (2) growth rate of *R. trifolii*, (3) growth of red clover, and/or (4) nodule formation by *R. trifolii* on clover roots are affected.

3. Material and Methods

3.1 Nanoparticles

TiO_2 NPs were P25 (80% anatase, 20% rutile, Sigma-Aldrich, USA, Art. No. 718467) and Hombitan FG, which we refer to as E171 (100% anatase, Sachtleben Pigments, Germany). Additionally, a NNM TiO_2 preparation (100% anatase, Sigma Aldrich, Art. No. 232033) was chosen as non-nano material (European Commission, 2011) containing less than 50% NPs (size distribution). All of these TiO_2 NPs and the NNM TiO_2 were uncoated. As a positive control, $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich) was used. Size distributions of primary TiO_2 NPs were measured by transmission electron microscopy (TEM). For this, TiO_2 NPs, i.e., P25, E171 and NNM TiO_2, were suspended in MQ water (Milli-Q Gradient A10, Millipore Corporation, Molsheim, France) by sonication in an ultrasonic bath (Sonorex digital 10 P, Bandelin, Germany) for 30 min at 720 W. A drop of the resulting suspension was then air-dried on a formvar/carbon coated TEM grid (Plano, Wetzlar, Germany) and visualized using a Tecnai G2 Spirit transmission electron microscope (FEI, Delmont, PA, USA). Electron micrographs were analyzed with ImageJ (Supplementary Information 1) (Rasband, 1997-2014). P25 particles were the smallest particles with an average diameter of 29±9 nm (n=92) confirming the manufacturer's specification of 21 nm. The size of E171 and NNM TiO_2 were on average

92±31 nm (n=52) and 145±46 nm (n=49), respectively. NPs, i.e., particles with at least one dimension below 100 nm, were 100% for P25, and 69% for E171. NNM TiO_2 contained 20% NPs and thus is referred to a non-nano material (European Commission, 2011). No larger particle sizes for the NNM TiO_2 control were chosen, because suspended particles needed to be stable over time for the exposure experiments. Using E171 and NNM TiO_2 allowed us to compare a nano-material with a non-nano-material.

3.2 Preparation of NPs

Because the growth media used needed to be sterile, surface sterilization of the NPs was performed. TiO_2 NPs (5 mg and 2.5 mg for liquid cultures and hydroponic system, respectively) were sterilized in 70% ethanol (0.4 ml) for 1h at 60 °C. TiO_2 NPs in ethanol were transferred antiseptically with a pipette to Schott bottles containing 100 ml yeast mannitol broth (YMB) for *R. trifolii* liquid cultures or Fåhraeus medium (FM) for hydroponic cultures (Somasegaran and Hoben, 1994). For controls without NPs, 0.4 ml 70% ethanol were added. Natural organic matter (40 mg l^{-1}, NOM, IHSS Suwannee River, RO isolation 2R101N, USA) was added to both media. The amount of NOM suitable for stabilization of the suspensions in our systems was tested using a concentration series of NOM in advance of the presented experiments. For better initiation of plant growth and assessment of nitrogen uptake in plants, KNO_3 (0.001 M, 4% ^{15}N, Cambridge Isotope Laboratories, USA) was added to the FM. Media were sonicated for 1 h at 720 W. The suspensions for the *R. trifolii* liquid cultures were sedimented for 24 h, and 50 ml of the supernatant was diluted to the final concentrations (1:0, 1:3, 1:9 and 1:27) and used for exposure experiments. For the hydroponic cultures, the NP containing medium (FM) was directly diluted (1:0 and 1:1 with FM) and used after sonication. The actual concentration of the undiluted NP suspensions was determined as total titanium from 3 ml of the suspensions (n=3) by ammonium persulfate digestion as described by Khosravi et al. (2012).

3.3 *Rhizobium trifolii*

The nitrogen fixing bacterium *R. trifolii* 30141 (DMSZ, Germany; NCBI Gen Bank AY509900.1) was used for the experiments. We selected for rifampicin resistance on yeast mannitol agar (YMA, (Somasegaran and Hoben, 1994)) by sequential plating on increasing concentrations of rifampicin up to 250 µg ml^{-1} (Glandorf et al., 1992). Resistant *R. trifolii* were grown in yeast mannitol broth (YMB) at 26 °C and 150 rpm for 5 d (Somasegaran and Hoben, 1994). *R. trifolii* were stored in 15% glycerol at -70 °C until use.

3.4 Exposure of *R. trifolii* in liquid cultures

Effects of TiO_2 NPs on *R. trifolii* growth rate in YMB were assessed similar as in the study of Bandyopadhyay et al. (2012) by measuring optical density (OD) at 620 nm using a spectrophotometer (Infinite F200, TECAN, Maennedorf, Switzerland). Controls without *R. trifolii* inoculation but the same NP concentrations as the treatments with *R. trifolii* were used for background OD determination. Background OD was subtracted from the OD of the samples with *R. trifolii* inoculation. Temperature was set to 26 °C for optimal growth of *R. trifolii* (150 rpm, dark conditions, n=4). *R. trifolii* was exposed for 32 h and subsamples for OD measurements were taken at t=0 and from 26 h on every second hour. From each exponential part of the growth curve (S1 Supplementary Information) a linear regression of ln-transformed OD over time was applied for determination of the growth rate.

3.5 Red clover

Red clover (*Trifolium pratense* var. Merula) was used for the hydroponic experiments. Seeds were surface sterilized (10 min in 3% bleach and 5 min in 70% ethanol) and put into a hydroponic system adapted from Tocquin et al. (2003). Seeds were germinated in 200 µl pipet tips from which the front part was removed, and which were filled with 0.65% agar and 100 µg ml^{-1} rifampicin in an autoclaved, water filled pipet tip box in a growth chamber for 7 d (day: 16 h at 20 °C and 250 µmol m^{-2} s^{-2} light, night: 8 h at 15°C, humidity 95%). Seedlings of similar height and root length were selected for the hydroponic experiment.

3.6 Exposure in the hydroponic system

Effects of TiO_2 NPs on red clover and symbiosis with *R. trifolii* were assessed in a hydroponic system (n=6) consisting of test tubes (16 mm x 150 mm) containing 20 ml of the TiO_2 NP suspensions in FM. All of the used TiO_2 NP concentrations caused turbidity of the medium (S1 Supplementary Information). Treatments with *R. trifolii* were inoculated with 1 ml of an overnight culture in YMB ($2x10^7$ cells ml^{-1}). Seedlings of red clover were transferred to the hydroponic system, and fixed with cotton. A cannula was inserted to allow addition of water and air with a syringe. Tubes were wrapped in aluminum foil to exclude light and hydroponic cultures were placed in a growth chamber for 28 days (16 h 20 °C and 250 µmol m^{-2} s^{-1} light day and 8 h at 15°C night, humidity 95%). The medium was not mixed during exposure but was replaced weekly. Plants were watered with autoclaved water when the water level dropped below the end of the pipet tip. At harvest, roots were rinsed with deionized water and separated from the shoot. Main shoot and root length were measured, and the number of secondary roots, root tips, and nodules were counted. For determination of dry weight, shoots and roots were dried at 70°C until weight constancy. Shoots were ground in a ball mill (MM301, Retsch, Haan, Germany), and 2 mg shoot powder per sample were used for determination of ^{14}N and ^{15}N content (Isotope Ratio Mass Spectroscopy, Stable Isotope Facility of the University of Saskatchewan, Canada) as described by Arcand et al. (Arcand et al., 2013). In a further experiment, randomly selected nodules of six controls and six E171 treated red clover plants with and without inoculation of *R. trifolii* were surface sterilized and crushed on YMB agar (Somasegaran and Hoben, 1994). If colonies were formed, they were plated on YMB agar containing 150 µg ml^{-1} rifampicin.

3.7 Actual NP quality and concentrations in growth media

To verify whether the added quantities of TiO_2 corresponded to the calculated TiO_2 NP concentration, we measured the actual exposure of TiO_2 NPs in the growth media, both for liquid cultures with *R. trifolii* growth and the hydroponic culture experiment. Total elemental titanium was determined in three ml suspension. Ammonium persulfate digestion (Khosravi

et al., 2012) was used and concentration was determined with inductively coupled plasma optical emission spectroscopy (Khosravi et al., 2012) (ICP-OES: Spectro Arcos, Spectro, Germany). For the hydroponic system, this was repeated at every medium change, and for the *R. trifolii* liquid culture experiment, where the suspension was continuously mixed, the concentration was measured at the beginning of the experiment. Particle size and zeta potential (dynamic light scattering, DLS, Zetasizer Nano, Malvern Instruments, Germany) of the stock suspensions were determined at every medium change for the hydroponic experiment and at the beginning and end of the *R. trifolii* growth experiment to monitor agglomeration of NPs. Stability of the concentration of suspended TiO_2 NPs in the hydroponic system, was determined after 18, 24, 42, 114 and 162 h for the top part (17 ml), where the roots were growing, and the bottom part (3 ml).

Coverage of roots with TiO_2 NPs was estimated by analyzing scanning electron microscopy images (SEM) by applying a 3 µm raster and measuring the area of the TiO_2 NPs within each square (Adobe Photoshop CS4 Extended 11.02). In total, 1117 squares on 10 different SEM images of different E171 treated root sectors and 1324 squares of control roots were analyzed. Sample preparation for SEM is explained in the Supplementary Information (S1 Supplementary Information).

3.8 Statistics

All statistical analyses were performed with R (R Core Team, 2014). For comparing the growth rates of *R. trifolii* liquid cultures, and the plant growth variables in the hydroponic system, a generalized linear model (McCullagh and Nelder, 1989) was applied. P-values were adjusted for multiple testing according to Benjamini and Hochberg (1995). For *R. trifolii* liquid cultures each particle was tested in a separate experiment. Therefore relative growth rates were calculated to be able to compare these experiments. If the model assumptions for using a generalized linear model were not fulfilled (not normally distributed residuals (shapiro.test) and inhomogeneous variances (bartlett.test)), a Kruskal test (kruskal.test)

followed by a Mann-Whitney test (wilcox.test) was conducted. For presence and absence data (e.g., nodules), a test of equal proportions (prop.test) was applied.

4. Results

4.1 Characteristics of TiO_2 NPs in growth media

For assessing the agglomeration of TiO_2 NPs particle size and zeta potential were determined. In YMB the average hydrodynamic diameters of TiO_2 NPs determined with DLS were between 341 and 806 nm. Zeta potentials ranged between -29 and -33 mV (n=3, Table 1). Initial titanium concentrations in YMB stock suspensions ranged from 18 to 24 mg l^{-1} (n=3, Table 1)

Table 1: Analytical data of the TiO_2 NPs suspended in YMB medium. Suspensions for the *R. trifolii* exposure experiment were assessed at the start of the experiment (t=0) and at the end, i.e., after 34 h (n=3).

	initial t=0			after exposure t=34 h	
Treatment	**size [nm]**	**zeta potential [mV]**	**concentration [mg l^{-1}]**	**size [nm]**	**zeta potential [mV]**
P25	806±17	-29±1	23±5	879±174	-28±1
E171	341±3	-31±1	24±2	341±6	-32±2
NNM TiO_2	356±1	-33±1	18±1	346±4	-33±2

In FM average hydrodynamic diameters were between 383 and 1077 nm (n=3, Table 2). Zeta potential was between -21 and -30 mV (Table 2). The initial titanium concentrations of the stock suspensions in FM, ranged between 11 and 27 mg l^{-1} over four weeks (Table 2). While concentrations and zeta potentials revealed a moderate correlation of r=-0.48 (p=0.013), and particle size and zeta potential of r=0.55 (p<0.001), concentration and size of the NPs were not correlated r=-0.09 (p=0.647). A decrease in the starting concentrations was observed in weeks 3 and 4.

Table 2: Analytical data of the TiO_2 NP suspensions in Fåhraeus medium (FM). Suspensions of the hydroponic experiment were explored over four weeks (n=3).

Particle	exposure week	conc.[1] [mg l^{-1}]	average size DLS[2] [nm]	PDI	zeta-potential[2] [mV]
P25	Week 1	27±2	876±46	0.49	-26±0
	Week 2	21±1	1663±248	0.85	-26±0
	Week 3	11	961±182	0.74	-24±0
	Week 4	12±0	1077±98	0.64	-21±1
E171	Week 1	21±4	383±10	0.24	-28±1
	Week 2	23±1	477±14	0.28	-28±1
	Week 3	16±0	524±19	0.39	-29±1
	Week 4	17±3	392±5	0.25	-25±1
NNM TiO_2	Week 1	25±1	394±8	0.25	-30±0
	Week 2	17	446±16	0.29	-28±1
	Week 3	18±1	406±7	0.31	-27±0
	Week 4	17±1	467±21	0.33	-28±1

[1]Concentration of total titanium in the suspension at the beginning of the exposure week (n=3) for the highest concentrations of each particle (2). The lower concentration (1) was diluted 1:1 with FM medium. For weeks two and three, two P25 and two NNM TiO_2 samples were lost during digestion and thus only one sample could be used for determination of the concentration

[2]Stock suspension was measured at every medium change

Sedimentation of the two nanoparticles, P25 and E171, in FM was determined over a 7 day period to monitor how exposure was changing over time. The total amount of titanium in the top part (17 ml) in contact with the red clover roots decreased by 85% for E171 and 98% for P25, when compared to the initial titanium concentration (Figure 1, S1 Supplementary Information). In the bottom part (3 ml) at the end of the 7 d exposure, 59% of the initial amount of titanium was detected for E171 and 80% for P25. P25 sedimented faster than E171 compared to the respective control, which is consistent with the observation of the different particle sizes and zeta potentials (Table 2). Thus, compared to the initial titanium

amounts in both treatments, 26% of E171 and 18% of P25 were not detectable and were most likely attached on the root surface (Figure 2) or the glass tube. The analysis of SEM images of rinsed root samples showed that on average 0.01±0.005 μm^2 E171 covered 1 μm^2 root surface. Additionally, TiO_2 NPs formed a layer of white precipitate on the glass tube. However, its titanium content could not be quantified.

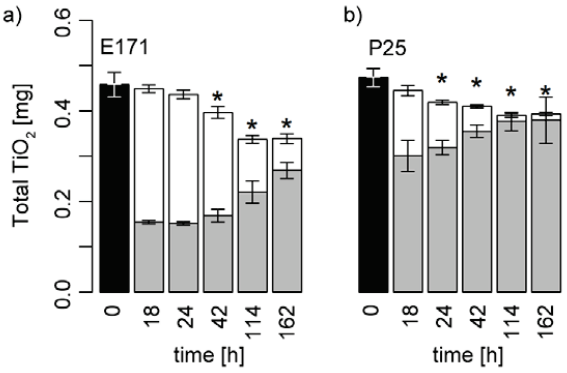

Figure 1. Total TiO_2 suspended or sedimented in the hydroponic system. Red clover was exposed (n=3) over 162 h to the two nanoparticles P25 and E171. TiO_2 amounts of the pooled stock suspension is shown at t=0 in black. TiO_2 amounts of the top (white, 17 ml, in contact with roots) and bottom part (grey, 3 ml, including precipitate) are shown. Differences of the total TiO_2 NP amount (bottom and top part together) to the total Ti amount at t=0 are indicated with asterisks ($p<0.05$). Error bars indicate standard deviations (n=3).

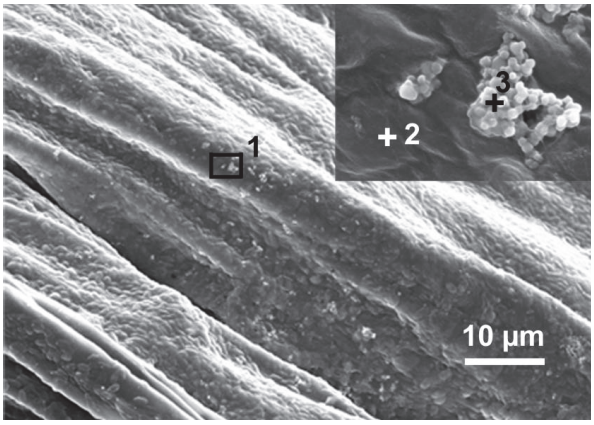

Figure 2. Scanning electron microscopy image of dried red clover root. Root surface from a 24 mg l^{-1} E171 treated plant is shown. The insert shows a magnification of 1, and from the spots 2 and 3 (+) X-ray fluorescence spectra were prepared revealing that spot 2 did not contain titanium while spot 3 contained titanium.

4.2 Effects on *R. trifolii* in liquid cultures

The growth rate of *R. trifolii* was differentially affected by additions of P25, E171 and NNM TiO_2 and was significantly reduced by 43% in average ($p<0.001$) by actual concentration of 23 mg l^{-1} E171 and by 23% ($p=0.035$) in 18 mg l^{-1} NNM TiO_2 treatment (Figure 3, S1 Supplementary Information). The $ZnSO_4*7H_2O$ treatment reduced the relative growth rate in average by 90%. Growth curves are shown in S1 Supplementary Information. The lower concentrations of all treatments did not affect the growth rate compared to the control.

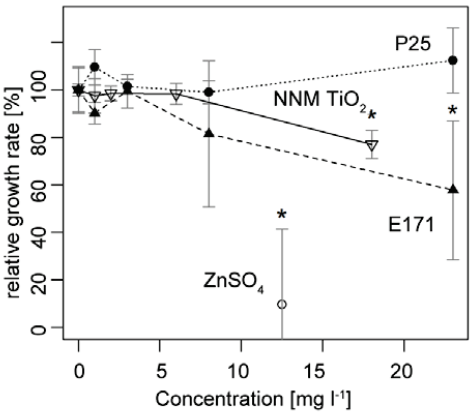

Figure 3: Relative growth rates of *R. trifolii* in YMB medium over a 32 h exposure assessed by optical density. *R. trifolii* growth rates were assessed in medium containing different actual concentrations and qualities of TiO_2 NPs (P25 filled diamond, E171 filled triangle, NNM TiO_2 inverted triangle, $ZnSO_4$*$7H_2O$ circle) during the 34 h (n=4). Stars indicate significant ($p<0.05$) differences from the control. Exponential growth curves are shown in S1 Supplementary Information.

4.3 Effects on red clover and *R. trifolii*

Shoot and root length of red clover plants significantly ($p<0.05$) decreased in all three TiO_2 NP treatments, i.e., P25, E171 and NNM TiO_2, as well as in the $ZnSO_4$ control regardless of the addition of *R. trifolii* (Figure 4). Growth reduction ranged between 41 and 62% for shoots and between 26 and 29% for roots, respectively (S1 Supplementary Information). Root and shoot dry weight significantly ($p<0.05$) decreased between 30 and 44% for roots and 27 and 53% for shoots in average over all TiO_2 NP treatments (S1 Supplementary Information). However, for the two NNM TiO_2 treatments with *R. trifolii*, this reduction of root weight was not significantly different from the control ($p=0.06$ and 0.08). Pearson's correlations between shoot weight and shoot length was moderate with $r=0.67$ ($p<0.001$) and $r=0.62$ ($p<0.001$) with and without *R. trifolii* inoculation, respectively. Root morphology, i.e., number of root tips

and secondary roots divided by main root length, was not affected by any of the treatments when compared to the controls (Table 3).

Table 3: Number of root tips and number of secondary roots of red clover in hydroponic system. Roots were assessed at the harvest (n=6, mean ± standard deviation). Exposure concentrations (1<2) are described in detail in Table 2.

root architecture	Rhizobia[1]	Control	P25 (1)	P25 (2)	E171 (1)	E171 (2)	NNM TiO_2 (1)	NNM TiO_2 (2)	$ZnSO_4$
number of root tips per root length	yes	2.2±0.6	2.7±0.9	3.6±0.4	2.5±0.3	2.3±0.7	2.7±0.8	2.6±1.0	2.2±1.0
	no	2.0±1.0	2.4±1.0	2.1±0.4	2.1±0.4	2.5±0.8	3.0±1.2	1.7±0.4	1.9±0.9
number of secondary roots per root length	yes	1.6±0.4	2.1±0.8	1.8±0.4	2.3±0.3	2.0±0.2	2.1±0.5	2.1±0.7	1.7±0.9
	no	1.6±0.5	2.1±0.7	1.7±0.4	1.8±0.2	2.0±0.6	3.0±0.9	1.5±0.3	1.6±0.7

[1]with or without inoculation of *R. trifolii*

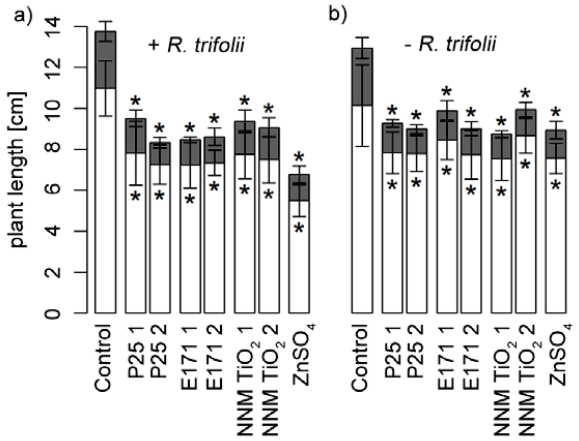

Figure 4: Length of the main root (white) and shoot (grey) at harvest (t=4 weeks). Lengths are shown for the control, the different TiO_2 NP treatments in two concentrations 1 (low) and 2 (high) that are described in Table 2, and the 16.1 mg l^{-1} $ZnSO_4$ treatment (a) in presence of *R. trifolii* or (b) without *R. trifolii.* Significant ($p<0.05$) differences to the respective control (n=6) are indicated with asterisks above the standard deviation error bars for shoots, and below the error bars for roots. The results of the same treatments with and without *R. trifolii* are not significantly different and neither were the controls, but the root length of $ZnSO_4$ with and without *R. trifolii* was different ($p=0.005$).

Red clover formed root nodules in all TiO_2 NP treatments when *R. trifolii* was added. However, the number of nodules decreased significantly ($p=0.02$) by 75% compared to the control when treated with $ZnSO_4$ (S1 Supplementary Information). No nodules were formed in 2 to 3 of the six replications when treated with E171 (2), NNM TiO_2 and $ZnSO_4$. Plants grown without *R. trifolii* also formed nodule-like structures, and at the lower concentration of P25, E171 and NNM TiO_2 their number increased significantly ($p<0.05$), by 120%, 80% and 90% compared to the control. Only one control plant without inoculation of *R. trifolii* formed nodule-like structures, and none was found in the $ZnSO_4$ treatment. To confirm if the nodules were colonized by *R. trifolii*, they were plated on YMB agar. Two control nodules and two

E171 treated nodules, with inoculation of *R. trifolii*, revealed bacterial growth on agar containing rifampicin. This confirms the presence of inoculated rifampicin resistant *R. trifolii*. However, nodule-like structures from treatments without inoculation revealed no bacterial growth on YMB agar without rifampicin. Both, 50% of control and E171 treated plants, which were inoculated with *R. trifolii*, revealed nodule-like structures which formed no bacterial colonies on the agar plates.

^{15}N contents in the shoots decreased in average by 49% in the TiO_2 NP treated plants with addition of *R. trifolii* and 57% without *R. trifolii* compared to the control ($p < 0.001$) (Figure 5). Because of too little biomass, not all replications could be assessed for ^{15}N content. Shoot content of ^{15}N decreased in $ZnSO_4$ treated plants with *R. trifolii* by 34% and by 52% without *R. trifolii* compared to the control (Figure 5). Pearson's correlation of ^{15}N content of shoots and the shoot dry weight was $r=0.61$ ($p<0.001$) for treatments with and $r=0.62$ ($p<0.001$) without inoculation of *R. trifolii*. Shoot length was correlated with the ^{15}N content in shoots and was $r=0.71$ ($p<0.001$) with inoculation and $r=0.88$ ($p<0.001$) without inoculation of *R. trifolii*. The ratio of ^{15}N content and biomass was only significantly decreased in the E171 19 mg l^{-1} treatment (S1 Supplementary Information).

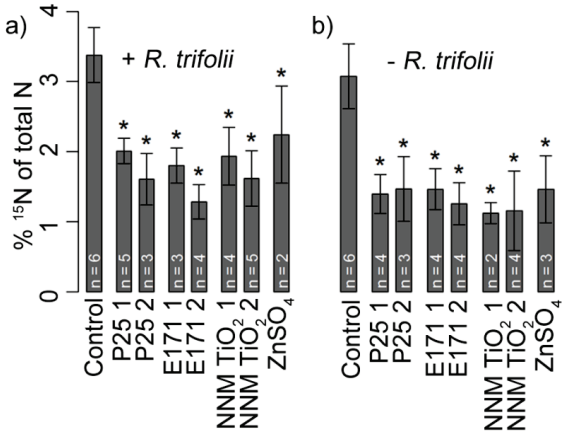

Figure 5: ^{15}N content (% of total N) of red clover shoots. Results are shown for the control, the different TiO_2 NPs in two concentrations (1=low, 2=high) as described in Table 1 and the $ZnSO_4$ treatment (a) with addition of *R. trifolii* and (b) without *R. trifolii* inoculation. Error bars indicate standard deviations, asterisks show significant differences compared to the control ($p<0.05$) and number of replications are indicated on the graph (n). Number of replications varied because not for all samples the required amount of shoot biomass for ^{15}N measurement was available.

5. Discussion

5.1 Nanoparticles in growth media

In this study we investigated the potential effects of two different TiO_2 NPs, i.e., P25, E171, and a non-nanomaterial TiO_2 referred to as NNM TiO_2, on *R. trifolii* growth in liquid cultures as well as on red clover growth and root nodulation in a hydroponic system. These experiments revealed that exposure concentration changed during the course of the incubation as previously reported for other growth media (French et al., 2009; Chowdhury et al., 2012). It has been reported that growth media for plants and bacteria promote agglomeration and thus sedimentation of TiO_2 NPs takes place (French et al., 2009;

Chowdhury et al., 2012). However, in these studies different media and conditions were used and we aimed at determining the actual Ti-concentrations and NP qualities over time in our system. In the *R. trifolii* liquid cultures, the medium was mixed constantly and therefore the concentrations of the suspensions were stable over time. However, in the hydroponic system the medium was not mixed and sedimentation was investigated. We addressed this by periodically changing the medium and determining the actual exposure concentration every week. Even though we applied the same method for the weekly preparations of NP suspensions, it did not always yield the same concentrations. The experimental variation ranged from 11 to 27 mg l^{-1}. Even though the exposure concentration was not constant and lower than the nominal concentration, we found effects on red clover plants in all treatments and were able to relate them to actual concentrations. Contrastingly to the primary particle size (P25<E171<NNM TiO_2) the results showed that P25 formed the largest agglomerates in the growth media while E171 revealed similar agglomerate sizes as NNM TiO_2 assessed by DLS (Tables 1 and 2). P25 sedimented faster than E171 in the hydroponic system (Figure 1 and S1 Supplementary Information). However, actual particle size and actual NP concentration of the suspensions were not correlated ($p=0.647$). Sedimentation of the NPs is also depended on the zeta potential (Fang et al., 2009). The measured zeta potentials were moderately correlated with the actual Ti-concentration ($r= -0.48$, $p=0.013$) and with the measured NP sizes ($r=0.55$, $p<0.001$) but did not explain the whole variation. P25 revealed a less negative zeta potential than E171 and NNM TiO_2 confirming the finding that P25 sedimented faster than E171. It is not known how stable TiO_2 agglomerates are in these systems and which proportion of free NPs are occurring. These free NPs potentially have stronger effects on plants and microbes than the agglomerates.

5.2 Effects of TiO_2 NPs on *R. trifolii* in liquid cultures

We first tested effects of TiO_2 NPs on the growth of *R. trifolii* in liquid cultures before we went to the more complex system with plants and bacteria. In liquid cultures with P25 (up to 23 mg l^{-1}, 806±17 nm, mixture of anatase and rutile) *R. trifolii* growth rate was not affected. The two

anatase preparations E171 and NNM TiO_2 with average agglomerate sizes of 341±3 nm and 356±1 nm, respectively, decreased the relative growth rate. E171 and NNM TiO_2 had different primary particle sizes, i.e., 92±31 and 145±46 nm, but did not reveal differences in affecting *R. trifolii* growth. This is in agreement with the similar agglomerate size of E171 and NNM TiO_2. The increase of OD of the medium containing P25 and controls was not different and therefore was indicative for bacterial growth. Different photocatalytic activities of the anatase particles and the mixture of anatase and rutile might affect plants and bacteria. However, our experiments were conducted under dark conditions and thus effects of reactive oxygen species were excluded. Lin et al. (2014) used another bacterial model species and reported that 25 mg l^{-1} anatase affected the viability of *Escherichia coli* stronger than the same concentration of a mixture of 93% anatase and 7% rutile. The viability has been reduced by 40 and 25%, respectively. However, in the study of Lin et al. (2014), the experiments were performed under natural light conditions and reactive oxygen species were released resulting in stronger effects of smaller anatase particles than larger particles or particles with a rutile crystal structure.

5.3 Effects of TiO_2 NPs on red clover and the formation of root nodules in a hydroponic system

Based on the information that E171 and NNM TiO_2 affected bacterial growth in liquid cultures, we performed experiments in a more complex hydroponic systems using red clover and *R. trifolii*. The shoot as well as root length and weight decreased significantly in TiO_2 NP treatments and the $ZnSO_4$ control. For the $ZnSO_4$ treated plants, this growth reduction was similar to other studies with comparable Zn^{2+} concentrations (Ebbs and Kochian, 1997; Kaya et al., 2000). Different plant species might be affected differently by TiO_2 NPs. In contrast to our investigations of decreased shoot length in TiO_2 NP treatments, no effects on shoot length have been reported for pea when exposed to 250 mg P25 l^{-1} (Fan et al., 2014) and for cucumber even an increase in shoot length at 4000 mg TiO_2 NPs l^{-1} has been observed (Servin et al., 2012). While shoot length, root length and shoot weight decreased in all

treatments in our experiments, root dry weight was not affected by NNM TiO_2 because there was a higher variance between replications. This might be because of the larger primary particle size of NNM TiO_2 compared to E171 and P25, which both decreased root weight. However, the mechanism how these NPs affect root weight is not known. In our experiment with red clover, we did not find effects on the number of secondary roots or the number of root tips as reported for pea (Fan et al., 2014).

In the hydroponic system it was not possible to determine nitrogen fixation because plants from the same treatment with and without nodules revealed the same ^{15}N signature. The number of plants, which did not form nodules when inoculated with *R. trifolii*, were increased in TiO_2 NP treatments, which might result from decreased bacterial growth observed in the liquid cultures. For another legume-rhizobium system (pea and *R. leguminosarum bv. viciae*) Fan et al. (2014) have reported that nodule formation was delayed. Nodulation could also be influenced by adhesion of TiO_2 NPs on root hairs, or TiO_2 NPs might interact with the signaling compounds (flavonoids, lipo-oligosaccharides). Further research is needed to understand the mechanism how TiO_2 NPs affect nodulation because reduction of root nodules would influence nitrogen fixation and thereby an important ecosystem function. Nodules, which revealed bacterial growth on YMB agar, grew also on agar containing rifampicin. This implies that these nodules were colonized by the inoculated rifampicin resistant *R. trifolii* and it was independent of the NP treatment. Interestingly, we found also nodule-like structures in the TiO_2 NP treatments without inoculation of *R. trifolii*. None of the tested nodule-like structures did reveal bacterial growth on YMB agar independent if they originated form E171 treated plants or controls. This implies, that these nodules were either not colonized or responded differently to the surface sterilization than the nodules, which revealed bacterial growth on YMB agar with rifampicin. It has been reported, that low concentrations of nitrogen in a medium can enhance the spontaneous production of nodule-like structures in white clover (Blauenfeldt et al., 1994). Red clover plants treated with TiO_2 NPs and ZnO_4 in our experiment revealed a reduced ^{15}N content in shoots. Therefore the

limitation of nitrogen in red clover might have induced these nodule-like structures in our experiment. However, more research is needed to understand the mechanism. We could use the ^{15}N content of shoots as a proxy of nutrient uptake. All treatments revealed a decreased ^{15}N content in shoots, which indicated a reduced nutrient uptake compared to the controls. However, due to insufficient quantities of biomass not all replications could be used for ^{15}N analysis. Therefore some of the treatments did not have enough replicates for giving enough statistical power. Nevertheless, the results clearly indicate a reduced nitrogen uptake of the red clover plants. For peas Fan et al. (2014) have reported that plants treated with TiO_2 NPs revealed impaired water uptake and Asli and Neumann (2009) have reported reduced transpiration in corn treated with 1 g l^{-1} P25. Adhesion of NPs on root surfaces has been discussed as possible mechanism (Asli and Neumann, 2009; Fan et al., 2014). Pores in the cell walls of plants are approximately 5 nm in diameter (Marschner, 2002) and thus might be blocked by the TiO_2 NPs and agglomerates. To investigate this further and to test whether the TiO_2 NP covered the roots, we performed scanning electron microscopy to assess TiO_2 NPs on the root surface. Only 1% of the root surface was covered by E171 agglomerates or single particles, indicating sparse or loose attachment of TiO_2 NPs to the roots. Investigation of uptake of TiO_2 NP into red clover plants was not the aim of this study which would have required larger plants yielding more biomass.

6. Conclusions

TiO_2 NPs agglomerated and revealed particle sizes larger than 100 nm in growth media. They tended to sediment in the hydroponic system and thereby decreasing the actual exposure concentrations, demonstrating the importance of determining the actual exposure concentration. Anatase TiO_2 NPs, i.e., E171 and NNM TiO_2, significantly reduced growth rate of *R. trifolii* and did not display a primary particle size dependent effect because they reduced bacterial growth in the same extent and revealed similar aggregate sizes. In the hydroponic system, red clover biomass significantly decreased in all TiO_2 NP treatments and NNM TiO_2. Red clover plants treated with TiO_2 NPs revealed reduced nitrogen (^{15}N) content,

indicating impaired nutrition and elevated stress. P25, E171 and NNM TiO_2 did affect red clover and *R. trifolii* in artificial hydroponic cultures, but it remains to be tested which mechanisms are responsible for these effects and whether these effects extend to plants grown in soil.

7. Acknowledgment

We thank Franziska Blum at Agroscope for her help with lab work and Beat Boller at Agroscope for providing red clover seeds. Furthermore we thank Kyle Hartman for comments on the manuscript and the Center of Microscopy at the University of Zurich for assistance with SEM analyses.

8. Supplementary Information

Text S1: Scanning electron microscopy of red clover roots.

Root samples of a repetition of the hydroponic experiment with 24 mg E171 l^{-1} were analyzed by scanning electron microscopy for coverage of titanium particles (Figure 2). Dried red clover roots were fixed on a conductive adhesive tape, mounted on an aluminum sample holder and sputtered with a 3 nm layer of platinum using a Bal-tec MED020 sputter coater (Bal-Tec, Liechtenstein). Samples were imaged using a Zeiss Supra 50VP scanning electron microscope (SEM) operating at 10kV. An energy dispersive x-ray (EDX) spectrometer (x-act, Oxford Instruments, United Kingdom) was employed for elemental analysis of selected areas on the root surface. The coverage of the TiO_2 NPs on the root surface was assessed.

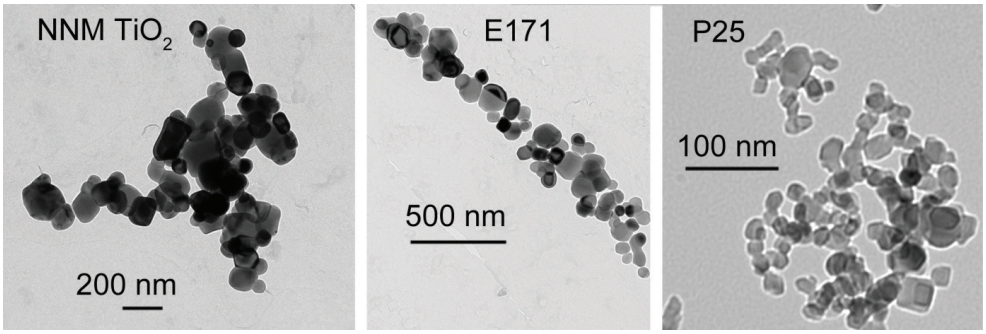

Figure S1: Transmission electron microscopy pictures of nanoparticles. From the left to the right: non-nanomaterial (NNM) TiO_2 particles, E171 and P25 nanoparticles.

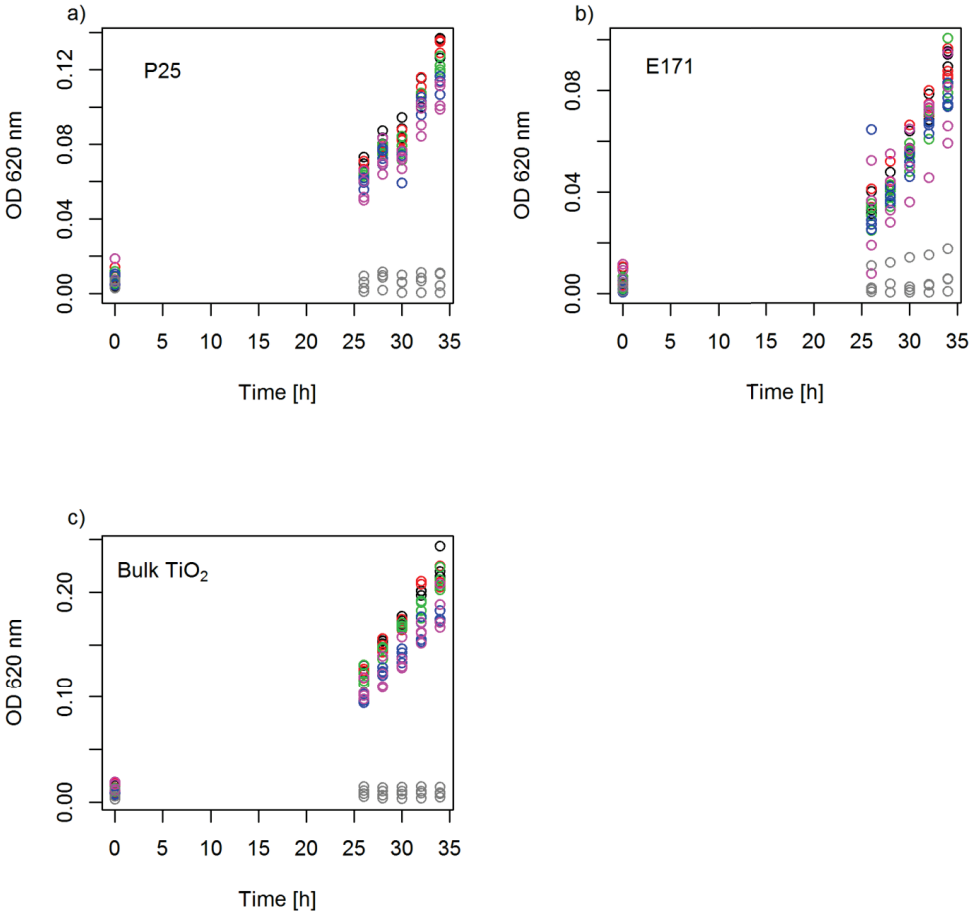

Figure S2: *Rhizobium trifolii* growth curves. Measured by optical density (OD) at 620 nm over time for a) P25, b) E171 and c) NNM TiO_2. Increasing concentrations of TiO_2 NPs are indicated in red, green, blue and cyan for 1, 3, 8 and 23 mg l^{-1} for P25 and E171 and 1, 2, 6 and 18 mg l^{-1} for NNM TiO_2. Each of the three experiments contained a control (black circles) and a positive control (gray circles), i.e. $ZnSO_4*7H_2O$ at 12.5 mg l^{-1}. Four replications of each treatment are shown. To remove the NP background of OD, we measured the same concentrations of NPs in YMB without *R. trifolii* and subtracted this value from the samples with *R. trifolii*.

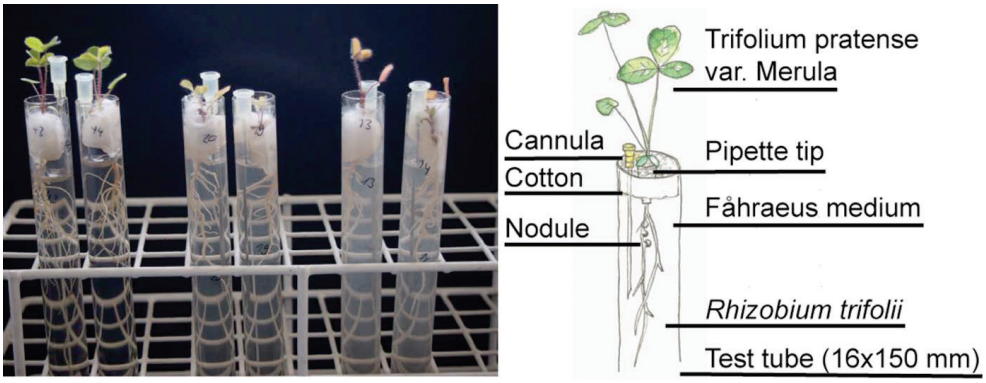

Figure S3: Hydroponic system after three weeks of growth. Shown are two replications of the control as well as the E171 1 and E171 2 treatments. On the drawing the setup of the hydroponic system is explained.

Table S1: Statistics for titanium concentration over time. Generalized linear model of total elemental titanium concentration over time in the Fåhraeus medium. Statistics are shown for the hydroponic system for the two particles E171 and P25 compared to the initial concentration. P-values were adjusted for multiple testing by Beniamini-Hochberg adjustment using the R command p.adjust.

glm	**E171**		**P25**	
time [h]	**t**	**p_{adj}**	**t**	**p_{adj}**
18	-0.6	0.591	-1.3	0.215
24	-1.3	0.276	-2.5	**0.034**
42	-3.6	**0.005**	-2.9	**0.020**
114	-7.1	**<0.001**	-3.8	**0.006**
162	-7.0	**<0.001**	-3.7	**0.006**

Table S2: Statistics for *R. trifolii* growth rates. Generalized linear model (glm) of the *R. trifolii* growth rates (slope of exponential growth phase) compared to the control. Statistics is shown for the different concentrations and NPs, i.e., P25, E171 and NNM TiO_2. Concentrations for P25 and E171 are shown and the ones for NNM TiO_2 are in parentheses. P-values were adjusted according to Benjamini-Hochberg using the R command p.adjust. In the case where the model assumptions were not fulfilled, a Kruskal-Wallis test was performed additionally.

glm	**P25**		**E171**[a]			**NNM-TiO_2**	
concentration [mg l^{-1}]	**t**	**p_{adj}**	**t**	**p_{adj}**	**Mann-Whitney p**	**t**	**p_{adj}**
1 (1)	1.8	0.181	-0.7	0.659	0.200	-0.5	0.758
3 (2)	0.3	0.867	0.0	0.971	0.886	-0.3	0.758
8 (6)	-0.2	0.867	-1.3	0.408	0.200	-0.4	0.758
23 (18)	2.3	0.132	-3.0	**0.034**	**0.029**	-5.0	**0.001**

[a]Resudials were not normally distributed, therefore an additiona Kruskal-Wallis test

Test was applied revealing a significant difference (p=0.029).

Table S3: Results of the statistical tests from the hydroponic experiment with *R. trifolii* inoculation. For each variable a generalized linear model (glm) was performed. In the case where the residuals were not normal distributed, a Mann-Whitney test was applied. P-values are adjusted for multiple testing. Exposure concentrations 1 and 2 (1=low, 2=high) are described in detail in Table 2.

	root length		shoot length		root weight		shoot weight		number of nodules		number of root tips per root lenght		secundary roots per length		^{15}N content shoot		^{15}N concent soot / plant weight	
	glm t-value	glm p-value	glm t-value	glm p-value	Mann - Whitney W	Mann-Whitney p-value	glm t-value	glm p-value	glm z-value	glm p-value	glm t-value	glm p-value	glm t-value	glm p-value	glm t-value	glm p-value	glm t-value	glm p-value
***R. trifolii* inoculation**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**
P25 9.0 mg l^{-1}	-4.9	**<0.001**	-4.5	**<0.001**	33	**0.039**	-4.6	**<0.001**	0.9	0.432	1.0	0.688	1.4	0.307	-5.7	**<0.001**	0.2	0.822
P25 18.0 mg l^{-1}	-5.7	**<0.001**	-7.1	**<0.001**	34	**0.027**	-4.6	**<0.001**	-1.2	0.302	1.9	0.249	1.2	0.307	-7.3	**<0.001**	-0.6	0.712
E171 9.5 mg l^{-1}	-5.8	**<0.001**	-6.3	**<0.001**	34	**0.027**	-5.2	**<0.001**	2.6	**0.001**	0.6	0.763	2.0	0.211	-6.2	**<0.001**	-0.2	0.822
E171 19.0 mg l^{-1}	-5.6	**<0.001**	-6.3	**<0.001**	33	**0.039**	-4.7	**<0.001**	-0.7	0.506	0.2	0.908	1.0	0.356	-9.0	**<0.001**	-1.6	0.300
NNM TiO_2 9.5 mg l^{-1}	-5.0	**<0.001**	-4.8	**<0.001**	31	0.062	-3.2	**0.002**	-1.6	0.178	1.1	0.687	1.2	0.307	-6.2	**<0.001**	-1.2	0.468
NNM TiO_2 19.0 mg l^{-1}	-5.4	**<0.001**	-5.1	**<0.001**	31	0.055	-3.8	**<0.001**	-2.1	0.057	0.8	0.688	1.3	0.307	-8.1	**<0.001**	-1.8	0.300
$ZnSO_4$ 16 mg l^{-1}	-8.5	**<0.001**	-6.2	**<0.001**	36	**0.017**	-5.0	**<0.001**	-2.8	**0.015**	-0.1	0.908	0.3	0.762	-3.9	**<0.001**	1.1	0.468

Table S4: Results of the statistical tests from the hydroponic experiment without *R. trifolii* inoculation. For each variable a generalized linear model (glm) was performed. In the case where the residuals were not normal distributed, a Mann-Whitney test was applied. P-values are adjusted for multiple testing. Exposure concentrations 1 and 2 (1=low, 2=high) are described in detail in Table 2.

	root length		shoot length		root weight		shoot weight		number of nodules		number of root tips per root lenght		secundary roots per length		^{15}N content shoot		^{15}N concent soot / plant weight	
without *R. trifolii* inoculation	**glm t-value**	**glm p-value**	**glm t-value**	**glm p-value**	**glm t-value**	**glm p-value**	**Mann - Whitney W**	**Mann-Whitney p-value**	**glm z-value**	**glm p-value**	**glm t-value**	**glm p-value**	**glm t-value**	**glm p-value**	**glm t-value**	**glm p-value**	**glm t-value**	**glm p-value**
P25 9.0 mg l^{-1}	-3.5	**0.002**	-6.5	**<0.001**	-2.8	**0.009**	34	**0.008**	3.4	**0.005**	0.9	0.726	1.5	0.363	-6.4	**<0.001**	-1.6	0.157
P25 18.0 mg l^{-1}	-3.6	**0.002**	-7.7	**<0.001**	-3.1	**0.006**	32	**0.030**	1.1	0.438	0.2	0.867	0.3	0.907	-5.6	**<0.001**	-2.0	0.115
E171 9.5 mg l^{-1}	-2.5	**0.017**	-6.6	**<0.001**	-2.6	**0.015**	32	**0.026**	2.9	**0.009**	0.2	0.867	0.4	0.907	-6.1	**<0.001**	-1.9	0.115
E171 19.0 mg l^{-1}	-3.5	**0.002**	-6.9	**<0.001**	-2.3	**0.025**	27	**0.040**	0.2	0.978	1.0	0.726	1.2	0.439	-6.9	**<0.001**	-2.8	**0.041**
NNM TiO_2 9.5 mg l^{-1}	-3.9	**<0.001**	-7.7	**<0.001**	-3.3	**0.005**	30	0.065	3.1	**0.008**	1.4	0.686	2.0	0.218	-5.8	**<0.001**	-1.5	0.160
NNM TiO_2 19.0 mg l^{-1}	-2.2	**0.031**	-7.3	**<0.001**	-3.1	**0.006**	32	**0.026**	0.8	0.565	-0.6	0.867	-0.4	0.907	-7.2	**<0.001**	-2.0	0.115
$ZnSO_4$ 16 mg l^{-1}	-3.0	**<0.001**	-6.8	**<0.001**	-5.3	**<0.001**	35	**0.004**	0.0	0.991	-0.2	0.867	0.0	0.98	-5.6	**<0.001**	0.5	0.601

Chapter III

Effects of nanoparticles on red clover and its symbiotic microorganisms

Janine Moll[1,2], Alexander Gogos[1], Thomas D. Bucheli[1], Franco Widmer[1], Marcel G.A. van der Heijden[1,2,3]

[1] Institute for Sustainability Sciences ISS, Agroscope, Zurich, Switzerland

[2] Plant-Microbe-Interactions, Department of Biology, Utrecht University, Utrecht, the Netherlands

[3] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland

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1. Abstract

Background: Nanoparticles are produced and used worldwide and are released to the environment, e.g., into soil systems. Titanium dioxide (TiO_2) nanoparticles (NPs), carbon nanotubes (CNTs) and cerium dioxide (CeO_2) NPs are among the ten most produced NPs and it is therefore important to test, whether these NPs affect plants and symbiotic microorganisms that help plants to acquire nutrients. In this part of a joint companion study, we spiked an agricultural soil with TiO_2 NPs, multi walled CNTs (MWCNTs), and CeO_2 NPs and we examined effects of these NP on red clover, biological nitrogen fixation by rhizobia and on root colonization of arbuscular mycorrhizal fungi (AMF). We also tested whether effects depended on the concentrations of the applied NPs.
Results: Plant biomass and AMF root colonization were not negatively affected by NP exposure. The number of flowers was statistically lower in pots treated with 3 mg kg^{-1} MWCNT, and nitrogen fixation slightly increased at 3000 mg kg^{-1} MWCNT
Conclusions: This study revealed that red clover was more sensitive to MWCNTs than TiO_2 and CeO_2 NPs. Further studies are necessary for finding general patterns and investigating mechanisms behind the effects of NPs on plants and plant symbionts.

Keywords

Nanomaterials, agriculture, crop, beneficial soil microbes, ecosystem services

2. Background

Titanium dioxide (TiO_2) nanoparticles (NPs), carbon nanotubes (CNTs) and cerium dioxide (CeO_2) NPs are among the ten most produced NPs worldwide (Keller et al., 2013). The production and use of these NPs leads to increasing concentrations in the soil system. Estimated material-flow in sludge treated soils for Europe are 2380 t^{-1} y^{-1} and 0.771 t y^{-1} for TiO_2 and CNTs, respectively (Sun et al., 2014). For CeO_2 1,400 t y^{-1} are assumed to end up in sludge treated soils worldwide (Keller et al., 2013). Thus, all of these three NP types are unintentionally released into the soil ecosystem. One NP type that needs special attention regarding risk assessment in soils is TiO_2 because these NPs are listed in patents and publications targeted as additives of plant protection products (Gogos et al., 2012; Khot et al., 2012). Thus, if such products were released to the market and applied in the fields, higher concentrations of TiO_2 NPs would be expected in soils. Due to the potential for increasing amounts of NPs that enter the soil system, it is important to test, whether these NPs affect plants and beneficial soil microorganisms that associate with plant roots and assist plants to acquire nutrients.

Several studies investigated effects of TiO_2 NPs, CNTs and CeO_2 NPs on either plants or microorganisms with variable results. For TiO_2 NPs, contrasting results were found and plant biomass was either decreased or not affected when grown in soil with enhanced TiO_2 NP concentrations (Du et al., 2011; Song et al., 2013; Burke et al., 2014). Soil microbial community structures were shown to be altered when treated with TiO_2 NPs (Ge et al., 2011; Ge et al., 2012; Burke et al., 2014). Also CNTs affected plants and soil microbial community structures: the number of flowers and fruits of tomatoes increased, and bacterial community structure changed (Khodakovskaya et al., 2013). In contrast, in another study with much higher CNT concentrations, soil microbial community structure was not affected (Shrestha et al., 2013). Most often, ecotoxicological tests with NPs (TiO_2, CeO_2 and CNTs) in soil systems are either performed with plants, or with microorganisms, but the symbiosis of plants and soil microorganisms has rarely been investigated. Plant symbionts provide important

ecosystem functions as e.g., nitrogen-fixation by rhizobia in legumes or phosphorus acquisition by arbuscular mycorrhizal fungi (AMF) (van der Heijden et al., 2015a). One example is red clover which is used for animal feeding and as green manure. Red clover associates with nitrogen-fixing rhizobia bacteria (rhizobia) (Somasegaran and Hoben, 1994; Heidstra and Bisseling, 1996). Up to 373 kg N ha^{-1} y^{-1} can be fixed by these bacteria in root nodules of red clover plants (Carlsson and Huss-Danell, 2003). Additionally, red clover performs a second symbiosis with AMF (Smith and Read, 2008; Smith and Smith, 2011; van der Heijden et al., 2015a; van der Heijden et al., 2015b). These fungi provide plants with soil nutrients, especially immobile nutrients such as phosphorus. Up to 90% of plant phosphorus is provided by AMF (van der Heijden et al., 2015b). The two microbial symbionts, AMF and rhizobia, conduct important ecosystem functions (van der Heijden et al., 2015a), and thus it is important to assess whether nitrogen fixation and root colonization by AMF are affected by NPs

Earlier studies showed that NPs had adverse effect on the legume-rhizobia symbiosis. For soybeans it has been reported that CeO_2 NPs diminished nitrogen fixation (Priester et al., 2012), and no effects of TiO_2 and Fe_3O_4 NPs on nodule colonization were found (Burke et al., 2015). For barrel clover it has been reported that the number of nodules was decreased and gene expression altered when exposed to biosolids containing Ag, ZnO and TiO_2 NPs (Chen et al., 2015; Judy et al., 2015). Peas revealed a delayed nitrogen fixation when exposed to TiO_2 and ZnO in hydroponic systems (Fan et al., 2014; Huang et al., 2014), and for faba beans, nodulation and nitrogenase activity were delayed by Ag NPs (Abd-Alla et al., 2016). AMF root colonization has been reported to not being affected in soybeans exposed to TiO_2 and Fe_3O_4 NPs (Burke et al., 2015), while colonization of white clover roots was increased by Ag and FeO NPs (Feng et al., 2013). Because of these effects on legume-rhizobia and AMF systems, it is important to assess whether root colonization by AMF and nitrogen fixation in soil-grown red clover are affected by NPs, e.g. TiO_2, CeO_2 and CNTs,

because these effects might be species and NP dependent. To our best knowledge, there are no studies available on the effects of CNTs on legume-rhizobia-AMF systems.

In the present study, we investigated the effects of three different NP types, i.e., TiO_2 NPs, multi-walled CNTs (MWCNTs) and CeO_2 NPs, on red clover growth, biological nitrogen fixation with rhizobia and on root colonization of AMF in a soil system. We investigated if these NPs affect (1) plant growth, (2) biological nitrogen fixation in plants, (3) AMF root colonization, and (4) phosphorus uptake by red clover. As positive control we chose $ZnSO_4 \cdot 7H_2O$ because Zn^{2+} was reported to decrease plant growth and affect nitrogen fixation of legumes (Vesper and Weidensaul, 1978). Effective soil elemental titanium and MWCNT (black carbon) concentrations, their vertical translocation and plant uptake were investigated in detail in a companion paper (Gogos et al., 2016).

3. Results

Red clover plants were exposed for 14 weeks to agricultural soil spiked with different concentrations of NPs, i.e., TiO_2 NPs (P25), a bigger non-nanomaterial (European Commission, 2011) TiO_2 particle (NNM-TiO_2, 20% particles < 100 nm), MWCNTs, CeO_2 NPs and a $ZnSO_4$ treatment. The biomass of red clover plants did not differ between NP spiked substrate and controls without NP addition, both for root and shoot dry weight separately and for total plant dry weight (Figure 1, Table S1). Total plant dry weight and effective titanium content per pot were correlated explaining 20% of variance (Pearson's correlation: $p=0.041$, $r = 0.45$). The root-shoot ratio was 0.49±0.04 on average, and was also not affected by the presence of NPs ($p>0.05$). The number of flowers decreased in the 3 mg MWCNT kg^{-1} soil treatment by 34% ($p=0.049$, Figure 1, Table S1). The higher concentration of 3000 mg MWCNT kg^{-1} exhibited a similar decrease in mean number of flowers (33%), but the variation was higher and therefore the number of flowers was not significantly different from the control plants ($p=0.160$).

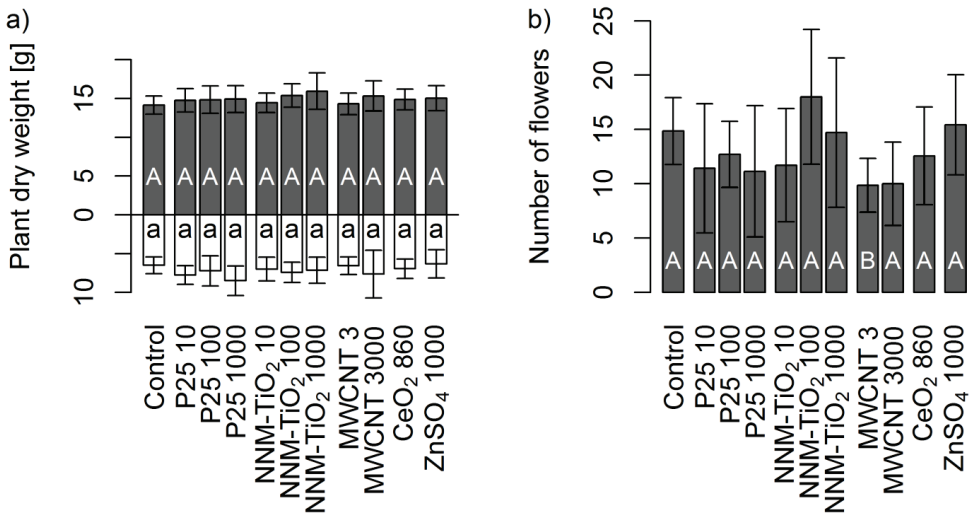

Figure 1: Plant weight and flowers. a) Red clover plant dry weight divided in shoot (grey) and root (white), and b) number of flowers per pot at the end of the three month exposure for control, TiO_2 (P25, non-nanomaterial NNM), MWCNT, CeO_2 NPs, and $ZnSO_4 \cdot 7H_2O$. The number behind the treatment name is the nominal concentration in mg kg^{-1}. Error bars show the standard deviations (n=7). Capital letters show significant differences for shoot biomass and number of flowers, and small letters for root biomass compared to the control plants ($p \leq 0.05$). The two blocks of starting time were included in the statistical model.

In addition to plant performance, the interaction of red clover with rhizobia was investigated. All harvested red clover plants contained root nodules and the root nodules had a reddish color which indicates that they fixed nitrogen (Somasegaran and Hoben, 1994). In addition, the percentage of fixed nitrogen was assessed based on the ^{15}N concentrations of clover and a reference plant (rye grass; see formula 1 in the methods). The percentages of fixed nitrogen of control red clover plants and NP treated plants were compared, and confirmed that biological nitrogen fixation took place (Figure 2). All of the treated red clover plants fixed nitrogen and NP application did not affect nitrogen fixation levels in most of the treatments. Only in the 3000 mg MWCNT kg^{-1} treatment, biological nitrogen fixation was increased by

8% (p=0.016). Pearson's correlation revealed a correlation of nitrogen fixation and total biomass of r=0.28 (p=0.012).

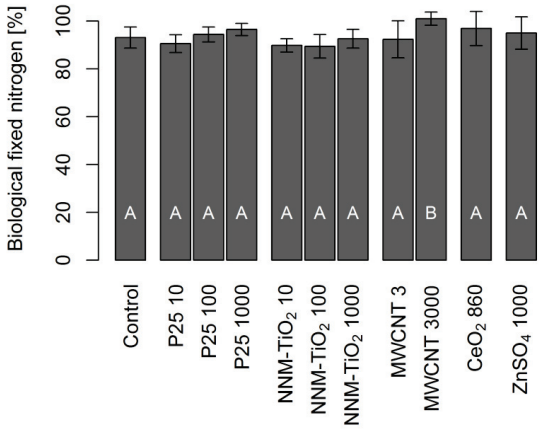

Figure 2: Biological nitrogen fixation. Percentage of atmospheric nitrogen derived from biological nitrogen fixation in red clover shoots for the control, P25 and NNM-TiO_2, MWCNTs, CeO_2 NPs, and $ZnSO_4 \cdot 7H_2O$. The number behind the treatment name is the nominal concentration in mg kg^{-1}. Rye grass was used as non-nitrogen fixing plant and the B value was assumed to be zero (see text). Error bars show the standard deviations (n=7). Capital letters show significant differences compared to the control plants ($p \leq 0.05$).

The second symbiotic partner of red clover, AMF, was assessed by determining root colonization by staining fungal tissue and counting fungal structures by microscopy (McGonigle et al., 1990; Vierheilig et al., 1998). In addition the phosphorus content of red clover shoots was assessed, as AMF can contribute significantly to plant P nutrition. Total root colonization by AMF, i.e., % arbuscules, vesicles and hyphae per investigated root intersection, was similar in all treatments (on average 51±4%; Figure S1). Also the arbuscular and vesicular colonization revealed no differences between the control and NP treatments (average 23±3% and 6±2%, respectively; Table 1). Phosphorus concentrations of

the red clover shoots were not affected in any of the treatments (Figure S1 b, Table S1). Plant phosphorus content and total root colonization by AMF were not correlated (Pearson correlation coefficient: p=0.199; r=0.15).

Table 1: Mean values and standard deviation of the arbuscular and vesicular root colonization.

	arbuscular colonization [%]		vesicular colonization [%]	
	mean	sd	mean	sd
Control	23	8	4	2
P25 10 mg kg^{-1}	22	7	3	3
P25 100 mg kg^{-1}	22	8	6	9
P25 1000 mg kg^{-1}	21	10	8	7
NNM-TiO_2 10 mg kg^{-1}	19	10	7	5
NNM-TiO_2 100 mg kg^{-1}	24	11	6	3
NNM-TiO_2 1000 mg kg^{-1}	25	11	7	8
CNT 3 mg kg^{-1}	20	9	2	2
CNT 3000 mg kg^{-1}	29	7	5	5
CeO_2 860 mg kg^{-1}	24	9	8	5
$ZnSO_4 \cdot 7H_2O$ 1000 mg kg^{-1}	27	8	6	4

4. Discussion

In the present study effects of different NPs, i.e., TiO_2 NPs, MWCNTs and CeO_2 NPs, on red clover and its symbiosis with rhizobia and AMF were assessed in a soil system. Both tested TiO_2 treatments (i.e. P25 and NNM-TiO_2) in all concentrations did not affect plant biomass in our experiment. The absence of effects of TiO_2 NPs on plant biomass are in agreement with other studies, using different plant species. For example plant growth was not affected when soybeans and corn were exposed to 200 mg TiO_2 NP kg^{-1} (Burke et al., 2014) and when tomatoes were exposed to concentrations between 1000 and 5000 mg P25 TiO_2 NP kg^{-1} (Song et al., 2013). However, in wheat 90 mg TiO_2 NPs kg^{-1} was shown to decrease plant biomass by 13% (Du et al., 2011). MWCNTs did not affect red clover biomass in our experiment. Contrary to our findings, MWCNTs have been reported to increase biomass of tomatoes exposed to 50 and 200 µg ml^{-1} MWCNTs per plant (Khodakovskaya et al., 2013).

In our experiment red clover biomass did not respond to the CeO_2 NP treatment, which is in agreement to a study using CeO_2 NPs at concentrations between 0.1 and 1 g kg^{-1} in an experiment with soybeans (Priester et al., 2012). Thus, effects on plant biomass might be influenced by plant species (as shown for the TiO_2 NPs and MWCNTs) as well as by NP type. All of the above cited studies used different soils. Depending on soil properties, NPs might be differently bound to soil particles (Fang et al., 2009) which could influence the exposure and the effects of NPs on plants.

The number of flower heads was not affected in both TiO_2 and CeO_2 NP treatments at all tested concentrations. However, MWCNTs decreased number of flowers by 34% ($p=0.049$) at the lower concentration (3 mg kg^{-1}). The higher MWCNT concentration showed a similar decrease of flower number (33%), but the variance between the samples was higher and there was no statistically significant difference ($p=0.16$). Our results indicate that the number of flowers is sensitive to MWCNTs. Khodakovskaya et al. (2013) showed that the number of flower increased significantly, when watered weekly with 50 ml of 50 and 200 µg ml^{-1} MWCNTs per pot for 9 weeks (Khodakovskaya et al., 2013). The direction of the effect was in contrast to our observations. Nevertheless, the number of flowers was affected and further research is needed to determine the mechanism responsible for the effects of MWCNT on flowering.

To test effects of NPs on biological nitrogen fixation, the natural abundance of ^{15}N was determined in the red clover shoots and in a reference plant (rye grass) and subsequently the fraction of biological fixed nitrogen in red clover was assessed (see methods). No nitrogen was added to the pots because increasing the availability of mineral nitrogen has been reported to decline nitrogen fixation rate progressively (Unkovich et al., 2008). The percentage of fixed nitrogen was high and ranged between 89 and 100% and was not affected by the TiO_2 NPs in our experiment. These results contrast those of another study performed in a hydroponic system using pea and rhizobia (Fan et al., 2014). This study

showed that nodulation was negatively affected and that the nitrogen fixation was delayed when TiO_2 NPs were present. However, it needs to be tested whether the results from hydroponic systems can be directly extrapolated to soil systems. In soils, TiO_2 NPs interact with soil particles and are probably heteroaggregated with soil particles such as clay minerals (Fang et al., 2009). Thus, the plant roots in soils might be less exposed to the NPs than in hydroponic systems and therefore roots and nodules might be less affected in soils, as indicated by the limited transport of TiO_2 NPs in soils in our experiment (Gogos et al., 2016). For the higher concentration of MWCNTs (3000 mg kg^{-1}), nitrogen fixation increased by 8% ($p=0.01$) compared to the control and 100% of the nitrogen content in the shoots originated from nitrogen fixation. Even though the biomass and total nitrogen content of these MWCNT treated plants were not different from those in the control treatment, correlation between biologically fixed nitrogen and total biomass over all treatments was significant but only 8% of the variation could be explained ($R^2= 0.08$; $p=0.012$). This indicates that enhanced nitrogen fixation had only a small beneficial effect on plant growth. In our experiment, nitrogen fixation was not affected by CeO_2 NPs. For soybeans however, the CeO_2 NPs have been reported to decrease nitrogen fixation potential up to 80% (Priester et al., 2012). This reference investigated a different plant species and effects of NPs might be plant and rhizobia species specific (Priester et al., 2012). Also the use of different soils with different soil characteristics might influence the results. Further experiments are needed to consolidate our understanding of the mechanisms of how NPs affect nitrogen fixation.

Total arbuscular, as well as vesicular root colonization of red clover by AMF were not affected in any of the treatments. In support of this finding, but again with another plant species, Burke et al. (2015) reported no effects of TiO_2 NPs on AMF root colonization in soybeans using a DNA based approach instead of counting the root colonization. AMF provide plants with nutrient, such as phosphorus (Smith and Read, 2008; van der Heijden et al., 2008). Therefore we assessed phosphorus content in red clover shoots at the harvest. Phosphorus content of red clover shoots was not affected in any of the treatments and there

was no correlation between plant phosphorus content and total AMF root colonization ($p=0.2$). Again, for TiO_2 NPs this is in agreement with Burke et al. who did not find differences in phosphorus content of soybean leaves (Burke et al., 2015). Even though root colonization was not affected by the tested NPs in our experiments, community structure of AMFs in soils might change as shown in Burke et al. (Burke et al., 2014).

Contrary to our expectations, the $ZnSO_4$ control did not affect any of the measured endpoints. It is known that Zn^{2+} availability is limited at high soil pH conditions (Lindsay, 1972). Soil pH was 7.7 (Gogos et al., 2016) and the concentration added was probably not high enough to release enough free Zn^{2+} to cause harmful effects.

The amount of NPs applied to the soil was high and partly outside the exposure range expected in the field. They were chosen to represent a potential agricultural application scenario, where fluxes between several micrograms to grams of NPs per kilogram of soil are estimated (Gogos et al., 2012). The highest concentration also simulates accidental spill during transport or pollution in industrial areas or in the field. In our experiment also lower concentrations, i.e. 10 and 100 mg kg^{-1} soil, were tested. This approach ensures that potential negative effects can be detected before a NP is widely used and applied. This approach also facilitates the detection of potential harmful NPs in comparison to non-toxic or less harmful NP. Moreover, in order to be able to detect and measure concentrations of some NPs in the environment (e.g. titanium oxides for this study), high amounts have to be added because element like titanium occur naturally in the soil and the concentrations added need to be higher as natural background levels. For instance, for TiO_2 NPs the lowest concentration of 10 mg kg^{-1} is realistic in comparison with estimations for soils treated with NP containing plant protection products, while the highest tested concentration (1000 mg kg^{-1}) rather represents a worst case scenario (Gogos et al., 2012). For MWCNTs, yearly increases of estimated environmental concentrations are estimated to range from 5 to 990 ng kg y^{-1} (Sun et al., 2014). Hence, both tested concentrations in our experiment are above

natural values and represent an upper limit. The addition of these high concentrations was necessary to distinguish the added MWCNTs from the black carbon background of the soil (Sobek and Bucheli, 2009; Gogos et al., 2016). New methods are currently being developed to distinguish NPs from natural backgrounds as reviewed by others (Farré et al., 2011; Von der Kammer et al., 2012). Further research is needed to measure and characterize NPs in soils at predicted environmental concentrations, both for fate and behavior studies, and to accompany environmentally relevant ecotoxicological tests.

5. Conclusions

The investigated TiO_2 NPs and CeO_2 NPs did not affect red clover growth, biological nitrogen fixation and AMF root colonization. Opposite to other studies with TiO_2 and CeO_2 that observed effects on N fixing legumes, no effects were observed here with red clover. Further research is needed to search for general patterns and investigate the mechanisms behind such effects. MWCNTs increased nitrogen fixation and decreased the number of flowers compared to the control treatment, which might affect fitness of red clover. However, these effects occurred at concentrations much higher than expected in the environment.

6. Materials and Methods

6.1 NPs used for the experiment

P25 (Sigma Aldrich, USA, art. No. 718467) with a particle size of 29±9 nm (Gogos et al., 2016) was used as representative for TiO_2 NPs. In addition, NNM-TiO_2 (Sigma Aldrich, USA, Art. No. 232033) with an average particle size of 145±46 nm (Gogos et al., 2016) was used as non-nano-material, i.e. less than 50% NPs (European Commission, 2011). MWCNTs were purchased from Cheap Tubes Inc. (USA). They had a length of 10-30 μm, outer diameter of 20-30 nm, a purity of >95% and an elemental carbon content of >98% (Table S2) (Gogos et al., 2016). CeO_2 NPs (Sigma Aldrich, USA, art. No. 700290) had a diameter of less than 50 nm with cubic crystal structure according to the manufacturer's specifications.

6.1 Mixing NPs into the soil

For preparing the substrate, soil classified as brown earth with a sandy loamy to loamy fine fraction was collected from an agricultural field at Agroscope Institute for Sustainability Sciences in Zurich, Switzerland (coordinates N47° 25' 39.564" E8° 31' 20.04"). For this, the top 5 cm were removed and the underlying 15 cm soil were collected and sieved (<0.5 cm). The soil was mixed with quartz sand (50% v/v) and then characterized as described by Gogos et al. (2016) (Table S3). Nutrient contents in the mixture were 37.6 mg kg^{-1} phosphorus and 85.3 mg kg^{-1} potassium determined by ammonium acetate EDTA extraction (Stünzi, 2006). Soil pH was 7.7. Each of the different NPs was premixed in 300 g substrate (soil and sand) on an overhead mixer (Turbula T2F, Switzerland) in 500 ml Schott bottles by adding 0.3, 3 and 30 g of P25 or NNM-TiO_2, 90 mg and 88 g MWCNTs, 25 g CeO_2 NPs and 30 g $ZnSO_4 \cdot 7H_2O$ (Sigma Aldrich, USA, art. No. Z0251), respectively. P25 (30 g) and MWCNTs (88 g) revealed a too large volume for the 500 ml Schott bottles, necessitating the division of the soil and additives into several bottles (300 g of substrate for each bottle). For P25 15 g were added to two Schott bottles, and for MWCNTs 22 g were added to four bottles. Each of these pre-mixtures was diluted with substrate to a total volume of 30 kg and mixed in a cement mixer for 6h.

6.3 Experimental setup

Pots were prepared by gluing PVC-sewer pipes (15 cm diameter, 20 cm long) on a plastic board with a ball valve as draining device (Figure 3). A plastic mesh (Propyltex 500 µm, Sefar, Switzerland) was placed on the top of the valve to prevent blockage of the valve by the substrate. Pots were filled with a 500 g quartz sand layer as drainage and 3.3 kg spiked substrate or control substrate. Seven replications per treatment were prepared, i.e., control, P25, NNM-TiO_2, MWCNT, CeO_2 NPs, and $ZnSO_4 \cdot 7H_2O$. Total elemental titanium, black carbon (BC, for MWCNT treatments) and elemental cerium concentrations were determined in the substrate as described in the accompanying study (Gogos et al., 2016). Average total elemental titanium concentration of the highest tested concentrations was determined at the

end of the experiment using X-ray fluorescence (XRF) and was 1332±100 for the control treatment without titanium, 2059±105 for 1000 mg kg^{-1} (nominal) P25 and 2007±79 mg kg^{-1} for NNM-TiO_2 treated soils, respectively (Gogos et al., 2016). For MWCNT the background of BC in control soils was on average 0.50±0.06 mg g^{-1} and BC concentration in MWCNT 3000 mg kg^{-1} treated soil was 2400±100 mg kg^{-1} as quantified by chemothermal oxidation (Gogos et al., 2016). Average elemental cerium concentration in the 830 mg kg^{-1} CeO_2 treatment was 416±19 mg kg^{-1} determined with XRF at the end of the experiment.

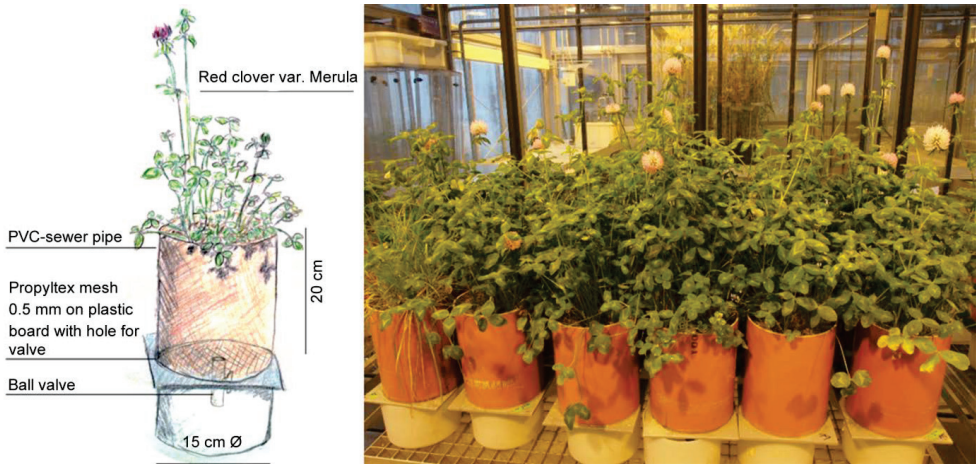

Figure 3: Experimental setup. Sketch of the experimental setup of the pots and picture of a part of the pots in the greenhouse 12 weeks after the start of the experiment. All of the pots were randomly arranged in the greenhouse.

6.4 Cultivation of red clover in NP spiked substrate

Red clover (*Trifolium pratense* var. Merula) was germinated on filter paper for 5 d. Thereafter, seven seedlings of equal size were transferred to the pots with substrate spiked with NPs or control soils in a greenhouse (16 h 25 °C 300W m^2, and 8 h 16 °C in the dark). In addition seven pots with ryegrass (*Lolium perenne* var. Arolus) were prepared in the same way. These plants were grown because a non-nitrogen-fixing plant was needed to estimate

biological fixed nitrogen in red clover (see below). The experiment was started in two blocks (n=4 and 3, respectively), time-shifted with one week difference. All pots were regularly watered to keep the water holding capacity between 60 and 70% (controlled by weighing and adding every time the same amount of water to all of the pots). Clover was fertilized after 6 and 9 weeks with 10 ml of a solution containing KH_2PO_4 (5 mM), $MgSO_4 \cdot 7H_2O$ (1 mM), KCl (50 µM), H_3BO_3 (25 µM), $MnSO_4 \cdot H_2O$ (1.3 µM), $ZnSO_4 \cdot 7H_2O$ (2 µM), $CuSO_4 \cdot 5H_2O$ (0.5 µM), $(NH_4)6Mo_7O_{27} \cdot 4H_2O$ (0.5 µM), and Fe(III) EDTA (20 µM). This is comparable to a phosphorus addition of 1.7 kg P ha^{-1}.

After 14 weeks NP exposure of red clover, the number of flowers (flower heads) was determined and the plant shoots were harvested. Soil cores were taken to assess NP concentration as described in Gogos et al. (2016). Roots were separated from the soil and washed. Then the roots were cut in 1 cm pieces, mixed in water and a randomized root subsample of approximately 2 g was taken for determining the AMF colonization. Roots were padded with a paper towel and weighed. The subsample was weighed separately and then stored at 4°C in 50% ethanol in Falcon tubes until the colonization was determined. The remaining roots as well as the red clover and ryegrass shoots were dried at 70 °C until they reached constant dry weight and dry weight of roots, shoots and total biomass (root+shoot weight) were determined. The dry weight of the AMF colonization root sample was calculated using the dry/wet weight ratio of the root sample. This AMF sample dry weight was added to the total root dry weight. Shoots of red clover and ryegrass were ground with a centrifugation mill (0.2 mm sieve, Retsch ZM200, Germany) and 2 mg samples were sent for ^{15}N analysis by isotope ratio mass spectrometry at the stable isotope facility at Saskatchewan University (Canada). Root colonization of AMF was analyzed by microscopy following the protocols of Vierheilig et al. (1998) for staining the roots and McGonigle et al. (1990) for counting the AMF structures. In short, roots were rinsed with deionized water, and transferred to 10 ml 10% KOH for 20 min at 80 °C. Roots were rinsed again with water and stained in 5% (v/v) ink (Parker Quink, black) in vinegar for 15 min at 80 °C. After rinsing the stained roots, they were

transferred to 50% glycerol for storage until root colonization was assessed. For microscopy, the root pieces were aligned in parallel onto a glass slide, covered with 50% glycerol, and the roots were covered with a cover slip (McGonigle et al., 1990). AMF structures in plant roots, i.e., hyphae, arbuscules, and vesicles, were counted for 100 intersections as described by McGonigle et al. (1990). Phosphorus content of shoots was assessed by ICP-OES using a hydrochloric acid digestion of the ashed residues (Bassler, 1993).

Nitrogen fixation [%] was calculated using equation (1) where B is the value of $\delta^{15}N$ of shoots of plants, that are fully dependent upon nitrogen fixation (Unkovich et al., 2008). For our experiment, a B value of 0 was assumed which reflects $\delta^{15}N$ of plants that are totally dependent on nitrogen fixation. The reference plant $\delta^{15}N$ was derived from the ryegrass shoots.

$$\%\ \text{Nitrogen fixation} = \frac{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of } N_2\text{fixing plant}}{\delta^{15}\text{N of reference plant} - \text{B}} \times \frac{100}{1} \quad (1)$$

6.5 Statistics

All statistical analyses were performed with R (R Core Team, 2014). A generalized linear model with Gaussian distribution was applied to determine differences of each treatment to the control. Thereby the two blocks of the different starting dates of the pot experiment were included as error term. The model was analyzed for homogeneity (Bartlett test) and normality (Shapiro test). Additionally a Dunnett test was performed (R library SimComp) using adjusted p-values for multiple testing (Hasler and Hothorn, 2011) when normality and homogeneity were fulfilled. For non-normal residuals or inhomogeneous data, a Mann-Whitney test was used and p-values were adjusted for multiple testing according to Benjamini and Hochberg (1995). Pearson's correlations were calculated with the R command cor.test.

7. Acknowledgements

We thank Florian Klingenfuss for providing assistance for harvesting the experiment.

8. Supplementary Information

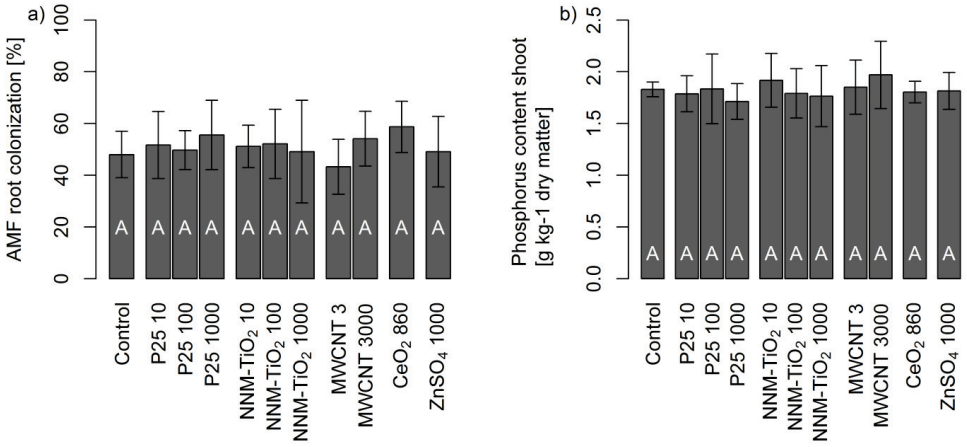

Figure S1: Arbuscular mycorrhizal root colonization and phosphorus content. a) Total root colonization (percentage of arbuscules, vesicles and hyphae per assessed root intersection) of red clover roots and b) phosphorus concentration in red clover shoots for the control, P25 and NNM-TiO_2, CNTs, CeO_2 NPs, and $ZnSO_4 \cdot 7H_2O$. The numbers after the treatment name indicate the nominal concentrations in mg kg^{-1}. Error bars show the standard deviations (n=7). Different letters indicate statistically significant differences between the treatments ($p \leq 0.05$).

Table S1: Summaries of the statistical tests applied. A generalized linear model (glm) was applied to test the model assumptions and to check if the two blocks of the starting times of the experiment were significant different from each other. If the residuals were normal and homogeneous, a Dunnett's test was performed and p values were adjusted for multiple comparisons. For not normal residuals, a Mann-Whitney test was applied.

	shoot dry weight [g]				root dry weight [g]				root shoot ratio				number of flowers				biological nitrogen fixation	
	glm		Dunnett		glm		Dunnett		glm		Dunnett		glm		Dunnett		Mann-Whitney	
	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	W	p_{adj}
Block	6.4	**<0.001**			-0.9	0.730			-3.3	**0.008**			2.1	0.186				
P25 10 mg kg^{-1}	0.9	0.453	0.8	0.977	1.4	0.681	2.1	0.354	1.1	0.872	1.7	0.563	-1.3	0.335	-1.4	0.781	34	0.437
P25 100 mg kg^{-1}	1.0	0.419	0.9	0.971	0.8	0.730	0.9	0.975	0.5	0.918	0.5	1.000	-0.8	0.486	-1.3	0.808	19	0.654
P25 1000 mg kg^{-1}	1.1	0.419	1.0	0.943	2.2	0.208	2.4	0.237	2.0	0.183	1.5	0.668	-1.5	0.335	-1.4	0.726	11	0.357
NNM-TiO_2 10 mg kg^{-1}	0.4	0.724	0.5	1.000	0.5	0.759	0.7	0.994	0.5	0.918	0.5	1.000	-1.2	0.335	-1.4	0.771	33	0.437
NNM-TiO_2 100 mg kg^{-1}	1.8	0.217	1.7	0.548	1.0	0.730	1.4	0.728	0.4	0.918	0.5	0.999	1.2	0.335	1.2	0.859	34	0.437
NNM-TiO_2 1000 mg kg^{-1}	2.6	**0.044**	1.8	0.501	0.7	0.730	0.8	0.977	-0.2	0.976	-0.3	1.000	-0.1	0.956	-0.1	1.000	25	1.000
CNT 3 mg kg^{-1}	0.2	0.807	0.2	1.000	0.0	0.965	0.1	1.000	0.0	0.981	0.0	1.000	-2.0	0.186	-3.3	**0.049**	23	0.992
CNT 3000 mg kg^{-1}	1.7	0.217	1.4	0.754	1.2	0.681	0.9	0.960	0.5	0.918	0.4	1.000	-1.9	0.186	-2.6	0.160	2	**0.013**
CeO_2 860 mg kg^{-1}	1.0	0.419	1.1	0.919	0.5	0.759	0.7	0.993	0.1	0.981	0.2	1.000	-0.9	0.486	-1.1	0.904	15	0.437
$ZnSO_4 \cdot 7H_2O$ 1000 mg kg^{-1}	1.3	0.397	1.2	0.864	-0.2	0.924	-0.2	1.000	-0.8	0.918	-0.9	0.955	0.2	0.899	0.3	1.000	16	0.437
Ryegrass control																	49	**0.006**

	%nitrogen in shoots				AMF total colonization				Ratio Ndfa / total biomass				Phosphorus [g kg^{-1} dry matter]			
	glm		Dunnett		glm		Dunnett		glm		Dunnett		glm		Dunnett	
	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}	t	p_{adj}
Block	-5.9	**<0.001**			-4.0	**0.001**			-2.6	0.120			-3.7	0.003		
P25 10 mg kg^{-1}	0.9	0.716	0.9	0.967	0.6	0.793	0.6	0.996	-1.9	0.220	-2.5	0.193	-0.3	0.944	-0.6	0.999
P25 100 mg kg^{-1}	-1.9	0.850	-0.1	1.000	0.3	0.846	0.4	1.000	-0.8	0.701	-0.8	0.978	0.1	0.944	0.0	1.000
P25 1000 mg kg^{-1}	-0.4	0.843	-0.4	1.000	1.3	0.607	1.2	0.824	-1.6	0.266	-2.6	0.172	-0.9	0.944	-1.6	0.670
NNM-TiO_2 10 mg kg^{-1}	1.1	0.696	1.1	0.882	0.5	0.793	0.7	0.993	-1.3	0.390	-1.7	0.593	0.7	0.944	0.8	0.982
NNM-TiO_2 100 mg kg^{-1}	-0.3	0.850	-0.2	1.000	0.7	0.793	0.7	0.993	-2.4	0.120	-3.3	0.051	-0.3	0.944	-0.4	1.000
NNM-TiO_2 1000 mg kg^{-1}	0.9	0.716	0.6	0.995	0.2	0.846	0.1	1.000	-1.8	0.220	-1.8	0.537	-0.5	0.944	-0.6	0.999
CNT 3 mg kg^{-1}	0.6	0.799	0.6	0.999	-0.8	0.793	-0.9	0.962	-0.3	0.977	-0.4	1.000	0.3	0.944	0.2	1.000
CNT 3000 mg kg^{-1}	0.7	0.759	0.6	0.998	1.0	0.720	1.2	0.862	0.0	0.977	0.0	1.000	1.2	0.912	1.0	0.938
CeO_2 860 mg kg^{-1}	0.4	0.843	0.6	0.998	1.8	0.292	2.1	0.317	-0.2	0.977	-0.2	1.000	-0.2	0.944	-0.5	1.000
$ZnSO_4 \cdot 7H_2O$ 1000 mg kg^{-1}	1.8	0.236	1.4	0.709	0.2	0.846	0.2	1.000	-0.1	0.977	-0.2	1.000	-0.1	0.944	-0.2	1.000
Ryegrass control	-21.8	**<0.001**	-27.9	**<0.001**												

Statistical significant p values ($p<0.05$) are indicated in bold.

Table S2: Characteristics of the MWCNTs.

		Value
Nominal TOC[1] g/kg dw		983.5
Length[1] (µm)		10-30
Od[1] (nm)		20-30
Id[1] (nm)		5-10
Functionalization[1] (wt%)		pristine
Elemental content (Quantified by energy dispersive X-ray spectroscopy EDX)	**C[1] (wt%)**	98.35
	Cl[1] (wt%)	0.45
	Fe[1] (wt%)	0.26
	Ni[1] (wt%)	0.94
Purity[1] (wt%)		>95
Ash[1] wt%		<1.5%
Specific surface area (BET)[1] (m^2/g)		>110
Electrical conductivity[1] (S/cm)		>100
TOC[2] (g/kg_{CNT})		977 ± 24
BC 375°C[2] (g/kg_{CNT})		658 ± 28
C after CTO-375[2] (wt%)	**Average**	67.36
	Stdev	28
Cd[2,3] (µg/kg)		<285
Co[2,3] (mg/kg)		11329.78
Cr[2,3] (mg/kg)		17.14
Cu[2,3] (mg/kg)		3.8
Fe[2,3] (mg/kg)		2629.17
Ni[2,3] (mg/kg)		927.17
Pb[2,3] (mg/kg)		1.03
Zn[2,3] (mg/kg)		<7.5
Mn[2,3] (mg/kg)		1.6
Mg[2,3] (mg/kg)		30.2
Al[2,3] (mg/kg)		14.7
Ca[2,3] (mg/kg)		258.6
Sum (mg/kg)		15213.1
Sum g/kg		15.2
Metals (%)		1.5

[1]Data provided by the manufacturer

[2]Own measurements

[3]Determined using microwave acid digestion and ICP-MS

Table S3: Characterization of the soil substrate used in the experiments analyzed by the companion study of Gogos et al. (2016).

Parameter	Value	SD
Org. C	0.55	0.03
CEC mmol+/kg	6	
$CaCO_3$ %	2.6	
pH	7.7	
max. WHC g H_2O/g dry soil	0.308	
Sand/silt/clay %	86.1/6.3/6.7	0/0/0.5

Chapter IV

Assessing the effects of titanium dioxide nanoparticles on soil microbial communities and wheat growth

Janine Moll[1,2], Florian Klingenfuss[1], Franco Widmer[1], Alexander Gogos[1], Thomas D. Bucheli[1], Martin Hartmann[3], Marcel G.A. van der Heijden[1,2,4]

[1] Institute for Sustainability Sciences ISS, Agroscope, Zurich, Switzerland

[2] Plant-Microbe-Interactions, Department of Biology, Utrecht University, Utrecht, the Netherlands

[3] Forests Soils and Biogeochemistry, Swiss Federal Research Institute WSL, Birmensdorf, Switzerland

[4] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland

1. Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) are the most produced NPs worldwide and have great potential to be utilized in agriculture as additives for plant protection products. However, concerns have been raised that some NPs may negatively affect crops and soil microbial communities, including beneficial microbes such as arbuscular mycorrhizal fungi. Here we tested two different TiO_2 NPs (P25, E171) and a bulk TiO_2 (particle size >100 nm) for their effects on the diversity and community composition of soil microorganisms. In addition we tested whether increasing concentrations of TiO_2 NPs had effects on wheat growth and yield. Microbial diversity was analyzed using Illumina Miseq paired-end sequencing of ribosomal markers (prokaryotic $16S_{V3V4}$ and eukaryotic ITS2 of the ribosomal RNA operon). Application of TiO_2 NPs altered the detected prokaryotic but not micro-eukaryotic community structure. The largest shift compared to the control treatment was observed for the positive control ($ZnSO_4$), but treatments E171, P25 and bulk TiO_2 revealed a significant different prokaryotic community structure compared to the control treatment. No negative effects on wheat growth and on arbuscular mycorrhizal root colonization were detected, and no evidence for a dose-response relationship between wheat performance and TiO_2 NP concentration was found. Overall, these results reveal that prokaryotes were more sensitive than eukaryotes to the TiO_2 NP treatments, which might potentially be based on NP interaction with their different surface structures, e.g., murein (bacteria) and chitin (fungi).

Keywords

Nanoparticle, plant, symbiotic microorganisms, soil, exposure, community structure

2. Introduction

Nanoparticles (NPs) are increasingly being used in electronics, composite materials, paints, cosmetics, food additives, and a wide range of other applications (Piccinno et al., 2012; Heiligtag and Niederberger, 2013). For instance, TiO_2 NPs reveal favorable properties, e.g. good covering power of pigments, UV-light attenuation and photocatalytic qualities. Nowadays TiO_2 NPs are manufactured worldwide with an estimated production of 88,000 t y^{-1} (Keller et al., 2013). Because of the substantial fabrication and usage of TiO_2 NP containing products, NPs get unintentionally released into the environment. For example in the US, approximately 760 t TiO_2 NPs y^{-1} are released into soils by application of sewage sludge (Gottschalk et al., 2009). Because of their properties as photocatalysts or UV protectors, TiO_2 NPs have a potential to be used in plant protection products to enhance their effectiveness and reduce the application amounts (Gogos et al., 2012; Kah et al., 2013). However, systematic application of such products would dramatically increase the estimated inputs of TiO_2 NPs to soils (Gogos et al., 2012). Soils have a geogenic background of TiO_2 on average of 0.5 %, suggesting a certain evolutionary adaptation of soil organisms to TiO_2 (Scheffer F. et al., 2002). However, TiO_2 in its nano-scale form might affect soil organisms differently than the natural occurring TiO_2 in soils, and potentially affect ecosystem functioning at various trophic levels (Gardea-Torresdey et al., 2014). Hence, there is a need to investigate potential non-target effects of TiO_2 NPs on soil organisms, plants, and plant-associated organisms.

Soil microorganisms conduct important ecosystem functions. For example they are important for soil carbon cycling, nitrogen fixation, and nutrient acquisition for plants (Carney and Matson, 2005; Hättenschwiler et al., 2005; van der Heijden et al., 2008). A key group of soil organisms that associate with two thirds of all terrestrial plants are arbuscular mycorrhizal fungi (AMF) of the phylum Glomeromycota (Smith and Read, 2008; Smith and Smith, 2011; van der Heijden et al., 2015b). AMF acquire limiting nutrients, especially immobile nutrients, such as phosphorus, for plants and can enhance plant growth. Wheat, which is of particular

importance for human nutrition, is one of the plant species that can benefit from a symbiosis with AMF(Pellegrino et al., 2015). Even though soil microorganisms play a crucial role in cropping systems, there are only a few studies that investigate the effects of TiO_2 NPs on soil microbial community structure, plants and the symbiosis between AMF and plants (Du et al., 2011; Ge et al., 2011; Song et al., 2013; Burke et al., 2014; Simonin and Richaume, 2015). For instance, soils planted with maize and soybean had been exposed for six weeks to 200 mg TiO_2 NPs kg^{-1} soil (Burke et al., 2014). While plant biomass and soil bacterial community structure were not affected by TiO_2 NPs, AMF communities were altered (Burke et al., 2014). In another study assessing AMF communities on soybean roots, no effects were found (Burke et al., 2015). Even less studies used high-throughput sequencing tools to investigate the effects of TiO_2 NPs on soil microbial communities. In one study using bar-coded pyrosequencing, Ge et al.(2012) observed that soil bacterial community structure was altered when treated with 0.5 to 2 mg TiO_2 NPs g^{-1} soil. However, that study only focused on bacteria and so far there is no study that simultaneously investigated effects of different TiO_2 NPs on bacteria, fungi, wheat and AMF root colonization in one experiment.

The current study was conducted to evaluate whether different concentrations and qualities (primary particle size and crystal structures) of TiO_2 NPs in agricultural soil affect (1) the diversity of soil prokaryotic and micro-eukaryotic communities, (2) root colonization by AMF and phosphorus uptake of wheat, and (3) the performance (yield) of wheat. For this purpose, we used industrially relevant TiO_2 NPs, i.e., P25 (anatase/rutile) and E171 (anatase), and a bulk anatase control (>100 nm) for E171. We assumed that the smallest NP, i.e. P25, would reveal the strongest effects on microorganism composition and plant growth, and that the effect size decreases with increasing particle size (E171< bulk TiO_2). Different photo-reactivity (ROS production) due to the different crystal structures (anatase and rutile) were assumed to have low influence, because in soils dark conditions prevail.

3. Material and Methods

3.1 Nanoparticles used

Two different TiO_2 NPs (P25 and E171) and bulk TiO_2 with increasing primary particle diameters were used. P25 (Sigma Aldrich, USA, Art. No. 718467) had the smallest primary particle diameter of 29±9 nm, E171 (Hombitan FG, Sachtleben Pigments, Germany) had a diameter of 92±31 nm, and bulk TiO_2 (Sigma Aldrich, USA, Art. No. 232033) had a diameter of 145±46 nm (Gogos et al., 2016). The size of bulk TiO_2 is taller than the nano-range (>100 nm) and is used as a non-nano control for E171.

3.2 Soil

Brown earth soil with a sandy loamy to loamy fine fraction was collected from an agricultural field near Agroscope, Institute for Sustainability Sciences, in Zurich, Switzerland (coordinates N47° 25' 39.564" E8° 31' 20.04") (Gogos, 2015). The soil was mixed with sand (50% v/v). Soil properties were described by Gogos et al.(2016) and were: pH 7.7, 86% sand, 6% silt, 7% clay, and nutrient contents were 37.6 mg kg^{-1} phosphorus and 85.3 mg kg^{-1} potassium determined by ammonium acetate EDTA extraction (Stünzi, 2006).

3.3 Spiking of substrate with NPs and preparation of pots

The effects of the TiO_2 NPs E171 and P25 were tested at three different concentrations (1, 100, and 1000 mg kg^{-1}) while the effect of bulk TiO_2 was tested at the highest concentration (1000 mg kg^{-1}). Three different amounts (0.03, 3, and 30 g) of each TiO_2 NP (E171, P25) and 30 g of bulk TiO_2 were added to 300 g substrate (50% v/v sand and soil) in a 500 ml Schott bottle and shaken in a powder mixer (Turbula T2F, Switzerland) for 30 min. In order to prepare the highest concentration of 30 g TiO_2 NPs, two bottles with 15 g NPs and 300 g substrate each were mixed. These premixed soil-particle mixtures were then diluted in 30 kg sand-soil substrate in a cement mixer for 6 h. This was done separately for each concentration (1, 100, and 1000 mg kg^{-1} for TiO_2 NPs E171, P25, and of bulk TiO_2. Control

substrate was treated as the spiked substrate but without adding NPs. Pots (15 cm diameter, 20 cm high, Figure S1) were filled with a drainage layer of sand (520 g) at the bottom, and then covered with 3.3 kg spiked substrate per pot. The total titanium concentration in the soils was determined by X-ray fluorescence spectroscopy at the end of the experiment to verify the exposure concentrations. Titanium concentrations of the soils were measured in three depth layers of 0-5, 5-10 and 10-15 cm and are shown in detail by Gogos et al. (2016). In short, the control soil contained on average 1024±284 mg Ti kg^{-1} (n=16), while soils spiked with 1000 mg kg^{-1} TiO_2 contained significantly ($p \leq 0.001$) more elemental Ti with values averaging 1720±280 (n=18), 1659±347 (n=18), and 2064±71 (n=17) mg kg^{-1} for P25, E171, and bulk TiO_2, respectively (Gogos et al. 2016). The treatments with the lower TiO_2 NP concentrations (1 and 100 mg kg^{-1}) did not differ significantly from the high natural background concentration of titanium in the soil.

3.4 Experimental design

Wheat (*Triticum ssp.* var. Fiorina) was exposed to P25 and E171 in three concentrations (1, 100, and 1000 mg kg^{-1} soil) as well as bulk TiO_2 (1000 mg kg^{-1} soil). A control treatment without NP addition was also included. As a positive control we used $ZnSO_4 \cdot 7H_2O$ (1000 mg kg^{-1} soil, Sigma-Aldrich, Art. No., Z0251), because it has been shown to affect wheat growth as well as soil microbial community structures (Frostegård et al., 1993; Frostegård et al., 1996; Warne et al., 2008; Rousk et al., 2012). All nine treatments were replicated 7 times, resulting in a total of 63 pots. The experiment performed temporary shifted in two blocks with one week between the blocks to allow harvesting one block within one week. Pots had fully randomized positions in the greenhouse (16 h 25 °C 300W m2, 8 h 16 °C dark).

Six wheat seeds per pot were directly sown into the pots and after germination thinned to three seedlings per pot. The plants were watered three times a week and the moisture content was kept between 50 and 60% by weighing. Weeds were removed regularly. Wheat was fertilized every week, starting after week 3, with 7.9 ml of (KNO_3 60 mM,

$Ca(NO_3)_2 \cdot 4H_2O$ 40mM, NH_3NH_4 7.5 mM, $NH_4H_2PO_4$ 5 mM, $MgSO_4 \cdot 7H_2O$ 10 mM, in 990 ml water with addition of 10 ml micro nutrient solution (KCl 37 µM, H_3BO_3 25 µM, $MnSO_4 \cdot H_2O$ 2 µM, $ZnSO_4 \cdot 7H_2O$ 2 µM, $CuSO_4 \cdot 5H_2O$ 0.5 µM, $(NH_4)_6Mo_7O_{27} \cdot 4H_2O$ 0.5 µM, Fe(III) EDTA 20 µM)). This corresponds to a total of 23 kg N ha^{-1} $year^{-1}$ and 1.6 kg P ha^{-1} y^{-1}. In the last week before harvest, 50 kg N ha^{-1} were added for measuring N_2O production (data not shown here). During plant growth, the chlorophyll content of wheat was measured by a chlorophyll meter (SPAD-502, Konica Minolta, Japan) after 14, 30, 45, 60, and 75 days.

3.5 Harvest

Wheat was harvested after 12 weeks, and plant weight (shoot and root separately) and yield (grain weight) were determined. The number of inflorescences (ears) was counted, shoots were cut and dried at 70 °C until constant weight and dry weight was determined. Grains were weighed, ground in a ball mill (MM400, Retsch, Germany) and phosphorus content was determined (Supplementary Information Text S1). Roots were washed with tap water, cut in 1 cm pieces, mixed in water, and a random subsample of approximately 1 g wet weight was taken to assess AMF colonization. The root samples for determination of AMF colonization were weighed and then stored in a falcon tube containing 10 ml 50% ethanol. The remaining roots were weighed wet and then dried as the shoots. The dry weight of root samples used for AMF quantification was later calculated using the wet/dry weight ratio of the remaining roots and was added to the total root dry weight. Soil cores (n=2) from each pot were taken and separated in three depths (0-5, 5-10 and 10-15 cm) for assessing titanium concentrations as shown by Gogos et al. (2016). The remaining soil was mixed, sieved through a 2 mm sieve and a subsample of 500 mg was put into 1.2 ml DNA extraction buffer in 2 ml Eppendorf tubes with 0.5 g glass beads (0.1 mm) as described by Bürgmann et al. (2001) but without using dithiotreitol. Samples were then stored at -20 °C until DNA extraction. Soil samples of 10 g of each pot were taken to determine soil dry weight.

3.6 Root colonization by arbuscular mycorrhizal fungi

Root samples that were stored in 50% ethanol, were stained to determine AMF colonization according to Vierheilig et al. (1998). Shortly, roots were rinsed with deionized water, and transferred to 10 ml 10% KOH for 20 min at 80 °C. Roots were rinsed again with water and stained in 5% ink (Parker Quink, black) in vinegar for 15 min at 80 °C. After rinsing the stained roots, they were transferred to 50% glycerol for storage until root colonization was assessed.

Glass slides were prepared by lining the root pieces parallel to each other onto the slide, some drops of 50% glycerol were added, and the roots were covered with a glass slide (McGonigle et al., 1990). AMF colonization was counted for 100 root intersections. Thereby hyphae, arbuscules, vesicles and both arbuscules and vesicles were counted. Vesicular, arbuscular and total colonization were assessed for 5 randomly chosen replicates of each treatment.

3.7 DNA extraction

DNA of the soil samples treated with 1000 mg kg^{-1} TiO_2 NPs, bulk TiO_2, and controls were extracted as described by Bürgmann et al. (2001) with some adjustments as outlined below. The soil samples were extracted three times. The first time with the extraction buffer as described by Bürgmann et al. (2001) followed by two times extraction with 9.78 µl Sodium-Phosphate buffer (mp biomedicals, USA, Art. No. 116560205) and 122 µl MT buffer (mp biomedicals, USA, Art. No. 116511202). The supernatant of the three extractions were pooled and 2 ml chloroform were added, samples were mixed and centrifuged for 5 min at 13000 x g. The aqueous phase was transferred into a new tube and 3 ml precipitation solution (20% PEG 6000 and 2.5 M NaCl) was added, mixed and incubated for 1h at 37 °C followed by 15 min centrifugation at 13000 x g. The supernatant was refused and the pellet was washed with 70% cold ethanol. Pellets were air-dried and 1 ml TE-buffer (10 mMTris, 1 mMEDTA, pH 8) was added per g soil dry weight. DNA was cleaned using the Nucleo Spin

gDNA cleanup kit (Marcherey-Nagel, Germany) and quantified by fluorescence spectroscopy (Cary Eclipse, Varian, Australia) using Quant-iT PicoGreen (Invitrogen, USA). DNA concentration was adjusted to a final concentration of 2 ng μl^{-1} and stored at -20 °C.

3.8 PCR and DNA Sequencing

The V3-V4 region of the prokaryotic 16S rRNA gene (bacteria and archaea) was amplified with variants of primers 341F (CCTAYGGGDBGCWSCAG) and 806R (GGACTACNVGGGTHTCTAAT) recently published by Frey et al. (2016), while the ITS2 region of the eukaryotic ribosomal operon (fungi and some protists) was amplified with degenerate versions of primers ITS3 (CAHCGATGAAGAACGYRG) and ITS4 (TCCTSCGCTTATTGATATGC) recently published by Tedersoo et al. (2014). The 16S rRNA primers, initially designed for targeting bacteria, were modified in order to maximize detection of archaeal sequences without compromising detection of bacterial sequences; however, due to the low coverage in the databases, archaeal sequences might still be underrepresented. The ITS2 primers, initially designed for fungi, also target some protistan groups, which is why we refer to micro-eukaryotes, but without claiming to recover the majority of these non-fungal groups. The 5' ends of the primers were tagged with the CS1 (forward primers) and CS2 (reverse primers) adapters required for multiplexing samples using the Fluidigm Access Array™ System (Fluidigm, South San Francisco, CA, USA). The PCR conditions to amplify the 16S rRNA gene fragments consisted of an initial denaturation at 95°C for 10 min, 36 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 40 s and elongation at 72°C for 1 min followed by a final elongation at 72°C for 10 min. The PCR conditions to amplify the ITS2 fragments consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation (95°C for 40 s), annealing (58°C for 40 s) and elongation (72°C for 1 min) steps with a final elongation step at 72°C for 10 min. PCR was performed on a C1000 Touch Thermal Cycler (BIO-RAD, USA) in a volume of 50 µl. First, 3.5 µl ddH2O, 1.5 µl BSA and 10 µl DNA (2 ng µl) were incubated for 5 min at 90°C to bind PCR-inhibiting substances. Subsequently, 23.1 µl ddH2O, 5 µl 10x Buffer (15mM $MgCl_2$, Quiagen, Germany), 2 µl $MgCl_2$

(25 mM), 1 µl dNTP-Mix, 1 µl primer forward (µM), 1 µl primer reverse (10 µM), 1.5 µl BSA and 0.4 µl Quiagen HotStarTaqPlus (5U μl^{-1}, Quiagen, Germany) were added to the solution. The quality of the PCR product was confirmed by electrophoresis in 1.4% (v/w) agarose gels using ethidium bromide for staining. Each PCR was repeated four times and technical replicates were pooled for sequencing. Amplicon pools were sent to the Génome Québec Innovation Center at McGill University (Montréal, Canada) for barcoding using the Fluidigm Access Array technology (Fluidigm, South San Francisco, CA, USA) and paired-end sequencing on the Illumina MiSeq v3 platform (Illumina Inc., San Diego, CA, USA).

3.9 Bioinformatics

Quality filtering and clustering into operational taxonomic units (OTUs) was performed using a customized pipeline(Frey et al., 2016) based on USEARCH v.8 (Edgar, 2010, 2013) and other tools. In brief, paired-end reads were merged using the USEARCH (Edgar and Flyvbjerg, 2015) and primers were trimmed using Cutadapt (Martin, 2011) allowing for one mismatch. Reads not matching the primers or with read lengths below 300 ($16S_{V3V4}$) or 200 bp (ITS2) were discarded. Trimmed reads were quality-filtered in USEARCH using a maximum expected error threshold of one. After strict dereplication, singleton reads were removed and the remaining sequences were clustered into OTUs at 97% sequence identity using the USEARCH *cluster_otu* function including an “on-the-fly” chimera detection (Edgar, 2013; Edgar and Flyvbjerg, 2015). OTU centroid sequences, i.e. seed sequences being representative of each OTU, were subjected to an additional round of chimera filtering by running UCHIME (Edgar et al., 2011) against customized versions of the GREENGENES (DeSantis et al., 2006) and UNITE (Nilsson et al., 2015) database, respectively. The remaining centroid sequences were tested for having prokaryotic or eukaryotic ribosomal signatures using V-Xtractor (Hartmann et al., 2010) or ITSx (Bengtsson-Palme et al., 2013), and centroid sequences with no ribosomal signatures were discarded. All quality filtered reads that remained after the filtering step were mapped to the final centroid sequences using the *usearch_global* algorithm (*maxrejects 0*, *maxaccepts 0*, *top_hit_only*) in

USEARCH. Centroid sequences were queried against selected reference databases for taxonomic assignment using the naïve Bayesian classifier (Wang et al., 2007) implemented in MOTHUR (Schloss et al., 2009) and a minimum bootstrap support of 60%. Prokaryotic $16S_{V3V4}$ sequences were queried against GREENGENES (DeSantis et al., 2006; McDonald et al., 2012), whereas eukaryotic ITS2 sequences were first queried against a custom-made ITS2 reference database retrieved from NCBI GenBank (Benson et al., 2005) and sequences assigned to fungi were subsequently queried against UNITE (Abarenkov et al., 2010). The 16S rRNA primers potentially amplify ribosomal DNA from eukaryotic organelles (chloroplast, mitochondria), whereas the ITS2 primers potentially amplify ribosomal DNA from plants (Viridiplantae) and soil animals (Metazoa). OTUs assigned to these taxonomic groups as well as OTUs not classified beyond the eukaryotic superkingdom level were removed from further analysis. Raw sequences have been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB13134.

3.10 Statistics

Between-treatment variation in prokaryotic and micro-eukaryotic community structure (β-diversity) were measured by Bray-Curtis similarities calculated from OTU abundances using a 1000-fold iterative subsampling approach implemented in MOTHUR (Frey et al., 2016). As recommended by Andersen et al. (2003), unconstrained as well as constrained multivariate statistical tests were applied to measure differences in community structure (Anderson et al., 2011). More precisely, as outlined in previous studies (Hartmann et al., 2014; Hartmann et al., 2015), principle coordinate analysis (PCO), canonical analysis of principal coordinates (CAP), permutational analysis of variance (PERMANOVA), analysis of multivariate dispersion (PERMDISP), and analysis of similarity (ANOSIM) were applied with 10^5 permutations using the homonymous routines in Primer7 (Clarke and Gorley, 2015). P values were adjusted for multiple testing using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) using the R function *p.adjust* (R Core Team, 2014). Effects on phylum level were assessed on the sum of relative abundances per phylum per pot using

PERMANOVA. Correlation-based indicator species analysis was performed with the R package *indicspecies* (De Cáceres and Legendre, 2009) with 10^5 permutations and 5 orders of group combinations (De Cáceres et al., 2010). Indicator status for rare OTUs is difficult to assess. Therefore OTUs with an abundance > 10 were used for the *indicspecies* test. P values were not adjusted for multiple testing, but a significance level of $p<0.01$ was chosen. Taxonomic trees were constructed in Cytoscape (Shannon et al., 2003) based on the taxonomic path of each OTU using the app *Allegro layout*. Fort treatment association network, a force-directed layout was used with correlations (*indicspecies*) as forcing variable. Estimates of alpha diversity (within treatment diversity) was assessed by observed richness S_{obs} and Smith-Wilson evenness $E_{var.}$ These values were calculated from the OTU abundance table using a 1000-fold iterative subsampling approach implemented in MOTHUR. Univariate PERMANOVA was performed for both richness and evenness based on Euclidean distances using Primer7.

The statistical analyses for plant and AMF endpoints were performed with the program R (R Core Team, 2014). A generalized linear model with block as error variable was applied for comparing differences to the control treatment, if the residuals were normally distributed and the data was homogenous. In the cases where these assumptions were not fulfilled, a Mann-Whitney U test was applied. For count data, e.g., the number of inflorescences, a generalized linear model with Poisson distribution was used. P values were corrected for multiple testing according to Benjamini et al. (1995) using *p.adjust* in R.

4. Results

4.1 Effects of NPs on variation of community structures and α-diversity

In total 581,576 $16S_{V3V4}$ and 2,363,759 ITS2 high-quality sequences were obtained. These sequences clustered into 3,603 prokaryotic OTUs as well as 1,295 micro-eukaryotic OTUs

(raw data ENA No. PRJEB13134). Average number of sequences (± standard deviation, n=35) per sample were 16,616±6,232 and 67,536±24,702 for the prokaryotic and micro-eukaryotic datasets, respectively. Average number of OTUs was 1,919±268 and 560±46, respectively. Application of TiO_2 NPs did not affect the prokaryotic and micro-eukaryotic α-diversity (i.e. richness and evenness) when compared to the control treatment (Table S1). Only the $ZnSO_4$ treatment (positive control) decreased the prokaryotic richness when compared to the control (Table S1). In contrast to these minor effects on α-diversity, TiO_2 NP application significantly altered the community structure of prokaryotes ($p<0.001$) but not of micro-eukaryotes ($p=0.61$, Figure 1 and S2, Tables S2 and S3). The largest shift compared to the control treatment was observed for the positive control ($ZnSO_4$), but treatments E171, P25 and bulk TiO_2 revealed significantly different community structures as assessed by ANOSIM and PERMANOVA (Tables S2 and S3).

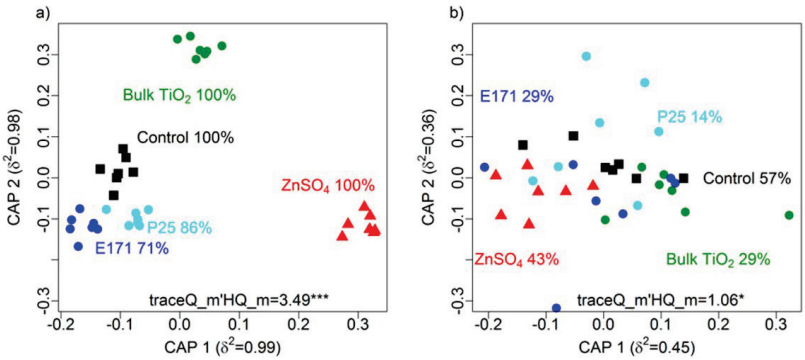

Figure 1: Effects of NP application at the highest concentration (1000 mg NPs kg^{-1}) on prokaryotic (a) and micro-eukaryotic (b) community structures. *CAP* ordinations based on Bray-Curtis similarities calculated from OTU abundances maximize discrimination among the different NP treatments. The canonical correlation (δ^2) of each *CAP* axis is given in parentheses. The *CAP* reclassification rates (%) for each treatment are provided next to the treatment label. The traceQ_m'HQ_m statistics (sum of canonical eigenvalues) is given in the plots and significant p-values are indicated by asterisks (*≤0.05 and ***≤0.001). Treatments include the negative control (black squares), the positive control $ZnSO_4$ (red triangles), the bulk TiO_2 control (green circles), as well as the two TiO_2 NPs P25 (turquoise circles) and E171 (blue circles). The circles stand for TiO_2 treatments.

Among the 3,603 prokaryotic and 1,295 micro-eukaryotic OTUs, a total of 3,563 and 1,253 OTUs, respectively, were assigned at the phylum level. For prokaryotes 3,495 (97%), 3,005 (83%), 1,843 (51%), 667 (19%) and 63 (2%) OTUs could be assigned at the taxonomic levels of class, order, family, genus and species, respectively. 40 (1%) OTUs remained unclassified. For ITS2, these values were 1,150 (89%), 1,068 (82%), 983 (76%), 900 (69%) and 774 (60%). 42 OTUs (3%) remained unclassified. The abundances of these OTUs were tested for correlation with the treatments. A total of 153 prokaryotic and 28 micro-eukaryotic OTUs could be associated to a treatment or a group of treatments (Figure 2). Visual inspection of the taxonomic trees (Figure 2) suggests that members of Actinobacteria were

more frequently affected than those of other phyla. Sensitive to NPs only (P25 and E171) were 25 OTUs. Sensitive to P25 was one Nocardioidaceae (family) OTU, one Acidobacteria OTU (Sva0725, order), one OD1 OTU (ZB2, class), one Gemmatimonadetes OTU (Ellin5301, family), one Euryarchaeota OTU (E2, order), 3 Ascomycota OTUs (Sordariomycetes, Cistella_sp_KUS_F52527, Hypocrea), one Basidiomycota OTU (*Leucogyrophana pinastri*), one Glomeromycota OTU (Diversisporaceae, family), one Zygomycota OTU (*Mortierella exigua*), and one unknown bacterium OTU. For E171 sensitive OTUs were two Ascomycota OTUs, i.e., Eurotiomycetes (class) and Penicillium (genus), one Basidiobolus OTU (genus), one Planctomycetes OTU (WD2101, order), one Agromyces (genus) OTU, and one unknown bacterium OTU. Associated to both, E171 and P25, were one Streptomyces (genus) OTU, one OD1 OTU (ZB2, class), one Rhodospirillaceae (family) OTU, one Ellin6529 (class) OTU, one Dolo 23 (family) OTU, one Anaeromyxobacter (gensu) OTU, and one Bacillales (order) OTU. Treatment effects at phylum level were assessed for the ten most abundant prokaryotic phyla (Figure 3). Actinobacteria and Chloroflexi revealed a shift in relative abundances from the controls for P25 and bulk TiO_2. Verrucomicrobia were sensitive to bulk TiO_2 and $ZnSO_4$. For the micro-eukaryotic phyla, no differences to the control could be detected (Figure S3). Focusing on the phylum Glomeromycota, which contains the ecological important group of AMF, 74 OTUs including 13,549 sequences were found (0.6% of the total sequences). One of these OTUs was associated with P25 and was of the family Diversisporaceae and contained 255 sequences.

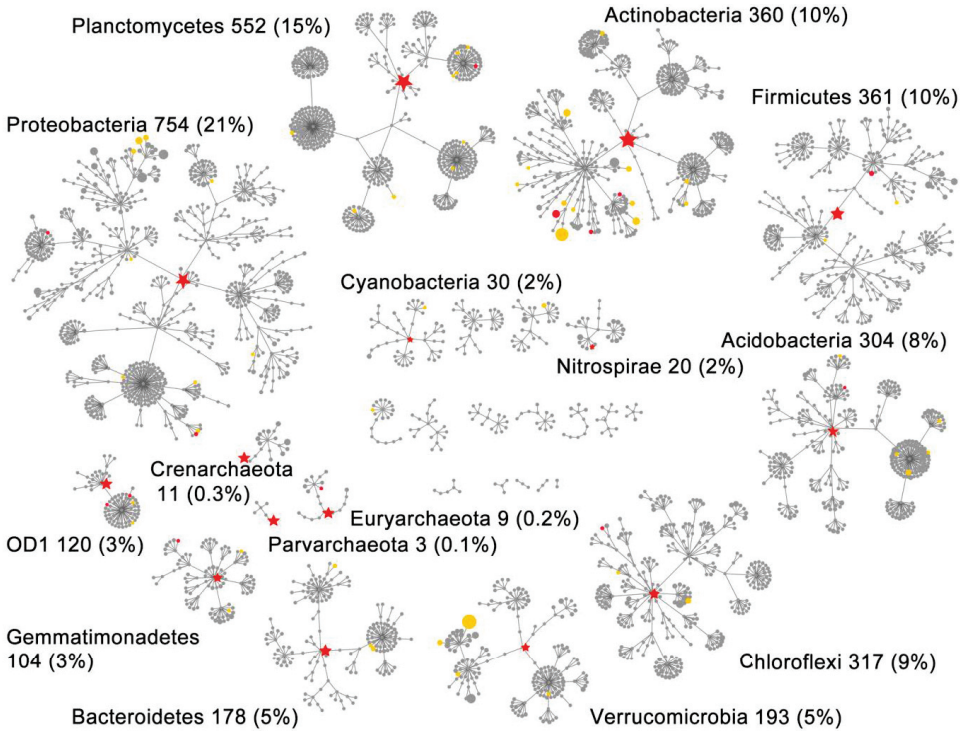

Figure 2: Taxonomic dendrograms of the detected prokaryotic OTUs excluding OTUs with abundance ≤10. Phyla are separated as single trees labeled with names, OTU counts, and percent relative abundance in brackets (including rare OTUs with read count <10). Terminal nodes represent the OTUs and their size is in proportion to their relative abundance (square root). Red asterisks indicate the root of the phylum and lines indicate connections from phylum to class, order, family, genus, and species level. OTU nodes associated with NPs are indicated in purple and OTUs associated to a group of treatments, containing at least one NP, are indicated in orange ($p<0.01$).

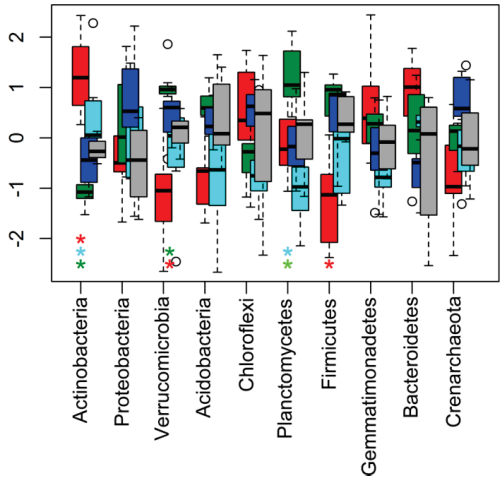

Figure 3: Treatment effects on the prokaryotic community at phylum level. Relative abundances were z-transformed and analyzed per phylum using PERMANOVA. Asterisks indicate statistically significant ($p<0.05$) differences, compared with the control, in the color of the treatment which is different. Controls are shown in grey, P25 in turquoise, E171 in blue, bulk TiO_2 in green and $ZnSO_4$ in red.

4.2 Symbiosis with AMF

Focusing on one group of microorganisms associated with plants, the wheat root colonization of AMF was determined as well as phosphorus content of wheat grains as a measure of nutrient acquisition by plant roots and AMF. The application of TiO_2 NPs did not influence the ability of wheat to form a symbiosis with AMF. Total root colonization did not differ between plant roots of the control treatments and those in soils treated with TiO_2 NPs (Figure 4). The phosphorus content of the wheat grains treated with TiO_2 NPs and $ZnSO_4$ were also not significantly different from the control treatment (Figure 4).

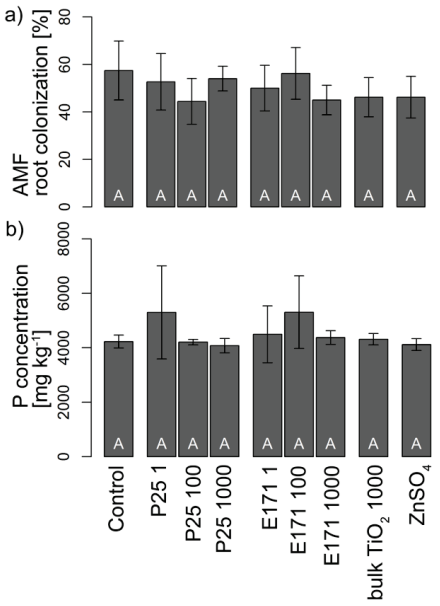

Figure 4: a) Total root colonization (%) by arbuscular mycorrhizal fungi (n=5), and b) phosphorus content of wheat grains (n=7) at the end of the three month exposure for the control, P25 and E171 in three concentrations (1, 100 and 1000 mg kg^{-1} soil), Bulk TiO_2 and positive control $ZnSO_4*7H_2O$ at 1000 mg kg^{-1}. Error bars show standard deviations and significant differences are shown with letters ($p<0.05$).

4.3 Biomass and plant health

In addition to the effects on microbial communities, the impact of NP application on plant performance was investigated. Root and shoot dry weight of wheat were unaffected by the different TiO_2 NPs (Figure 5, Table S4). The positive control with 1000 mg kg^{-1} $ZnSO_4*7H_2O$ increased the shoot dry weight significantly ($p<0.001$) by 14%. Average chlorophyll contents of wheat leaves of E171 at 1 and 100 mg kg^{-1} and P25 at 1 and 1000 mg kg^{-1} were significantly ($p<0.001$) increased after 30 d by 6%; however, after 45 days, chlorophyll contents of plants grown in TiO_2 NP treated pots, did not differ anymore from control pots

(Figure S4). The number of ears and their dry weight were not affected by any of the TiO_2 NP treatments (Table S4).

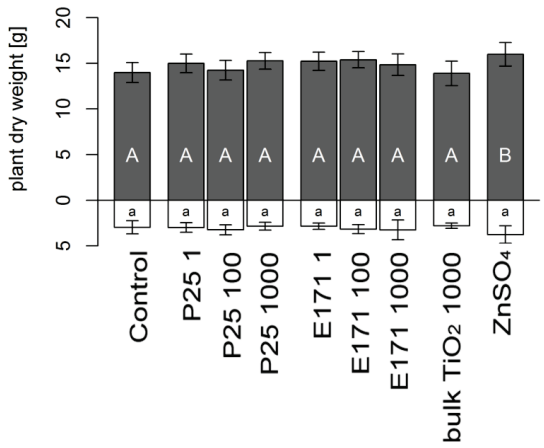

Figure 5: Wheat plant dry weight divided in shoot (grey), and root (white) at the end of the three month exposure. Results are shown for control, P25 and E171 in three concentrations, i.e., 1, 100 and 1000 mg kg^{-1} soil, Bulk TiO_2 and the positive control $ZnSO_4*7H_2O$ at 1000 mg kg^{-1}. Error bars show the standard deviations (n=7). Capital letters show significant differences for shoots, and small letters for roots compared to the control ($p<0.05$).

5. Discussion

This study demonstrates that the addition of nanoparticles to the soil changed the community structure of prokaryotes but not micro-eukaryotes. This suggests that prokaryotes are more sensitive to TiO_2 NPs and $ZnSO_4$ than micro-eukaryotes, at least during the three month of exposure. NPs have been reported to interact with bacterial surfaces (Neal, 2008). The difference in susceptibility of bacteria and fungi might potentially be attributed to different interactions of NPs with their surfaces, e.g., cell walls containing chitin (fungi) or murein (bacteria). However, more research is needed to understand the mechanisms. The observed

effects on prokaryotes are in agreement to other studies testing TiO_2 NPs (Ge et al., 2011; Ge et al., 2012; Shah et al., 2014). However, in contrast to no effects on micro-eukaryotic community structure in our experiment, effects of TiO_2 NPs (40-60 nm) on AMF community structure (18S rRNA, TRFLP) have been found in a previous study (Burke et al., 2014). In these studies other crops were used, i.e., soybean and maize, which might influence soil microorganisms and interactions with NPs due to their different root exudates. For bacteria communities, it has been reported that they were differently affected by NPs when exposed with or without soybeans growing in the soil (Ge et al., 2014). However, the mechanisms of how plant exudates, NPs and bacteria interact with each other are not known yet.

TiO_2 NPs had a negative effect on the abundance of four prokaryotic OTUs and increased the abundance of 15 prokaryotic and six micro-eukaryotic OTUs. Three micro-eukaryotic OTUs belonged to the phylum Ascomycota. However, when treatment effects were investigated on phylum level, Ascomycota showed no differences between the NP treatments and the control. For prokaryotes, treatment associated OTUs belonged to the phyla Actinobacteria, Acidobacteria, Firmicutes, Gemmatimonadetes, Chloroflexi, Cyanobacteria, OD1, Planctomycetes, Proteobacteria, TM7, Euryarchaeota, Tenericutes, WS3, and Verrucomicrobia (Figure 2). For Actinobacteria and OD1, these TiO_2 NP effects were also found at the phylum level (Figure 3). The causes for decline or increase of these taxa are unknown; it might be related to the abiotic environment (see below) or because of biotic effects such as altered root biomass or root exudation patterns (e.g. the wheat biomass was slightly but not significantly higher in most NP treatments). Long-term exposure experiments are required to draw more solid conclusions regarding the susceptibility of microorganisms to increased NP loads in soil. In this respect it is important to mention that other studies demonstrated that duration of exposure influences the effects on bacterial community structure, with higher effects after 60 days compared to 15 days of exposure (Ge et al., 2011; Ge et al., 2012). Notably, our observation that 25 microbial taxa were significantly affected by TiO_2 NP applications suggest that such taxa could be used as “bio-

indicators" for TiO_2 NP applications if further studies confirm that they are indeed sensitive to TiO_2 NP applications. Future studies also need to investigate the ecological role of such microbes and how they function in microbial networks (van der Heijden and Hartmann, 2016).

Factors such as primary particle size and crystal structure of the particles potentially drive the effects of NPs on microbial communities. Assessing the influence of particle size and crystal structure using multivariate statistical techniques in our study revealed that the treatment response of the prokaryotic communities was more similar for E171 and P25 compared to bulk TiO_2 (Figure 1). Bulk TiO_2 particles have a larger diameter than E171 and P25, which might be one reason why prokaryotes responded differently. Moreover, these particles differ in their crystal structure, with E171 and bulk TiO_2 consisting of 100% anatase and P25 being a mixture of 21% rutile and 79% anatase. *PERMANOVA* (Table S2) revealed that the similarity of prokaryotic communities between P25 and bulk TiO_2 was smaller than the one of E171 and bulk TiO_2, which suggests a potentially different effect of the crystal structure. However, the similarity between bulk TiO_2 and E171 was the same as between E171 and P25. Therefore our findings suggest that both primary particle size and crystal structure might trigger the community shift with primary particle size being the more important factor (Figure 1, Tables S2 and S3). Shah et al. found that the different crystal structures of TiO_2 NPs, i.e. anatase and rutile, affected bacterial community structure assessed by pyrosequencing. Rutile (55 nm) revealed stronger effects compared to the control than the smaller anatase particles (5-10 nm) (Shah et al., 2014). Physical interactions between NPs and other surfaces, such as heteroaggregation with soil particles, are also potentially important factors determining the impact on the microbial community. The NPs can potentially interact with the surrounding surfaces and, thus, differences in soil texture could also explain the different findings compared to Shah et al. (2014). For example, it has been reported that soil types determine the effect size of TiO_2 NPs on microbial abundance as assessed by quantitative PCR (Simonin et al., 2015). In loamy soils, bacterial abundance

decreased, while it remained unaffected in sandy loam and silty clay soils. Additionally to different soil types, also farming systems approaches with, e.g., different fertilization levels, have been reported to change microbial communities (Alguacil et al., 2008; Lumini et al., 2010; Verbruggen et al., 2010; Leff et al., 2015). Further research is needed to test how and if farming system type interacts with NP effects on soil microorganisms.

In addition to the micro-eukaryotic community structures, we specifically assessed the effects of TiO_2 NPs on one important group of fungi, i.e. AMF, in more detail by counting root colonization and determining phosphorus content of grains (Figure 4). No effects of TiO_2 NPs on the colonization of roots and phosphorus content of wheat grains were observed ($p=0.56$). These results suggest that the functionality of phosphorous acquisition of the AMF was not affected by the TiO_2 NPs. In agreement to our study Burke et al (2015) found no effects of TiO_2 NPs on AMF communities colonizing roots of soybeans.

Shoot biomass of the wheat plants was not affected by TiO_2 NPs (Figure 5). In contrast, Du et al. showed that wheat shoot biomass decreased by 13% after TiO_2 NP treatment (90 mg kg^{-1}, 20±5 nm) for six months (Du et al., 2011). Several reasons may account for the deviating results in the two studies, such as the use of aged TiO_2, different soil properties, different wheat varieties and extended exposure time in the study of Du et al. (2011). The $ZnSO_4$ treatment increased the wheat biomass. It is known, that with increasing pH, the solubility of Zn in soils decreases (Lindsay, 1972), which might be the reason why in our experiment with a high soil pH of 7.7 the 1000 mg kg^{-1} $ZnSO_4$ treatment acted as a fertilizer for wheat rather than a toxin. However, for prokaryotes the $ZnSO_4$ treatment worked as positive control and community structure was significantly different from the negative control.

Applied NP concentrations in our experiment (Gogos et al., 2016) were relatively high compared to expected environmental concentrations (Sun et al., 2014). However, in our experiment vertical transportation of the NPs in the pots was not statistical significant (Gogos

et al., 2016) and thus it is probable that the TiO_2 NPs accumulate over time. Therefore, it will be important to investigate more long-term effects on the plant-microbiome system before we can draw more solid conclusions on the impact of TiO_2 NPs on the environment.

6. Acknowledgments

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7. Supplementary Information

Text S1: Phosphorus content of wheat grains

To determine the phosphorus content of wheat grains, 200 mg ground grains were digested by heating them together with 15 ml 70% HNO_3 at 120 °C for 90 min in a digestion block (DigiPREP, SCP Science, Quebec, Canada). When the samples reached room temperature, 3 ml 30% H_2O_2 was added and the digestion block was heated again to 120 °C for 90 min. The solution was diluted with Milli-Q water to 50 ml and phosphorus was measured by inductively coupled plasma optical emission spectrometry (Acros FHS 16, Spectro Analytical Instruments, Kleve, Germany). For quality control, we analyzed a plant reference material (IPE 198 2014.3, WEPAL, Wageningen, Netherlands). Analytical recoveries for P were >95%.

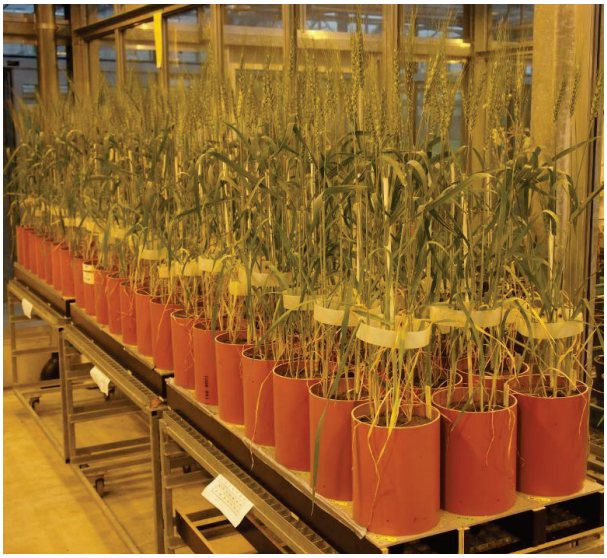

Figure S1: Picture of the wheat exposure experiment two weeks previous to the harvest.

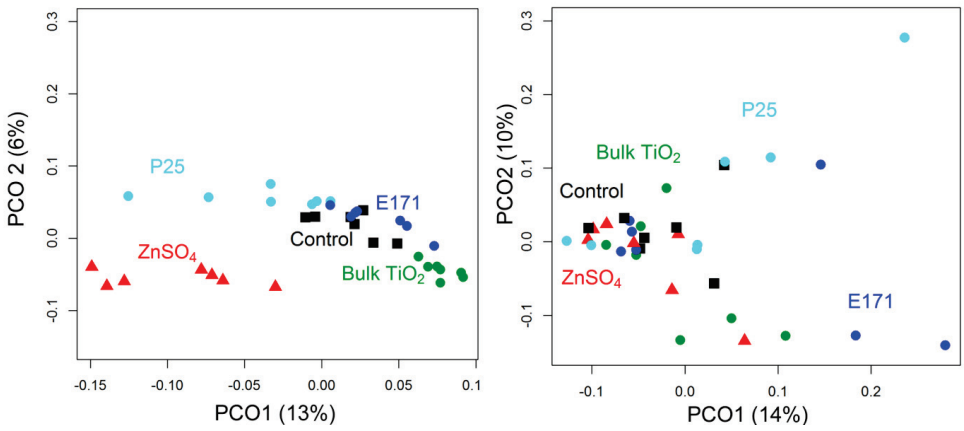

Figure S2: Effects of NP application at the highest concentration (1000 mg NPs kg^{-1}), on between treatment variation of prokaryotes (a) and micro-eukaryotes (b). Unconstrained *PCO* ordinations of Bray-Curtis similarities calculated from OTU abundances are presented. The variance explained by each *PCO* axis is given in parentheses.

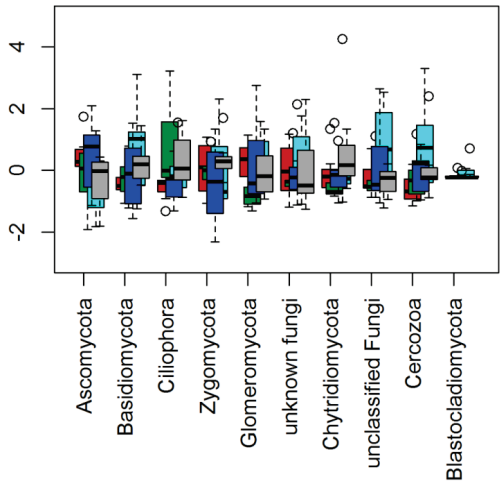

Figure S3: Treatment effects on the micro-eukaryotic community at phylum level. Controls are shown in grey, P25 in turquoise, E171 in blue, bulk TiO_2 in green and $ZnSO_4$ in red.

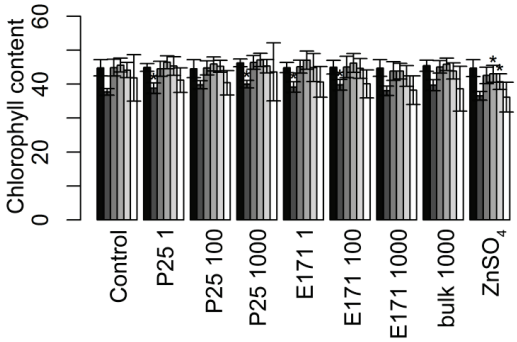

Figure S4: Chlorophyll content (SPAD) over time of wheat in control pots, TiO_2 NP treated pots, e.g., P25 and E171 in three concentrations, bulk TiO_2, and in $ZnSO_4 \cdot 7H_2O$ treatment. For each treatment measurements at days 14, 30, 45, 60, 75 and 82 are shown in grey levels with increasing brightness. Statistical significant differences between the control plants and the TiO_2 NP treated plants at the same time point are indicated by asterisks and standard deviations are shown by the error bars.

Table S1: Effects of the different treatments on alpha-diversity of prokaryotes and micro-eukaryotes.

Main test[a]	Prokaryotes		Micro-eukaryotes	
	Richness (S_{obs}) F(p)	Evenness (E_{var}) F(p)	Richness (S_{obs}) F(p)	Evenness (E_{var}) F(p)
Block (F_1, 25)	1.3 (0.258)	0.3 (0.612)	0.4 (0.532)	0.3 (0.599)
Treatment (F_4, 25)	17.7 (**<0.001**)	2.5 (0.066)	0.9 (0.488)	3.7 (**0.016**)
Block*Treatment (F_4, 25)	2.2 (0.099)	1.1 (0.378)	0.3 (0.884)	0.6 (0.655)
Treatments[b]	mean±SD	mean±SD	mean±SD	mean±SD
Control	1459±18^{A}	0.191±0.006^{A}	476±32^{A}	0.487±0.006^{AB}
P25	1453±22^{A}	0.185±0.008^{A}	470±31^{A}	0.482±0.008^{A}
E171	1460±26^{A}	0.186±0.007^{A}	461±48^{A}	0.487±0.004^{A}
bulk TiO_2	1473±19^{A}	0.191±0.005^{A}	447±26^{A}	0.493±0.002^{B}
$ZnSO_4$	1392±22^{B}	0.196±0.006^{A}	478±19^{A}	0.488±0.003^{A}

[a]Effects of main factors, i.e. block (1 and 2, error factor) and Treatment (control, P25, E171, BulkTiO_2 and $ZnSO_4$) assessed by analysis of variance (PERMANOVA on Euclidean dissimilarities). Degrees of freedom and corresponding error term are given in brackets. Values represent F ratio (F) and the level of significance (p).

[b]Average richness and evenness (mean±SD, n=7) for each treatment. Different letters indicate statistical significant differences ($p<0.05$) with adjusted p values.

Table S2: Effects of the different treatments on microbial community structure, assessed by *PERMANOVA*.

Main test[a]	Prokaryotes			Micro-eukaryotes		
	F	P	VC	F	P	VC
Block (F_1, 25)	1.3	**0.051**	8.6	0.9	0.611	-6.3
Treatment (F_4, 25)	2.3	**<0.001**	26.7	1.2	0.072	12.7
Block*Treatment (F_4, 25)	1.0	0.426	1.8	1.1	0.245	11.9
Treatments[b]	t	P_{adjust}	Øsim [%]	t	P_{adjust}	Øsim [%]
control vs. P25	1.2	**<0.001**	74	0.93	0.870	63
control vs. E171	1.1	**0.008**	75	0.96	0.691	64
control vs. bulk TiO_2	1.3	**<0.001**	75	1.13	0.148	67
control vs. $ZnSO_4$	1.7	**<0.001**	73	1.20	0.073	67
P25 vs. E171	1.3	**<0.001**	74	0.95	0.737	58
P25 vs. Bulk TiO_2	1.7	**<0.001**	72	1.18	0.148	61
bulk TiO_2 vs.E171	1.3	**<0.001**	74	1.02	0.575	63
$ZnSO_4$ vs. P25	1.4	**<0.001**	73	1.15	0.138	61
$ZnSO_4$ vs. E171	1.8	**<0.001**	72	1.14	0.184	62
$ZnSO_4$ vs. Bulk TiO_2	2.0	**<0.001**	71	1.28	0.073	66

[a]Effects of main factors and their interactions as assessed by multivariate permutational analysis of variance (PERMANOVA; degrees of freedom for each factor and the corresponding error term are given in brackets). Main factors are treatment (Control, P25, E171, bulk TiO_2 and $ZnSO_4$) and block (1, 2). Pseudo-F ratio (F), permutation-based level of significance (P) and the estimation of the variance component (VC, %) are shown. Statistical significant p values are indicated in bold ($p<0.05$).

[b]Pairwise comparison between the treatments. Univariate t-statistics and the average between-group Bray-Curtis similarities (Øsim) are shown. A Benjamini-Hochberg adjustment of the p values was performed because of multiple comparisons.

Table S3: ANOSIM and PERMDISP.

	ANOSIM				PERMDISP			
	Prokaryotes		Micro-eukaryotes		Prokaryotes		Micro-eukaryotes	
Main test[a]	R	P	R	P	F	P	F	P
Treatment	0.677	<0.001	0.044	0.069	1.8	0.187	4.30	0.0336
Treatments[b]	R	P_{adjust}	R	P_{adjust}	t	P_{adjust}	t	P_{adjust}
control vs. P25	0.5	0.001	0.0	0.740	2.06	0.031	2.74	0.070
control vs. E171	0.3	0.006	0.0	0.740	0.91	0.557	2.72	0.118
control vs. bulk TiO_2	0.8	0.001	0.1	0.250	0.69	0.557	0.47	0.873
control vs. $ZnSO_4$	0.9	0.001	0.1	0.140	1.57	0.376	0.31	0.873
P25 vs. E171	0.4	0.001	0.0	0.841	1.52	0.376	0.39	0.873
P25 vs. Bulk TiO_2	0.8	0.001	0.1	0.240	1.59	0.376	2.32	0.118
bulk TiO_2 vs.E171	0.8	0.001	0.0	0.541	0.15	0.880	2.19	0.163
$ZnSO_4$ vs. P25	0.7	0.001	0.1	0.143	0.92	0.557	2.50	0.118
$ZnSO_4$ vs. E171	0.9	0.001	0.1	0.250	0.77	0.557	2.41	0.163
$ZnSO_4$ vs. Bulk TiO_2	1.0	0.001	0.2	0.140	0.87	0.557	0.18	0.886

[a]Effects of main factors assessed by analysis of similarity (ANOSIM) and permutational analysis of dispersion (PERMDISP). Main factor is treatment (Control, P25, E171, bulk TiO_2 and $ZnSO_4$). Global test statistics (R) is given for the ANOSIM and pseudo-F ratio for PERMDISP.

[b]Pairwise comparison between the treatments. Pairwise test statistics is shown by R value for ANOSIM and univariate t-statistics for PERMDISP. A Benjamini-Hochberg adjustment of the p values was performed because of multiple comparisons.

Table S4: Statistical data of all measured variables of wheat, i.e., shoot, root and ears dry weight, number of inflorescences, total AMF root colonization and phosphorus content of grains. A glm model with block as error term was used when residuals were normal and homogenous. Otherwise a Mann-Whitney-test was applied. The calculated p-values were adjusted for multiple testing with a Benjamini-Hochberg adjustment. Statistical significant p values are indicated in bold ($p<0.05$).

	Shoot dry weight [g]		root dry weight [g]				Ears dry weight [g]		number of flowers[a]		% AMF total colonization		Phosphorus content [mg kg^{-1}]			
	t	p adj	t	p adj	Mann-Whitney	p adj	t	p adj	z	p adj	t	p adj	t	p adj	Mann-Whitney	p adj
P25 1 mg l^{-1}	1.7	0.140	0.1	0.948	18	0.911	1.2	0.505	0.1	1.00	-0.8	0.533	2.3	0.076	12	0.641
P25 100 mg l^{-1}	0.4	0.741	0.8	0.833	16	0.848	1.1	0.505	-0.2	1.00	-2.2	0.111	0	0.963		
P25 1000 mg l^{-1}	2.2	0.070	-0.3	0.833	25	1.000	1.9	0.286	-0.2	1.00	-0.6	0.646	-0	0.961	25	0.641
E171 1 mg l^{-1}	2.1	0.070	-0.3	0.833	26	1.000	1.3	0.505	0.0	1.00	-1.3	0.307	0.6	0.961	22	0.945
E171 100 mg l^{-1}	2.4	0.060	0.6	0.833	15	0.848	0.8	0.539	0.0	1.00	-0.2	0.842	2.3	0.076	16	0.747
E171 1000 mg l^{-1}	1.5	0.188	0.8	0.833	22	1.000	0.9	0.539	0.3	1.00	-2.2	0.111	0.3	0.961	14	0.641
Bulk TiO_2 1000 mg l^{-1}	-0.2	0.877	-0.5	0.833	24	1.000	0.7	0.580	-0.3	1.00	-1.9	0.125	0.2	0.961	19	0.945
$ZnSO_4$ 1000 mg l^{-1}	3.4	**0.006**	2.3	0.116	28	0.128	0.0	0.970	0.4	1.00	-1.9	0.125	-0	0.961	21	0.219

[a]glm with Poisson distribution

Chapter V

Different susceptibility of prokaryotic and eukaryotic microorganisms to carbon nanotubes and mineral nanoparticles

Janine Moll[1,2], Franco Widmer[1], Marcel G.A. van der Heijden[1,2,3], Martin Hartmann[4]

[1] Institute for Sustainability Sciences ISS, Agroscope, Zurich, Switzerland

[2] Plant-Microbe-Interactions, Department of Biology, Utrecht University, Utrecht, the Netherlands

[3] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland

[4] Forests Soils and Biogeochemistry, Swiss Federal Research Institute WSL, Birmensdorf, Switzerland

1. Abstract

Nanoparticles (NPs) are produced and used in a wide range of applications, thereby getting unintentionally released into the environment including soil systems. However, we currently have a poor understanding of how NPs impact soil microorganisms and associated ecosystem functions. Here, we evaluated the effects of three of the most produced NPs worldwide, i.e., TiO_2 NPs, carbon nanotubes and CeO_2 NPs, on soil microbial community structure using high-throughput sequencing of prokaryotic and eukaryotic ribosomal markers. Application of carbon nanotubes altered prokaryotic but not micro-eukaryotic community structures. The other NPs showed no effects on soil microbial diversity. Our results raise awareness of the effects of certain nanoparticles on the soil microbiota with currently unknown implications for associated ecosystem functions.

Keywords

Nanoparticle, carbon nanotubes, ecotoxicity, soil microorganisms, community structure

2. Introduction

Nanoparticles (NPs) are produced and used worldwide in a variety of different applications including electronics, composite materials, paints, and catalysts (Heiligtag and Niederberger, 2013). As a side-effect, these NPs get unintentionally released into the environment including the soil system (Keller et al., 2013; Sun et al., 2014). Titanium dioxide (TiO_2) NPs, carbon nanotubes (CNTs) and cerium oxide (CeO_2) NPs are among the ten most frequently manufactured NPs worldwide (Keller et al., 2013) and, therefore, high concentrations of these NPs are expected to get released into the environment. In 2010, the estimated environmental concentrations in soils were 38,200, 500, and 1,400 t y^{-1}, for TiO_2 NPs, CNTs and CeO_2 NPs, respectively (Keller et al., 2013). For Europe, Sun et al. (2014) estimated the yearly increase of NP concentrations in soils treated with sludge to be 1,200 µg kg^{-1} y^{-1} for TiO_2 NPs and 990 ng kg^{-1} y^{-1} for CNTs. NPs are in discussion to be used in plant protection formulation and fertilizers because they potentially can reduce application amounts and efficiency (Gogos et al., 2012; Khot et al., 2012; Liu and Lal, 2015). However, this additional application of NPs would lead to substantially higher concentrations of NPs in soils.

Soil microbial communities provide a range of important ecosystem functions including decomposition and transformation of organic materials and toxic compounds, nutrient cycling, or control of pests and disease (Carney and Matson, 2005; van der Heijden et al., 2008). Previous studies have raised concerns that NPs released into the environment could affect soil microbial communities and associated ecosystem functions (Ge et al., 2011; Ge et al., 2012; Shrestha et al., 2013; Burke et al., 2014). However, it is difficult to compare among these studies and to draw more universal conclusions, as they have used different NP qualities in different soil types and applied different technologies to assess microbial diversity. As far as we know, there is no study investigating the impact of CeO_2 NPs on soil microbial community structure. However, it was reported, that CeO_2 NPs negatively affected symbiotic nitrogen fixation of soybeans and rhizobia (Priester et al., 2012). It is not well understood how different types of NPs affect the soil microbiota and the industrially important

NP types TiO_2, CNT, and CeO_2 have never been tested simultaneously under the same conditions in the same system.

In this context, the current study was conducted to evaluate (1) whether and (2) which NPs applied to agricultural soil affect the microbiota, and (3) if there are specific taxa that are sensitive to any of these NPs. For this purpose, we mixed industrially relevant NPs, i.e. type P25 TiO_2, multiwall carbon nanotubes (MWCNT), and CeO_2, with an agricultural soil in a pot experiment and assessed changes in microbial community structures by sequencing different ribosomal markers on the Illumina MiSeq platform.

3. Methods

3.1 Nanoparticles used and mixing with soil

For TiO_2, P25 (Sigma Aldrich, USA, Art. No. 718467) was used as representative NP with average particle diameter of 29±9 nm (Gogos et al., 2016). Additionally, a non-nanomaterial TiO_2 (NNM-TiO_2, Sigma Aldrich, USA, Art. No. 232033) with average primary particle diameter of 145±46 nm (Gogos et al., 2016) was used as non-nanomaterial (European Commission, 2011) control. NNM are defined to contain less than 50% NPs (European Commission, 2011). Our TiO_2 NNM contained a fraction of 20% NPs. The applied MWCNTs (Cheap Tubes Inc., USA) revealed a length of 10-30 µm, outer diameter of 20-30 nm, a purity of >95% and an elemental carbon content of >98% (Gogos et al., 2016). The used CeO_2 NPs (Sigma Aldrich, USA, art. No. 700290) had a diameter of less than 50 nm with cubic crystal structure according to the manufacturer's specifications. TEM pictures and further characterization of the used NPs can be found in the study of Gogos et al. (2016). As positive control, with which effects on microbial community structure were expected, $ZnSO_4$*$7H_2O$ was chosen.

For the substrate, brown earth with a sandy loamy to loamy fine fraction was collected from an agricultural field at Agroscope Institute for Sustainability Sciences in Zurich, Switzerland

(coordinates N47° 25' 39.564" E8° 31' 20.04"). The top 5 cm were removed and the underlying 15 cm soil were collected, sieved (<0.5 cm), and mixed with quartz sand (50% v/v) and then characterized as described previously (Gogos et al., 2016). The substrate revealed a pH of 7.7, 86% sand, 6% silt, 7% clay, 0.55% organic carbon, 6 mmol+/kg soil cation exchange capacity and a maximal water holding capacity of 0.3 g H_2O/g dry soil (Gogos et al., 2016). Each of the different NPs was premixed in 300 g substrate on an overhead mixer (Turbula T2F, Switzerland) in 500 ml Schott bottles. Two bottles were filled with 15 g of P25, one bottle with 30g of NNM-TiO_2, four bottles with 22 g MWCNTs, one bottle with 25 g CeO_2 NPs and one bottle with 30 g $ZnSO_4 \cdot 7H_2O$ (Sigma Aldrich, USA, art. No. Z0251). Each of these pre-mixtures was diluted with substrate to a total volume of 30 kg and mixed in a cement mixer for 6h. These high NP concentrations were needed to being able to assess differences of titanium and black carbon content (BC) of the NP treatments from the control soil. The concentrations of total titanium, BC (for MWCNTs) and cerium were verified as described previously (Gogos et al., 2016) using x-ray fluorescence (XRF) and were 1332±100, 2059±105 and 2007±79 Ti mg kg^{-1} for the control, P25 and NNM-TiO_2 , respectively, 0.50±0.06 BC mg g^{-1} in the MWCNT treatments and 416±19 Ce mg kg^{-1} in the CeO_2 treatment at the end of the experiment.

3.2 Experimental setup and sample preparation

Cylindrical pots with a diameter of 15 cm and a length of 20 cm were filled with 500 g sand layer as drainage and 3.3 kg substrate for controls or substrate plus NPs for the different NP treatments. For each treatment, seven replications were prepared. Red clover (*Trifolium pratense* var. Merula) was germinated on filter paper for 5 days and 7 plants per pot were planted. The plants grew for 14 weeks in a greenhouse (16 h 25 °C 300W m^2, 8 h 16 °C dark) and watered regularly (water holding capacity kept between 50 and 60%). Because of the red clover plants, 10 ml fertilizer per pot (KH_2PO_4 (5 mM), $MgSO_4 \cdot 7H_2O$ (1 mM), KCl (50 µM), H_3BO_3 (25 µM), $MnSO_4 \cdot H_2O$ (1.3 µM), $ZnSO_4 \cdot 7H_2O$ (2 µM), $CuSO_4 \cdot 5H_2O$ (0.5 µM), $(NH_4)6Mo_7O_{27} \cdot 4H_2O$ (0.5 µM), and Fe(III) EDTA (20 µM)) was added after 6 and 9 weeks.

This resulted in a phosphorus addition of 1.7 kg P ha^{-1}. After 14 weeks, red clover plants were removed, the substrate of each treatment was mixed, sieved through a 2 mm sieve and a 500 mg sample was taken and put into a tube containing 0.5 g glass beads (0.1 mm) and DNA-extraction buffer as described by Bürgmann et al. (2001) without using dithiotreitol. These samples were stored at -20 °C until DNA extraction.

3.3 DNA extraction and PCR

DNA extraction was performed as described by Bürgmann et al. (2001) with some adaption. Soil samples were first extracted in extraction buffer as described by Bürgmann et al. (2001). The supernatant were collected in new tubes and the soil samples were extracted two times more using 9.78 µl Sodium-Phosphate buffer (mp biomedicals, USA, Art. No. 116560205) and 122 µl MT buffer (mp biomedicals, USA, Art. No. 116511202). 2 ml chloroform were added and samples were mixed and centrifuged for 5 min at 13000 x g. Then the aqueous phase was transferred into a new tube and 3 ml precipitation solution containing 20% PEG 6000 and 2.5 M NaCl in ddH_2O was added. After mixing the tubes they were incubated for 1h at 37 °C followed by 15 min centrifugation at 13000 x g. Supernatant was refused and the pellet was washed with 70% cold ethanol. Pellets were air-dried and 1 ml TE-buffer (10 mMTris, 1 mMEDTA, pH 8) was added per g soil dry weight. DNA was cleaned using the Nucleo Spin gDNA cleanup kit (Marcherey-Nagel, Germany). Then the DNA was quantified by fluorescence spectroscopy (Cary Eclipse, Varian, Australia) using Quant-iT PicoGreen (Invitrogen, USA). DNA concentration was adjusted to a final concentration of 2 ng $µl^{-1}$ and stored at -20 °C.

For prokaryotes, the V3-V4 region the 16S rRNA gene was amplified with variants of primers 341F (CCTAYGGGDBGCWSCAG) and 806R (GGACTACNVGGGTHTCTAAT) recently published by Frey et al. (2016). For eukaryotic microorganisms, the ITS2 region of the ribosomal operon was amplified with degenerate versions of the ITS3 (CAHCGATGAAGAACGYRG) and ITS4 (TCCTSCGCTTATTGATATGC) primers recently

published by Tedersoo et al. (2014). The 5' ends of the primers were tagged with the CS1 and CS2 adapters required for multiplexing samples using the Fluidigm Access Array™ System (Fluidigm, South San Francisco, CA, USA). PCR was performed on a C1000 Touch Thermal Cycler (BIO-RAD, USA) in a volume of 50 µl. First, 3.5 µl ddH2O, 1.5 µl BSA and 10 µl DNA (2 ng µl) were incubated for 5 min at 90°C to bind PCR-inhibiting substances. Then, 23.1 µl ddH2O, 5 µl 10x Buffer (15mM $MgCl_2$, Quiagen, Germany), 2 µl $MgCl_2$ (25 mM), 1 µl dNTP-Mix, 1 µl primer forward (µM), 1 µl primer reverse (10 µM), 1.5 µl BSA and 0.4 µl Quiagen HotStarTaqPlus (5U μl^{-1}, Quiagen, Germany) were added to the solution and PCR was conducted (95 °C 10 min, 36 cycles for 16S RNA gene or 40 cycles for ITS2 fragments of (95 °C 40 s, 58 °C 40 s, 72 °C 1 min) terminated by 72°C for 10 min). Electrophoresis in 1.4% (v/w) agarose gels using ethidium bromide for staining was performed. Each PCR was repeated four times and technical replicates were pooled for sequencing. Amplicon pools were sent to the Génome Québec Innovation Center at McGill University (Montréal, Canada) for barcoding using the Fluidigm Access Array technology (Fluidigm, South San Francisco, CA, USA) and paired-end sequencing on the Illumina MiSeq v3 platform (Illumina Inc., San Diego, CA, USA).

3.4 Bioinformatics

A customized pipeline (Frey et al., 2016) centered around USEARCH v.8 (Edgar, 2010) was used for quality filtering and clustering into operational taxonomic units (OTUs). In brief, paired-end reads were merged using the USEARCH *fastq_mergepairs* algorithm (Edgar and Flyvbjerg, 2015). Primers were detected and trimmed using Cutadapt (Martin, 2011) allowing for one mismatch and reads not matching the primers or with read lengths below 300 ($16S_{V3V4}$) or 200 bp (ITS2) were discarded. Trimmed reads were quality-filtered in USEARCH using a maximum expected error threshold of one. Singleton reads were removed prior to clustering. Sequences were clustered into OTUs at 97% sequence identity including a chimera detection algorithm using the USEARCH *cluster_otu* function (Edgar, 2013). Additionally, OTU centroid sequences were subjected to a reference-based chimera-filter by

running UCHIME (Edgar et al., 2011) against customized versions of the GREENGENES (DeSantis et al., 2006) and UNITE (Nilsson et al., 2015) database, respectively. The remaining centroid sequences were tested for having prokaryotic or eukaryotic ribosomal signatures using V-Xtractor (Hartmann et al., 2010) or ITSx (Bengtsson-Palme et al., 2013), and centroid sequences with no ribosomal signatures were discarded. All quality filtered reads that remained after the *fastq_filter* step were mapped to the final centroid sequences using the *usearch_global* algorithm (*maxrejects 0*, *maxaccepts 0*, *top_hit_only*) in USEARCH. Corresponding centroid sequences were queried against selected reference databases for taxonomic assignment using the naïve Bayesian classifier (Wang et al., 2007) implemented in MOTHUR (Schloss et al., 2009) and a minimum bootstrap support of 60%. Prokaryotic $16S_{V3V4}$ sequences were queried against GREENGENES (DeSantis et al., 2006; McDonald et al., 2012), whereas micro-eukaryotic ITS2 sequences were first queried against a custom-made ITS2 reference database retrieved from NCBI GenBank (Benson et al., 2005) and sequences assigned to fungi were subsequently queried against UNITE (Abarenkov et al., 2010). The prokaryotic primers potentially amplify ribosomal DNA from eukaryotic organelles (chloroplast, mitochondria), whereas the eukaryotic primers potentially amplify ribosomal DNA from plants (Viridiplantae) and soil animals (Metazoa). OTUs assigned to these taxonomic groups as well as OTUs not classified beyond the eukaryotic superkingdom level were removed from further analysis. Sequences assigned to bacteria, archaea, fungi, and protists were kept for further analysis. Raw sequences have been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB13135.

3.5 Statistics

Differences in community structure among the treatments (β-diversity) (Anderson et al., 2011) were assessed by calculating Bray-Curtis dissimilarities from OTU abundances using a 1000-fold iterative subsampling approach implemented in MOTHUR. This was performed to account for the differences in read counts of the different samples (Frey et al., 2016).

Principle coordinate analysis (PCO), canonical analysis of principal coordinates (CAP), permutational analysis of variance (PERMANOVA), analysis of multivariate dispersion (PERMDISP), and analysis of similarity (ANOSIM) were applied with 10^5 permutations using the homonymous routines in Primer7 (Hartmann et al., 2014; Clarke and Gorley, 2015). P-values were adjusted for multiple testing using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) using the R function *p.adjust* (R Core Team, 2014). Correlation-based indicator species analysis was performed with the R package *indicspecies* (De Cáceres and Legendre, 2009) with 10^5 permutations on relative abundances (De Cáceres et al., 2010). Raw p values are presented for the *indicspecies* assessment and no adjustment for multiple testing was applied. However, the significance level was defined to $p<0.01$ for the *indicspecies* assessment. The program Cytoscape (Shannon et al., 2003, version 3.2.1) was used for drawing taxonomic trees based on the taxonomic path of each OTU using the app *Allegro layout*. At phylum level, relative abundances per phylum per treatment were assessed using adonis (R package vegan). Estimates of alpha diversity (within treatment diversity) were assessed by observed richness S_{obs} and Smith-Wilson evenness E_{var} using a univariate PERMANOVA (Primer7) based on Euclidean distances.

4. Results

Sequencing yielded a total of 790,193 (18,814 ± 7,873) $16S_{V3V4}$ and 2,595,687 (61,802 ± 25,062) ITS2 high quality sequences corresponding to 3,945 (2,010±305) prokaryotic as well as 1,320 (504±38) micro-eukaryotic OTUs, respectively. Prokaryotic OTU richness was significantly ($p<0.001$) increased in the MWCNT-treated soils, whereas α-diversity of micro-eukaryotes in any of the treatments was not different from the control (Table S1). The MWCNT and $ZnSO_4$ treated soils revealed a distinct prokaryotic community composition compared to the other treatments and the control, whereas the micro-eukaryotic community structure was unaffected by the tested treatments (Table 1, Figure 1, Table S2).

Table 1: Results of PERMANOVA for prokaryotic and micro-eukaryotic communities.

Main test[a]	Prokaryotes			Micro-eukaryotes		
	F	P	VC	F	P	VC
Block (F_1, 25)	1.1	0.221	5.2	1.200	0.182	9.0
Treatment (F_4, 25)	1.7	**<0.001**	25.6	1.100	0.205	10.6
Block*Treatment (F_4, 25)	0.9	0.827	-12.0	1.000	0.516	-6.9
Pairwise test[b]	t	P_{adjust}	Øsim [%]	t	P_{adjust}	Øsim [%]
MWCNT vs. Control	1.5	**0.003**	72	1.0	0.662	55
MWCNT vs. P25	1.5	**0.003**	72	1.0	0.662	60
MWCNT vs. NNM-TiO_2	1.5	**0.003**	73	1.3	0.315	53
MWCNT vs. CeO_2	1.7	**0.003**	71	0.9	0.900	61
MWCNT vs. $ZnSO_4$	1.6	**0.003**	71	1.1	0.427	64
control vs. P25	1.0	0.757	75	0.9	0.900	52
control vs. NNM-TiO_2	1.0	0.266	75	0.9	0.816	46
control vs. CeO_2	1.0	0.311	74	0.9	0.900	53
control vs. $ZnSO_4$	1.2	**0.030**	73	0.9	0.816	55
P25 vs. NNM-TiO_2	1.1	0.156	75	1.2	0.427	49
P25 vs. CeO_2	0.9	0.819	74	0.8	0.975	57
P25 vs. $ZnSO_4$	1.2	**0.034**	73	1.0	0.662	60
NNM-TiO_2 vs. CeO_2	1.2	**0.034**	74	1.1	0.596	51
NNM-TiO_2 vs. $ZnSO_4$	1.4	**0.003**	74	1.5	0.302	51
CeO_2 vs. $ZnSO_4$	1.2	**0.041**	73	1.0	0.662	60

[a]Effects of main factors and their interactions as assessed by multivariate permutational analysis of variance (PERMANOVA; degrees of freedom for each factor and the corresponding error term are given in brackets). Main factors are Treatment (Control, P25, NNM-TiO_2, MWCNT, CeO_2 NPs and $ZnSO_4$) and block (1, 2). Pseudo-F ratio (F), permutation-based level of significance (P) and the estimation of the variance component (VC) are shown. Statistically significant test at $p<0.05$ are indicated in bold.

[b]Pairwise comparison between the treatments. Univariate t-statistics, Benjamini-Hochberg adjusted levels of significance (P_{adjust}), and the average between-group Bray-Curtis similarities (Øsim) are provided.

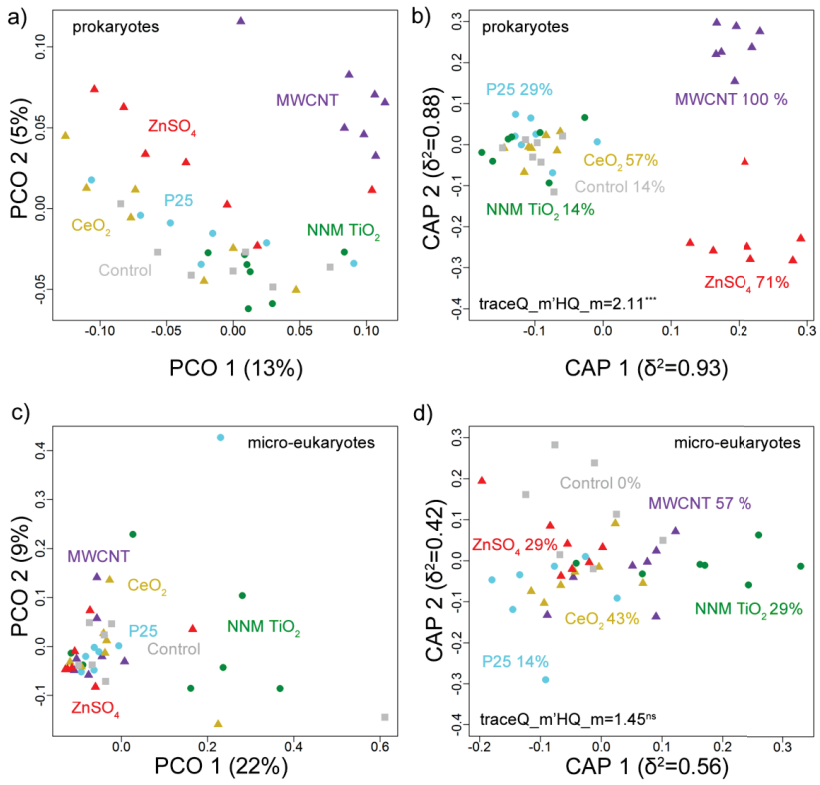

Figure 1: Nanoparticle effects on eukaryotic and prokaryotic community structures. a) and c) principle coordinate ordinations (PCO) based on Bray-Curtis distances calculated from relative OTU abundances for prokaryotes and micro-eukaryotes, respectively. The percentage explained by each axis is indicated in brackets. b) and d) show canonical analyses of principle coordinates (CAP) of prokaryotic and micro-eukaryotic communities, respectively. The canonical correlation of each axis is indicated in brackets and explains the association strength between the data points and the hypothesis of differences between different NPs. The reclassification rates (quantitative estimate of the degree of discrimination among the NP treatments achieved by the canonical axes) are indicated after the treatment name and traceQ_m'HQ_m (*** $p<0.001$) statistics are shown on the plots. In all four plots, treatments are indicated by color and are control in gray, P25 in turquoise, NNM-TiO_2 in green, MWCNT in purple, CeO_2 in yellow and $ZnSO_4$ in red.

Because both, the ordinations and PERMANOVA, revealed different prokaryotic community structures for MWCNTs compared to the control, we were interested, which prokaryotes were sensitive to this treatment. Indicator species analysis revealed 77 prokaryotic OTUs which were significantly ($p<0.01$) associated with MWCNTs (Figure 2). From these 77 OTUs, 22 revealed decreased relative abundances in the MWCNT treatment compared to the control and 55 OTUs revealed increased relative abundances. These MWCNT sensitive OTUs were spread across different phyla, i.e., Acidobacteria, Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Crenarchaeota, Euryarchaeota, Firmicutes, Gemmatimonadetes, OD1, Planctomycetes, Proteobacteria, TM7, and Verrucomicrobia (Figure 2). From the MWCNT sensitive OTUs, 19 could be assigned to the genus level. Genera of the affected OTUs included Streptomyces, Glycomyces, Mehanosarina, Nonomureae, Sediminibacterium, Shimazuella, Candidatus Nitrososphaera, Actinomadura, Planifilum, Pilimelia, Nocardioides and Clostridium. Strongest effects were found in Streptomyces where 100% of the OTUs of this genus containing 11'918 sequences revealed decreased relative abundances in MWCNT treatments. For Candidatus Nitrososphaera, relative abundances were increased in 48% of the OTUs within this genus and 2500 sequences were affected. At phylum level, the MWCNT treatment revealed significantly decreased relative abundances of Actinobacteria and "unclassified bacteria", while relative abundances of TM7, Crenarchaeota, OD1 and Euryarchaeota increased (Figure 3). For the other phyla, no statistical significant differences between treatments and control were detected.

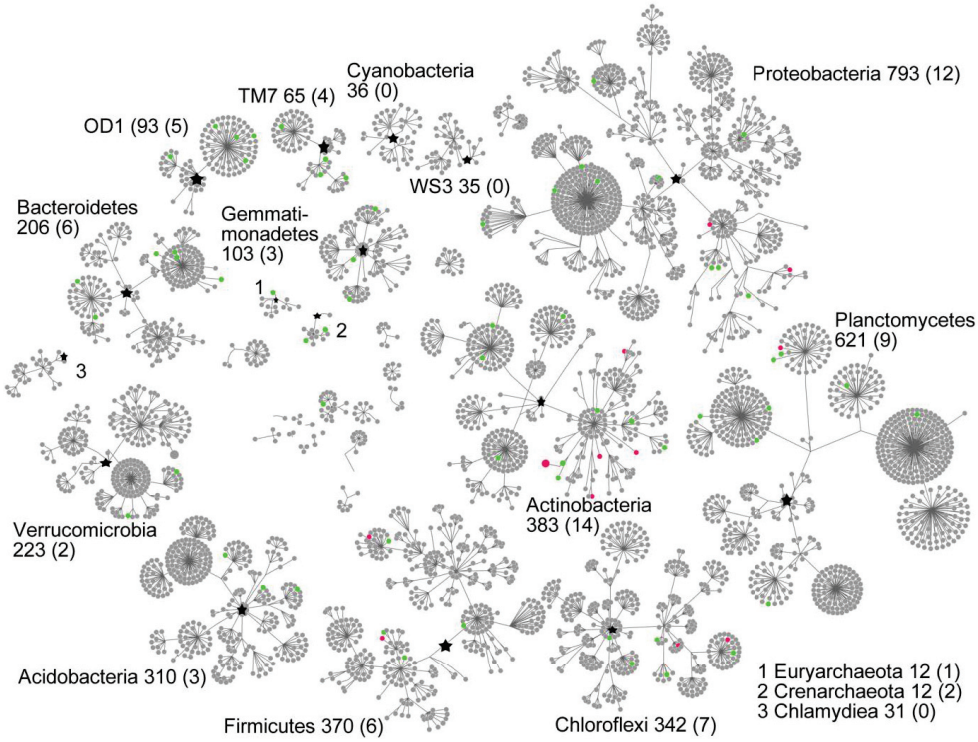

Figure 2: Taxonomic phylum-level trees of the prokaryotic OTUs detected in control and MWCNT treatments. Roots of the trees are indicated by black asterisks. The nodes indicate OTUs and their size corresponds to their relative abundance (square root). OTUs, which were significantly ($p<0.01$) assigned to a treatment by the indicator analysis, are highlighted in green (more abundant in MWCNT) and red (more abundant in control). The most abundant phyla are labeled including the total number of OTUs and the number of indicator OTUs in parentheses.

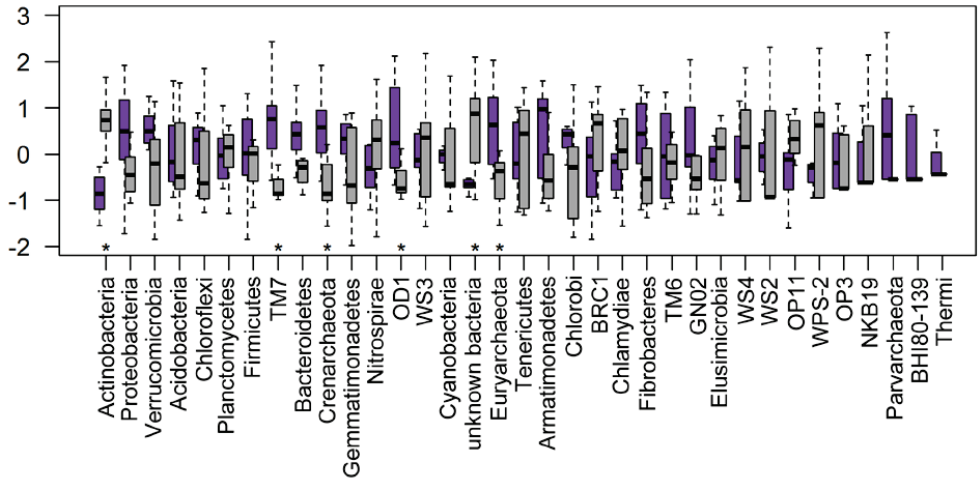

Figure 3: Change in relative abundance at the phylum level between MWCNT-treated and control soils. Phyla are shown in decreasing order of abundance for the control (gray) and MWCNT (purple) treatment. Asterisks indicate significant differences ($p<0.05$, PERMANOVA).

5. Discussion

With our experiment we could show that prokaryotes responded differently to the tested NPs than micro-eukaryotes. MWCNTs significantly affected the detected prokaryotes, while the detected micro-eukaryotes were largely insensitive (Table 1, Figure 1). Effects of MWCNTs on bacterial community structures have been reported by others when exposed to 10'000 mg kg^{-1} for 90 days (Shrestha et al., 2013), or to 200 µg/ml for 9 weeks (Khodakovskaya et al., 2013) as assessed by pyrosequencing. In contradiction with our study, a previous study showed that fungi were more sensitive to CNTs (500 mg kg^{-1}) than bacteria after 14 days of exposure (Rodrigues et al., 2012), but these findings were based on the application of single walled CNTs in a smaller amount of soil (40 g) measured after a shorter exposure time.

At phylum level, relative abundances of Actinobacteria decreased in MWCNT-treated soil compared to the controls, whereas the bacterial candidates OD1 and TM7 as well as the archaeal groups Crenarchaeota and Euryarchaeota increased (Figure 3). For Actinobacteria 71% of the MWCNT sensitive OTUs belonged to the order Actinomycetales. Most of the Actinomycetes are saprophytes and are able to decompose cellulose and lignin but there are also some pathogens and antibiotic producers within this order (Goodfellow and Williams, 1983). Streptomyces are one example of Actinomycetales, which we also found in our experiment, and are among the most abundant genera in soil (Nacke et al., 2011; Bontemps et al., 2013). Streptomyces are important for degradation of carbon compounds, such as cellulose, as well as production of antibiotics (Park et al., 2011; Bontemps et al., 2013).The phylum candidate OD1 is poorly investigated and contains anaerobic bacteria (Peura et al., 2012; Wrighton et al., 2012). There are indications that OD1 bacteria play a role in methane oxidation and sulfur reduction (Peura et al., 2012; Wrighton et al., 2012). Crenarchaeota were reported to play a major role in ammonium-oxidation in soils (Leininger et al., 2006). The genus *Methanosarcina* (Euryarchaeota), which was MWCNT sensitive in our experiment, has been reported to oxidize methane anaerobically (Knittel and Boetius, 2009). However, further research is needed for testing if ecosystem functions are affected by the community shifts caused by MWCNTs.

For the MWCNT sensitive OTUs it is not clear yet, how they are affected in their abundance by MWCNTs. A potential mechanism might be that MWCNTs might act as carbon source for certain bacteria as it has been shown that MWCNTs can be degraded by enzymes such as horseradish peroxidase. However, half-lives of MWCNTs are high and it is not known how and if microbial degradation of MWCNTs takes place in nature (Flores-Cervantes et al., 2014). Alternatively, surface interactions with MWCNTs, bacterial cell disruption and genotoxicity might be other potential explanations for the decreased abundances of certain bacterial OTUs (Kang et al., 2008; Neal, 2008). Therefore, the detected MWCNT-sensitive OTUs might be potential indicators for MWCNTs in soils, but need to be investigated closer

with quantitative assays (Hartmann and Widmer, 2006), and the ecological function of the indicator species and their role in microbial networks should be assessed in more detail (van der Heijden and Hartmann, 2016).

In contrast to the MWCNTs, the P25 and CeO_2 NPs as well as the NNM-TiO_2 did not affect the microbial community structures (Table 1, Figure 1). The lack of effects of TiO_2 NPs on bacterial community structure is in agreement with a previous study assessing the impact of 200 mg kg^{-1} TiO_2 NPs on bacterial community structure (using T-RFLP) after six weeks of exposure in soils cultivated with soybean or maize (Burke et al., 2014). However, in contrast to our observations, community structure of arbuscular mycorrhizal fungi changed compared to the controls (Burke et al., 2014). Other studies have found effects of TiO_2 NPs on bacterial community structure (Ge et al., 2011; Ge et al., 2012; Nogueira et al., 2012). Soil bacterial communities changed after exposure to 5g TiO_2 NPs per kg of soil for 30 days as assessed by 16S rRNA gene based denaturing gradient gel electrophoresis (Nogueira et al., 2012) or after exposure to 2 mg TiO_2 NPs per gram of soil after 60 days as assessed by 16S rRNA gene based pyrosequencing (Ge et al., 2012). It has been suggested that NP concentration, exposure time and soil chemistry such as pH and organic matter content are critical factors determining the effects on the soil bacterial community (Simonin et al., 2015).

For CeO_2 NPs we found no other study that investigated effects on soil microbial communities. However, Ma et al. (2015b) investigated the effects of CeO_2 NPs on microbial community structure in sequenced batch reactors (model for wastewater plants) and showed that CeO_2 in bulk and nano form did not affect bacterial communities at concentrations up to 20 mg L^{-1} (continuous flow) as assessed by pyrosequencing of 16S rRNA genes. The NP concentrations in our experiment were relatively high compared to expected environmental concentrations (Sun et al. 2014); however, such concentrations are not completely unlikely as the NPs in these pots were largely immobile (Gogos et al., 2016) and might accumulate over time. Moreover, due to the background concentrations of black carbon and titanium in

soils, relatively high amounts of NPs had to be added in order to properly quantify the added NPs in the soil substrate (Gogos et al., 2016).

6. Conclusions

Under the tested conditions MWCNTs pose a higher potential risk than the tested mineral NPs because they significantly changed the bacterial community structure and might potentially affect associated ecosystem functions. We found sensitive bacterial taxa that were either negatively or positively affected by MWCNT application; however, the underlying mechanisms are not yet understood. Prokaryotes were more sensitive than micro-eukaryotes, which might be related to different abilities of prokaryotic and eukaryotic cells to cope with nanoparticles. In conclusion, the impact on NPs on the soil microbiota is still poorly understood, as different studies provide equivocal results. Therefore, long-term surveys assessing different types and concentrations of NPs under different soil conditions are required to gain a better scientific basis for estimating potential loading thresholds below which there will be no harmful effect on the soil microbiota and associated ecosystem functions.

7. Acknowledgments

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8. Supplementary Information

Table S1: Treatment effects on observed richness and Smith-Wilson evenness of prokaryotes and micro-eukaryotes.

Main test[a]	Prokaryotes		Micro-eukaryotes	
	Richness (S_{obs}) F(p)	Evenness (E_{var}) F(p)	Richness (S_{obs}) F(p)	Evenness (E_{var}) F(p)
Block ($F_{1,25}$)	0.0 (0.871)	0.1 (0.751)	0.3 (0.588)	0.4 (0.536)
Treatment ($F_{4,25}$)	7.6 (**<0.001**)	0.8 (0.531)	1.9 (0.107)	2.5 (**0.048**)
Block*Treatment ($F_{4,25}$)	0.7 (0.651)	1.0 (0.450)	1.8 (0.132)	0.8 (0.588)
Treatments[b]	mean±SD	mean±SD	mean±SD	mean±SD
Control	1541±26^{A}	0.474±0.010^{A}	383±57^{A}	0.215±0.006^{AB}
P25	1519±27^{A}	0.471±0.010^{A}	397±28^{A}	0.222±0.011^{AB}
NNM-TiO_2	1533±30^{A}	0.464±0.007^{A}	393±49^{A}	0.220±0.007^{B}
MWCNT	1591±27^{B}	0.475±0.003^{A}	431±18^{A}	0.214±0.008^{A}
CeO_2	1527±33^{A}	0.476±0.006^{A}	398±33^{A}	0.217±0.009^{A}
$ZnSO_4$	1504±26^{A}	0.475±0.004^{A}	422±30^{A}	0.218±0.009^{A}

[a]Effects of main factors and their interactions as assessed by univariate permutational analysis of variance (PERMANOVA; degrees of freedom for each factor and the corresponding error term are given in brackets). Main factors include Treatment (Control, P25, NNM-TiO_2, MWCNT, CeO_2 NPs and $ZnSO_4$) and block (1, 2). Values represent the pseudo-F ratio (F) and the permutation-based level of significance (p).

[b]Average richness and evenness (mean±SD, n=7) for each treatment. Different letters indicate statistically significant differences ($p<0.05$) with p-values adjusted according to Benjamini-Hochberg.

Table S2: Results of ANOSIM and PERMDISP analysis for prokaryotes and micro-eukaryotes.

	ANOSIM				PERMDISP			
	Prokaryotes		Micro-eukaryotes		Prokaryotes		Micro-eukaryotes	
Main test[a]	R	P	R	P	F	P	F	P
Treatment	0.337	**<0.001**	0.027	0.128	1.2	0.399	2.70	0.184
Pairwise tests[b]	R	P_{adjust}	R	P_{adjust}	t	P_{adjust}	t	P_{adjust}
MWCNT vs. Control	0.7	**0.003**	0.1	0.383	0.5	0.975	2.10	0.038
MWCNT vs. P25	0.6	**0.003**	0.0	0.503	0.3	0.975	1.40	0.632
MWCNT vs. NNM-TiO_2	0.7	**0.003**	0.1	0.394	2.1	0.056	3.80	**0.015**
MWCNT vs. CeO_2	0.8	**0.003**	0.0	0.503	0.2	0.975	2.20	0.055
MWCNT vs. $ZnSO_4$	0.7	**0.003**	0.1	0.491	0.4	0.975	0.00	0.998
control vs. P25	-0.1	0.882	0.0	0.953	0.2	0.975	0.80	0.663
control vs. NNM-TiO_2	0.0	0.369	0.0	0.503	2.2	0.272	0.20	0.969
control vs. CeO_2	0.0	0.468	0.0	0.688	1	0.748	1.20	0.663
control vs. $ZnSO_4$	0.3	**0.015**	0.0	0.491	0	0.975	2.00	0.438
P25 vs. NNM-TiO_2	0.1	0.119	0.1	0.394	1.8	0.419	1.30	0.638
P25 vs. CeO_2	-0.1	0.985	-0.1	0.953	0.6	0.975	0.20	0.969
P25 vs. $ZnSO_4$	0.4	**0.011**	0.0	0.491	0.1	0.975	1.20	0.632
NNM-TiO_2 vs. CeO_2	0.2	0.054	0.0	0.503	3.6	**0.01**	2.00	0.342
NNM-TiO_2 vs. $ZnSO_4$	0.6	**0.003**	0.2	0.330	1.6	0.586	3.10	0.105
CeO_2 vs. $ZnSO_4$	0.3	**0.033**	0.0	0.941	0.7	0.975	1.50	0.626

[a]Effects of main factors assessed by analysis of similarity (ANOSIM) and permutational analysis of dispersion (PERMDISP). Main factor is treatment (Control, P25, NNM-TiO_2, MWCNT, CeO_2 NPs and $ZnSO_4$). Global test statistics (R) is given for the ANOSIM and pseudo-F ratio for PERMDISP.

[b]Pairwise comparison between the treatments. Pairwise test statistics is shown by R value for ANOSIM and univariate t-statistics for PERMDISP. A Benjamini-Hochberg adjustment of the p values was performed because of multiple comparisons.

Chapter VI

General Discussion

1. Contributions of this thesis

Nanoparticles (NPs) are produced and used worldwide, which can result in their unintentional release into the environment (Sun et al., 2014). Three of the most produced NPs worldwide are TiO_2 NPs, carbon nanotubes (CNT) and CeO_2 NPs (Keller et al., 2013). A number of studies, including work presented in this thesis, have tested and reported effects of these NPs on soybean, wheat, and soil microorganisms (Tables 1-4, Chapters II-V). This thesis contributes knowledge about whether TiO_2 NPs, multiwalled CNTs (MWCNTs) and CeO_2 NPs affect plants and microorganisms. To our knowledge, these NPs have never been tested together in one system under the same conditions. Effects of TiO_2 NPs, were assessed in both a hydroponic (Chapter II, Moll et al., 2016b) and soil (Chapters II-V) testing system. The comparison of these systems merits the conclusion that experiments exposing plants and soil microorganisms to NPs should be conducted in soil because experiments in hydroponic and soil systems yielded different ecotoxicological results and cannot be compared. In this thesis, we assessed red clover as model plant, because of its relevance in agriculture, and because it is still unclear whether NPs affect the symbiosis of red clover with nitrogen-fixing *Rhizobium* bacteria and arbuscular mycorrhizal fungi (AMF). Additionally, wheat and its symbiosis with AMF was assessed because of its relevance for human nutrition, and because AMF assist wheat to acquire nutrients. Few studies have tested the sensitivity of eukaryotic soil microorganism to NPs assessed in this thesis (Table 3, Burke et al., 2014; Burke et al., 2015). In view of the ecological importance of many soil eukaryotic microorganisms further attention is required. With our exposure studies we have provided new insights about the sensitivity of soil micro-eukaryotes to TiO_2 NPs, CeO_2 NPs and multiwalled CNTs (MWCNT). In the hydroponic system, red clover growth was decreased and not all plants exposed to TiO_2 NPs formed nodules (Figure 1). In the soil system, exposure to TiO_2 NPs did not affect red clover (Chapter III, Moll et al., 2016a) or wheat (Chapter IV) biomass or AMF colonization of their roots. In the red clover experiment, MWCNTs and CeO_2 NPs did not affect biomass and AMF root colonization, but, MWCNTs decreased number of flowers and increased nitrogen fixation (Chapter III, Moll et al., 2016a).

In the wheat experiment, exposure to TiO_2 NPs altered the structure of the prokaryotic community. However, in the red clover experiment, no changes in prokaryotic community structure were observed in the TiO_2 NP treatment. However, prokaryotic community structure was altered by MWCNT. In both soil experiments, with wheat or red clover, micro-eukaryotic community structure was not affected by any of the tested NPs. However, individual microbial taxa were affected by NPs (Chapter IV). Our data adds to a growing body of findings related to NP effects on the environment and demonstrates a poor understanding of the underlying mechanisms. Therefore, it will be important to investigate more long-term effects on the plant-microbiome and assess the mechanisms causing these effects before we can draw more solid conclusions on the environmental impact of TiO_2 NPs, CNTs and CeO_2 NPs.

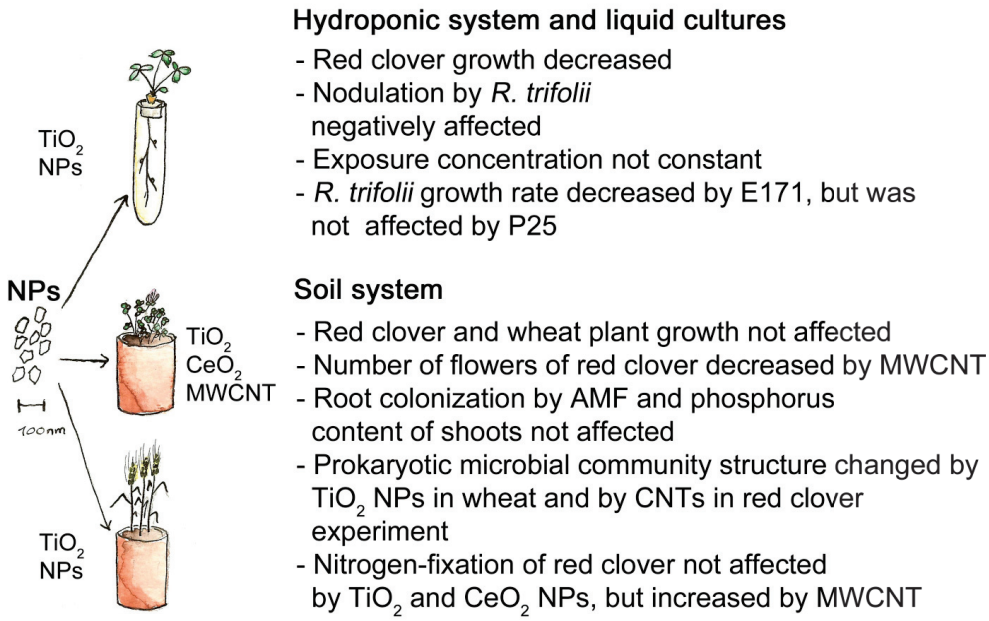

Figure 1: Overview of the results obtained in the experiments conducted in this thesis.

2. Testing NPs in hydroponic systems

Characterizing NPs in soils is very challenging (Von der Kammer et al., 2012; Cornelis et al., 2014). Therefore, we started with a hydroponic system in which we could quantify and characterize NPs in a liquid medium while simultaneously assessing effects of NPs on red clover and on root nodule formation by *R. trifolii.* For all of the NPs assessed in this thesis, i.e. TiO_2 NPs, CNTs and CeO_2 NPs, different studies have reported that NPs either increased (4 observations), decreased (5 observations) or did not affect (9 observations) plant length and biomass under certain conditions in hydroponic systems (Table 1). In the experiment presented in Chapter II (Moll et al., 2016b), we observed reduced shoot and root length of red clover exposed to TiO_2 NPs. However, the mechanisms behind how these NPs affect plant length and biomass are not known. For pea (Fan et al., 2014) and maize (Asli and Neumann, 2009), it was reported that plants took up less medium or exhibited reduced transpiration. Moreover, we observed reduced nitrogen (^{15}N) uptake from the medium when plants were exposed to TiO_2 NPs (Chapter II, Moll et al., 2016b). Fan et al. and Asli et al. hypothesized that NPs might attach to the root cell walls and block pores of maize and pea roots (Asli and Neumann, 2009; Fan et al., 2014). To assess whether red clover roots were covered by TiO_2 NPs, which could potentially block pores, we assessed scanning electron microscope images (Chapter II, Moll et al., 2016b) to visualize the location and attachment of NPs on plant roots. TiO_2 NPs covered on average only 1% of the surface of red clover roots. Therefore, in addition to pore blockage, other mechanisms might play a role in the observed decrease in plant growth in our experiment. The reported effects on medium uptake and transpiration may be plant dependent because in willow trees, wheat, and rapeseed transpiration was not affected by TiO_2 NPs (Seeger et al., 2009; Larue et al., 2012a; Larue et al., 2012b).

Besides effects of NPs on plant growth (biomass, length), effects on other measured endpoints have also been reported. For instance, changes in gene expression were reported in *Arabidopsis thaliana* and Tobacco plants exposed to NPs (Ma et al., 2013; Frazier et al.,

2014). For *Arabidopsis thaliana* exposed to CeO_2 NPs, expression of genes responsible for the induction of stress (sulfur assimilation and glutathione) was increased (Ma et al., 2013). In tobacco, expression of stress response genes coding for alcohol dehydrogenase and alcohol peroxidase production increased due to exposure to TiO_2 NPs (Frazier et al., 2014).

Effects of NPs on the interaction of plants with microorganisms have also been reported. For instance, pea exposed to TiO_2 NPs revealed a delayed root nodulation by rhizobia (Fan et al., 2014). Additionally, detection of NPs in plant tissue have been reported (Larue et al., 2012a; Larue et al., 2012b; Larue et al., 2012c; Jacob et al., 2013; Zhai et al., 2015). The mechanisms behind how these NPs cause effects on plants and symbiotic microorganisms are poorly understood. Reactive oxygen species released by the NPs can cause effects (Ma et al., 2015a), but also other potential mechanisms such as root pore blocking (Asli and Neumann, 2009) and the attachment of NPs onto surfaces of bacteria have been mentioned (Fan et al., 2014).

The particle size of the TiO_2 NPs in the hydroponic system, presented in Chapter II, was assessed by dynamic light scattering. Additionally, zeta-potential was measured, and elemental titanium concentrations were determined by ICP-MS (Chapter II, Moll et al., 2016b). Electron microscopy was used to characterize the NP sizes and shapes (Chapter II, Moll et al., 2016b). As in other studies (Allouni et al., 2009), TiO_2 NPs agglomerated and sedimented in our test system. We observed that the exposure concentration in the suspension decreased with time since addition (Chapter II, Moll et al., 2016b). This time-dependent decrease of exposure concentration makes it difficult to assess the effective concentrations of TiO_2 NPs. Compared to estimated environmental concentrations (Table 5), effects of CeO_2 NPs and CNTs on plant growth in hydroponic systems occurred at concentrations much higher than the expected environmental concentration in soils. However, for TiO_2 NPs, several studies reported effects at concentrations which are estimated to occur in soils treated with sludge (Tables 1, 2 and 5).

Hydroponic systems allow for the investigation of roots and their interactions with symbionts over the course of the whole experiment. We took advantage of this in our experiment (Chapter II, Moll et al., 2016b) and investigated nodule formation on red clover roots. Red clover plants treated with TiO_2 NP (E171 and P25) formed root nodules; however, up to 50% of the red clover plants treated with E171 and non-nanomaterial (NNM)-TiO_2 (average particle size >100 nm) did not form nodules (Chapter II, Moll et al., 2016b). To assess whether *R. trifolii* are affected by TiO_2 NPs, bacterial growth was measured in liquid cultures without red clover. E117 and NNM-TiO_2 reduced the relative growth rates of *R. trifolii* in liquid cultures. In contrast, P25 did not reduce the relative growth rate of *R. trifolii* (Chapter II, Moll et al., 2016b). Thus, the absence of root nodules in red clover plants exposed to E171 and NNM-TiO_2 may be a result of the observed effects on the *R. trifolii* growth rate. In pea, another leguminous plant, negative effects of TiO_2 NP on nitrogen fixation and on rhizobia were reported in a hydroponic system (Fan et al., 2014). Despite the advantages, our hydroponic test system presented in Chapter II, has also its disadvantages. For example, red clover plants were too small to assess nitrogen fixation, plant performance was not optimal compared to plants grown in soil, and the exposure concentration was not constant.

3. Testing NPs in soil systems

Several studies have been conducted to determine the effects of NPs on plants growing in soils (Table 2). For instance, CeO_2 NPs increased shoot length in coriander (Morales et al., 2013) but decreased dry weight of lettuce (Gui et al., 2015) and of tomato plants (Antisari et al., 2015). However, no effects on the biomass of wheat (Du et al., 2015), cucumber (Zhao et al., 2014) or red clover (Chapter III, Moll et al., 2016a) were reported. Tomatoes treated with MWCNTs had an increased shoot length and number of flowers (Khodakovskaya et al., 2013). In contrast to this finding, we found that number of flower-heads of red clover decreased in response to MWCNT application, while red clover biomass was not affected (Chapter III, Moll et al., 2016a). This suggests that the number of flowers may be a sensitive endpoint for assessing MWCNTs. However, the mechanism behind the reduced number

flowers is not yet known, and it remains to be tested if seeds collected from MWCNT-treated plants are able to germinate and produce viable plants.

Reported responses to CeO_2 NPs and MWCNTs (Table 2) were plant species dependent. However, different responses to NPs can also occur within the same plant species. In wheat, one study revealed a decrease in plant weight by TiO_2 NPs (Du et al., 2011), while in the experiment presented in Chapter IV, no effects on wheat biomass were observed. One possible explanation for this discrepancy could be the use of different soil types and wheat varieties. This was the case in a small scale soil experiment (Phytotoxkit F) in which cress root length was differently affected depending on NP type (TiO_2, ZnO and Ni NPs) and soil used (Jośko and Oleszczuk, 2013).

Soil microorganisms provide a wide variety of ecosystem functions, e.g., nutrient cycling (Altieri, 1999; Carney and Matson, 2005; van der Heijden et al., 2008). Thus, it is important to test whether NPs affect soil microorganisms. Several studies have reported changes in bacterial community structure or on ecosystem functions provided by microorganisms when exposed to different NPs (Table 3). Therefore, it was assessed if TiO_2 NPs, MWCNTs and CeO_2 NPs affect prokaryotic and micro-eukaryotic community structure soil (Chapter IV and V). Prokaryotic community structure changed when exposed to 1000 mg kg^{-1} TiO_2 NPs (P25, E171 and NNM-TiO_2,) in soil where wheat was growing (Chapter IV). In contrast, the community structure of micro-eukaryotes was not altered by spiking TiO_2 NP into the soil (Chapter IV). These changes in bacterial community structure by TiO_2 NPs are consistent with results reported by others (Table 3; Ge et al., 2011; Ge et al., 2012; Nogueira et al., 2012; Ge et al., 2013; Shah et al., 2014). We also assessed the effects of TiO_2 NPs on soil microorganisms in pots planted with red clover (Chapter V). In this experiment, no effects of TiO_2 NPs on prokaryotic and micro-eukaryotic community structure were observed (Chapter V). This might be because of the use of different plant species (i.e., red clover and wheat) or because of different exposure conditions. In the experiments with wheat (Chapter IV) and red

clover (Chapter V), the same soil was used. However, the NPs were spiked separately into soil for each experiment. Therefore, the response of both plant species to these NPs cannot be directly compared. In the wheat experiment, exposure concentration for elemental titanium was on average slightly lower than in the red clover experiment (P25 treatment: wheat 1720±280 and clover 2059±105 mg kg^{-1}, Chapters IV, V, Gogos et al. 2016). Therefore, the different plant species may have an influence on TiO_2 NP interaction with prokaryotic soil microorganisms. These differences in interactions between plant species and NPs might be caused by the release of root exudates and the altering NP mobility due to changes in pH or other soil characteristics. Soil properties such as pH, clay content, dissolved organic carbon, ionic strength and zeta potential have been reported to alter TiO_2 NP mobility in soil columns (Fang et al., 2009). If plants induce changes to the soil properties, NP mobility and their interaction with soil microorganisms may in turn change as well. Ge et al. (2014) reported that plants growing in soil can affect NP response of bacteria. In control soils without plants, bacterial community structure was not affected by CeO_2 NPs. However, in the same soil planted with soybean, community structure changed compared to the control (Ge et al., 2014). Changes in bacterial community structure have also been reported for CNT (Khodakovskaya et al., 2013). Under the experimental conditions in chapters IV and V, prokaryotes were more sensitive than micro-eukaryotes, and no changes in micro-eukaryotic community structure were observed. Contrary to our experiment, Burke et al. (2014) reported changes in arbuscular mycorrhizal community structure when exposed to TiO_2 NPs. The effects of TiO_2 NPs, CNTs and CeO_2 NPs on other microbial characteristics have been assessed, such as enzyme activity, microbial biomass, carbon mineralization and respiration, and effects of TiO_2 NPs on these variables have also been reported (Chung et al., 2011; Antisari et al., 2013; Simonin and Richaume, 2015).

Not only microbial community structure but also plant-microorganism interactions may be affected by NPs, (Table 4). For instance, Priester et al. (2012) investigated the effects of CeO_2 NPs on soybeans and their rhizobial symbiont .When subjected to CeO_2 NPs,

soybeans exhibited a decreased pod biomass and a decreased nitrogen fixation potential of symbiotic rhizobia (Priester et al., 2012). In contrast to this, we found that application of CeO_2 NPs did not influence plant biomass and nitrogen fixation of another legume, red clover (Chapter III, Moll et al., 2016a). However, MWCNTs increased nitrogen fixation in red clover (Chapter III). The mechanisms behind how nitrogen fixation is affected are not yet known. Because nitrogen fixation is an crucial ecosystem function, it is important to understand how, and under which conditions, NPs cause these effects.

In our experiment we did not find any effects of the tested NPs on AMF root colonization or plant phosphorus content of red clover and wheat shoots and grains, respectively. This is in agreement with another study assessing AMF (Burke et al., 2015) but in contrast to Burke et al. (2014). Burke et al. (2014) used a molecular technique to assess root colonization by AMF; thus the results are not directly comparable (Gamper et al., 2008). NP application also did not influence the occurrence of specific AMF taxa in the red clover experiment (Chapter III, Moll et al., 2016a). However, in the wheat experiment (Chapter IV), one out of 74 operational taxonomic units, that was differentially affected by NPs, belonged to AMF (a taxon of the *Diversisporaceae* was sensitive to P25).

Quantitative and qualitative characterization of NPs in soil is very challenging (Von der Kammer et al., 2012; Cornelis et al., 2014). Currently, it is not possible to determine the plant available fraction of NPs that is not bound to surfaces, for instance on soil particles. Therefore, indirect analytic methods, such as assessment of elemental titanium, are used in the experiments. Environmental concentrations are modelled based on studies which have assessed NPs in the environment and production rates of NPs (Keller et al., 2013; Sun et al., 2014) and are summarized in Table 5. The lowest applied TiO_2 concentrations we used (Chapters III to V) were similar to estimated environmental concentrations of soils treated with sludge as fertilizer. However, the highest concentrations used in these experiments represents a worst-case scenario, caused for instance by an accidental spill of NPs. For

MWCNTs and CeO_2 NPs, the applied concentrations in our experiments were much higher than the estimated concentrations in soils. In the case of MWCNT, the concentration in our experiment (Chapters III and V) was needed in order to be able to analytically assess the concentration (Gogos et al., 2016). For CeO_2 NPs, we wanted to determine if the previously reported negative effects of high CeO_2 NP concentration on nitrogen fixation in soybean were similar for red clover. Additionally, the lowest tested effect concentrations (LOEC) of TiO_2 NPs, MWCNTs and CeO_2 NPs from other studies (see Tables 2 and 4) were much higher than the expected environmental concentrations of these NPs. (Table 5).

4. Comparison of hydroponic and soil systems

Compared to the hydroponic system (Chapter II, Moll et al., 2016b), in which we found decreased plant growth and nitrogen uptake by red clover treated with TiO_2 NPs, no effects of TiO_2 NPs on red clover plant growth were found in soil (Chapter III and IV). In the hydroponic system, the red clover roots were surrounded by TiO_2 NPs, and a frequent and strong interaction between roots and NPs can be assumed. One percentage of the red clover root surface was covered by TiO_2 NPs (Chapter III, Moll et al., 2016a). In the soil experiments, however, elemental titanium concentrations over different depths of the soil core were constant (Gogos et al., 2016). This indicates that titanium was not mobile in the soil. Depending on soil characteristics, heteroaggregation of NPs with soil particles is likely and can influence the mobility and toxicity of NPs (Cornelis et al., 2014). Methods for assessing unbound NPs in soil matrices are still under development (Von der Kammer et al., 2012; Cornelis et al., 2014) and are of high importance because unbound NPs might interact more strongly with plant roots and soil microorganisms than heteroaggregates. Although the analytics of NPs in soils can only be conducted at a low resolution, it is very important to conduct ecotoxicological tests of NPs in soils. The aging of NPs may result in changes to their properties over the course of experiments in soil, as was shown for Ag NPs (Coutris et al., 2012; Nowack et al., 2012). These aging-induced changes may include altered bioavailability or the dissolving of ions, both which could potentially alter a NP's toxicity.

Assuming that unbound NPs are more toxic than soil bound NPs (heteroaggregates), effective exposure concentrations might be much lower than the spiked nominal concentration for NPs where no ions are dissolved, for instance TiO_2 NPs. As a result, plant roots and microorganisms in soil may have less direct contact with NPs than in hydroponic systems where they are entirely surrounded by NPs. Thus, in soils, much higher effective concentrations might be expected for NPs that do not release ions than in hydroponic systems.

In this thesis we first assessed NPs in hydroponic systems because we assumed that the analytics of NPs would be easier. However, because of the inconstant exposure concentration and sub-optimal plant growth conditions, we now recommend assessing effects of NPs on plants and microorganisms in soil systems rather than hydroponic systems.

5. Outlook for further research

Testing the ecotoxicity of NPs and the analytical tools for characterizing NPs in environmental samples is still in its infancy. For determining the risk of NPs, analytical methods are needed to determine environmental concentrations and exposure concentrations in ecotoxicological experiments. Several studies, including the experiments performed in this thesis, found effects of NPs on plants and soil microorganisms. Further research is needed to understand under which conditions these effects occur and how these effects were triggered. Additionally, long-term experiments, which test more than one generation of plants and investigate fitness of populations grown in NP spiked soil, should be conducted. For soil microorganisms, effects on community structure were reported in several studies (Table 3) and in our experiments (Chapter IV and V). However, further studies are needed to assess how these community changes affect ecosystem functions.

Table 1: Assessing effects of NPs on plants and symbiotic microorganisms in hydroponic systems. NP type, investigated plant species and family are listed, as well as the medium in which the test was performed. NOAEL indicates the highest tested not adverse effect level and LOEC the lowest tested concentration where effects were investigated. Effects on plants as well as symbiotic microorganisms are mentioned.

							Plant growth					
NP	NP properties	Organisms	Family	Medium	Concentration	exposure time	NOAEL	LOEC	Effect plant	Effect on microorganisms	other endpoints	Reference
CeO_2	CeO_2 10-30nm, In2O320-70nm	*Arabidopsis thaliana*	brassicaceae	Murashige Skoog medium	0-2000 ppm	25 d		250 mg/l	250 mg/l fresh biomass increased, 500 mg/l fresh biomass and root length decreased	na	Chlorophyll content decreased, gene expression altered	Ma et al. 2013
CNT	14C labeled MWCNTs, 2.65±1.55 and 2.90±1.99 nm in GA and HA	*Triticum spp*	poaceae	Hoagland	10-100 mg/l	7 d	100 mg/l		no effect	na	uptake of labeled MWCNTs, evapotranspiration and chlorophyll content not affected, H_2O_2 and TBARS content not affected	Larue et al. 2012
CNT	14C labeled MWCNTs, 2.65±1.55 and 2.90±1.99 nm in GA and HA	*Brassica napus*	brassicaceae	Hoagland	10-100 mg/l	7 d	100 mg/l		no effect	na	uptake of labeled MWCNTs, evapotranspiration and chlorophyll content not affected, H_2O_2 and TBARS content not affected	Larue et al. 2012
CNT	MWCNTs	*Curcubita pepo*	cucurbitaceae	25% Hoagland with 0 or 0.2% sodium dodecyl sulfate	1000 mg/l	15d		1000 mg/l	reduced biomass	na		Stampoulis et al. 2009
CNT	MWCNTs with different charge	*Glycine max*	fabaceae	hoagland	10-50 mg/l	18d	20 mg/l			na	uptake of MWCNT	Zhai et al. 2015
CNT	MWCNTs with different charge	*Zea mays*	poaceae	hoagland	10-50 mg/l	18d		20 mg/l	dry weight increased	na	cumulative transpiration increased, uptake of MWCNTs	Zhai et al. 2015
TiO_2	P25	*Pisum sativum, Rhizobium leguminosarum bv. Viciae*	fabaceae	Fahraeus Medium	100-1000 mg/l	14 d	800 mg/l		no effect	reduced number of bacteroids, delayed nitrogen fixation	reduced nutrient solution uzilization	Fan et al. 2014
TiO_2	<25 nm	*Nicotiana tabacco*	solanaceae	Murashige and skoog medium	0.01-5%	3 weeks		0.1%	root length, number of leaves and biomass decreased		microRNA expression altered, stress-related genes upregulated (APX, ADH)	Frazier et al. 2014
TiO_2	anatase, <25 nm	*Phaseolus vulgaris*	fabaceae	Harmens et al 1993	10-30 mg/l	4 weeks	30 mg/l		no effect	na	no translocation to shoot	Jacob et al. 2013

Continuation of Table 1.

							Plant growth					
NP	**NP properties**	**Organisms**	**Family**	**Medium**	**Concentration**	**exposure time**	**NOAEL**	**LOEC**	**Effect plant**	**Effect on micro-organisms**	**other endpoints**	**Reference**
TiO_2	anatase, <25 nm	*Elodea canadensis*	hydrocharitaceae	Harmens et al 1993	10-30 mg/l	4 weeks	30 mg/l		no effect	na		Jacob et al. 2013
TiO_2	anatase, <25 nm	*Triticum aestivum*	poaceae	Harmens et al 1993	10-30 mg/l	4 weeks	30 mg/l		no effect	na	no translocation to shoot	Jacob et al. 2013
TiO_2	anatase, <25 nm	*Phaseolus vulgaris, Triticum aestivum, rumex crispus, Elodea canadensis*	poligonaceae	Harmens et al 1993	10-30 mg/l	4 weeks	30 mg/l		no effect	na	translocation of Ti to shoot	Jacob et al. 2013
TiO_2	14 and 25 nm, P25 and self-synthesiced anatase NPs	*Brassica napus*	brassicaceae	Hoagland	100 mg /l	7 d		100 mg/l	no effect	na		Larue et al. 2012
TiO_2	anatase14,25,140nm, rutlie 655,22,36nm	*Triticum aestivum*	poaceae	Hoagland	100 mg/l	7 d	50 mg/l		increased root lenght, biomass unaffected	na	uptake of Ti, TBARS and H_2O_2 content not affected	Larue et al. 2012
TiO_2	14 and 25 nm, P25 and self-synthesiced anatase NPs	*Triticum aestivum*	poaceae	Hoagland	100 mg /l	7 d	50 mg/l		increased root lenght, biomass unaffected	na	uptake of Ti, evapotranspiration unaffected	Larue et al. 2012
TiO_2	P25, E171, bulk TiO_2	*Trifolium pratense*	fabaceae	Fahraeus Medium	12-24 mg/l	28d		12 mg/l	reduced root and shoot length and biomass	not all inoculated plants formed nodules	reduced ^{15}N content in shoots	Chapter II
TiO_2	25 (P25) and 100 nm (100%anatase)	*Salix*	salicaceae	distilled water	1-100 mg/l	250 h	100 mg/l		no effect	na	no effect on transpiration and water use efficiency	Seeger et al. 2009
TiO_2	82% anatase 18% rutile, 27 nm	*Cucumis sativus*	cucurbitaceae	Hoagland	100-4000 mg/l	7 d		250 mg/l	root length increased, shoot length increased at 4000 mg/l	na	uptake and translocation of Ti	Servin et al. 2012
TiO_2	P25	*Lactuca sativa*	asteraceae	Hoagland, soil Sunshine mix#5	100-5000 mg /l			5000 mg/l	root length decreased	na	chlorophyll content and oxidant capacity not affected, uptake of Ti	Song et al. 2013

Table 2: Studies assessing NP effects on plants soil systems. NP type, investigated plant species and family are listed. NOAEL indicates the highest tested not adverse effect level and LOEC the lowest tested concentration where effects were investigated. If microorganisms were assessed in the same system, they are also mentioned in the table.

			Plant growth			Microorganisms				
NP	**NP properties**	**Organisms**	**NOAEL**	**LOEC**	**Effect**	**NOAEL**	**LOEC**	**Effect**	**other endpoints**	**Reference**
CeO_2	8 nm	Triticum aestivum	400 mg/kg		no effect on biomass	na	na	na	chlorophyll content decreased, increased antioxidants, Ce detected in roots but not translocated to shoots, changed root and leave cell microstructures, starche content not affected, protei content increased	Du et al. 2015
CeO_2		Latuca sativa		100 mg/kg	fresh biomass increased (100 mg/kg), dry biomass decreased (1000 mg/kg)	na	na	na	antioxidants changed (SOD decreased and MDA increased in roots, shoot not affected), took Ce up, no effect on chlorophyll and protein content, increased nitrate content in plants, soluble sugar content decreased	Gui et al. 2015
CeO_2	8 nm	Coriandrum sativum		125 mg/kg	root and shoot growth increased, dry weight not different from control,	na	na	an		Morales et al. 2013
CeO_2	8±1 nm	Cucumis sativus	800 mg/kg		no effect on plant growth	na	na	na	400 mg CeO_2 NPs affected sucrose, globulin and glutelin content. No effects on macronutrients, reduced Mo content. Reduced fresh weight of cucmbers by 31% at 800 mg/kg CeO_2.	Zhao et al. 2014
CeO_2		Cucumis sativus	800 mg/kg		no effect on stem, root, leave and fruit biomass	na	na	na	no effect on chlorophyl content, and transpiration rate, uptake of Ce	Zhao et al. 2014
CeO_2/TiO_2	Ce: 50-105nm, TiO2: 20-160nm	Lycopersion esculentum		CeO_2 101 mg/kg; TiO_2 208 mg/kg	Ce decreased stem and leave weight, Ti decreased leave weight	na	na	na	nutrient contents in plant tissue altered by both NPs	Antisari et al. 2014

Continuation of Table 2.

			Plant growth			Microorganisms				
NP	**NP properties**	**Organisms**	**NOAEL**	**LOEC**	**Effect**	**NOAEL**	**LOEC**	**Effect**	**other endpoints**	**Reference**
CNT	MWCNTs	Lycopersion esculentum		50 µg/ml	plants grew higher and revealed more fruits, number of leaves not affected		200 µg/ml	shift in bacterial community structure	uptake of CNTs	Khodakovskaya et al. 2013
Mixture of TiO_2, ZnO and Ag NPs	na	Medicago trunculata			shoot length and fresh biomass decreased, dry weight not affected			change in microbial community structure	gene expression in Medicago changed, nodulation frequency decreased	Judy et al. 2015/ Chen et al. 2015
TiO_2	20 nm	Triticum aestivum		10 g / lysimeter	dry weight reduction		10 g/ lysimeter	soil enzyme activities changed (urease, catalase, peroxidase)		Du et al. 2011
TiO_2	P25, E171, bulk TiO_2	Triticum spp.	1000 mg/kg		no effect on plant growth		1000 mg/kg	prokaryotic community structure changed, eukaryotic-microorganisms not		Chapter IV
TiO_2, CeO_2, MWCNT		Trifolium pratense	1000 mg/kg TiO_2, CeO_2	3 mg/kg MWCNT	Number of flowers decreased by MWCNT, other NPs no effect	1000 mg/kg TiO_2, CeO_2	3000 mg/kg MWCNT	prokaryotic community structure changed by MWCNT, eukaryotic-microorganisms not. Other NPs no effect. Nitrogen fixation increased in 3000 mg/kg MWCNT treatment		Chapter III

Table 3: Studies assessing NP effects on soil microorganisms. The characteristics (type, size) of the used NPs are shown, NOAEL indicates the highest tested not adverse effect level and LOEC the lowest tested concentration where effects were investigated. If effects were found on microorganisms, they are briefly mentioned. Also other endpoints related to microorganisms are listed.

			Microorganisms				
NP	**NP properties**	**Organisms**	**NOAEL**	**LOEC**	**Effect**	**other endpoints**	**Reference**
CeO_2	50-105nm	soil microorganisms		10 mg/kg	microbial biomass not affected (Nmic Cmic), C/N ratio was altered, metabolic quotient increased shortly after addition of CeO_2	Chemical and physical properties of soil were not affected	Antisari et al 2013
CNT	MWCNTs, 15.1nm diameter, 10-20 µm long	soil microorganisms		500 mg/kg	different enzyme activities (e.g. Phosphatase, Glucosidase) decreased, microbial biomass decreased		Chung et al. 2011
TiO_2	20-30nm, 81%anatase, 19%rutile	bacterial communities		20 mg/g	community structure of bacteria changed	the dryer the soil, the stronger the effects. No changes in organic matter, total C, total N and CN ratio of soil	Ge et al. 2013
TiO_2	15-20 nm, 19% rutile 81% anatase	soil bacteria		0.5 mg/g	reduced extractable DNA, reduced substrate induced respiration and changed bacterial community structure		Ge et al. 2011
TiO_2	15-20 nm, 19% rutile 81% anatase	soil bacteria		0.5 mg/g	Changed bacterial community structure time and dose dependent	Protease activity increased	Ge et al. 2012
TiO_2	Ti <100 nm ,in water 296.2nm	soil bacteria		5 g/kg	community structure of bacteria changed		Nogueira et al. 2012
TiO_2	anatase 5-10nm, rutile 55nm, Cu 25nm, Ag 35 nm, ZnO 35 nm	soil microorganisms		0.06 mg/kg	Both TiO_2 crystal structures changed community structure with rutile having longer effects. With rutile richness decreased at 120d		Shah et al. 2014
TiO_2	80% anatase and 20% rutile, 21 nm	soil microorganisms		1 mg/kg	C mineralization decreased depending on soil type and concentration of NPs. Bacterial abundance decreased only in one soil type at 500 mg/kg		Simonin et al. 2015
TiO_2	P25, E171, NNM-TiO_2	soil microorganisms		1000 mg/kg	prokaryotic community structure changed, eukaryotic-microorganisms not. AMF colonization not affected		Chapter IV
TiO_2, CeO_2, MWCNT	P25, NNM-TiO_2, CeO_2	soil microorganisms	1000 mg/kg TiO_2, CeO_2	3000 mg/kg MWCNT	prokaryotic community structure changed by MWCNT, eukaryotic-microorganisms not. Other NPs no effect. Nitrogen fixation increased in 3000 mg/kg MWCNT treatment.		Chapter III

Table 4: Studies assessing NP effects on plant-microorganism interactions. NP type is mentioned as well as investigated plant species and symbiotic microorganisms. LOEC indicates the lowest tested concentration where effects were investigated.

			Plants			Microorganisms		
NP	**NP properties**	**Organisms**	**plant_kind**	**LOEC**	**Effect**	**LOEC**	**Effect**	**Reference**
CeO_2		soybean, bacterial communities	fabaceae	0.1 g/kg	reduced growth (Priester et al.)	0.1 g/kg	community structure of bacteria changed	Ge et al. 2014
CeO_2	8 nm	soybean, Bradyrhizobium japonicum	fabaceae	1 g/kg	reduced number of pots	0.5 mg/g	reduced nitrogen fixation potential (ethylene assay)	Priester et al. 2012
TiO_2	P25, E171, bulk TiO2	Triticum spp.	poaceae		no effect on plant growth	1000 mg/kg	prokaryotic community structure changed, eukaryotic-microorganisms not affected. AMF root colonization not affected	Chapter IV
TiO_2, CeO_2, MWCNT		Trifolium pratense	fabaceae	3 mg/kg MWCNT	Number of flowers decreased by MWCNT, other NPs no effect	3000 mg/kg MWCNT	prokaryotic community structure changed by MWCNT, eukaryotic-microorganisms not. Other NPs no effect. Nitrogen fixation increased in 3000 mg/kg MWCNT treatment. AMF root colonization not affected	Chapter III
TiO_2	P25 with different surface charges	Soy bean	fabaceae		lower biomass	200 mg/kg	AMF colonization decreased	Burke et al. 2014

Table 5: Estimated environmental concentrations of TiO_2 NPs, MWCNT and CeO_2 NPs in different environmental compartments for Europe and Switzerland (Sun et al. 2013), and worldwide (Keller et al. 2012). For the worldwide production percentage of NPs released to the environment are indicated (Keller et al. 2012).

Nanoparticle		Air	Water	Soil without sludge treatment	Soil with sludge treatment	land fill	suage treatment plant	effluent suage treatment plant	Sediment	sludge
TiO_2	Worldwide[1]	1'600 t/y	15'600 t/y	na	38'200 t/y	32'600 t/y	47'700 t/y			
	% of produced amount[1]	2	18		43	37				
	EU[2]	0.001 µg/m^3	0.53 µg/l	0.13 µg/(kg*y)	1200 µg/(kg*y)			16 µg/l	1.9 mg/(kg*y)	170 mg/kg
	Switzerland[2]	0.002 µg/m^3	0.67 µg/l	0.57 µg/(kg*y)				32 µg/l	2.3 mg/(kg*y)	320 mg/kg
CeO_2	Worldwide[1]	100 t/y	300 t/y	na	1'400 t/y	8'200 t/y	1'100 t/y			
	% of produced amount[1]	1	3		14	82				
CNTs	Worldwide[1]	43 t/y	33 t/y	na	500 t/y	2'700 t/y	200 t/y			
	% of produced amount[1]	1	1		16	84				
	EU[2]	0.02 ng/m^3	0.23 ng/l	5.1 ng/(kg*y)	0.99 µg/(kg*y)			4 ng/L	0.79 µg/(kg*y)	0.15 mg/kg
	Switzerland[2]	0.09 ng/m^3	0.35 ng/l	22 ng/(kg*y)				5.5 ng/l	1.2 µg/(kg*y)	0.27 mg/kg

[1] Keller et al. (2013), [2] Sun et al. (2014)

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Summary

Engineered nanoparticles (NPs) are small particles (< 100 nm) that are widely used in electronics, paints, cosmetics, and composite materials. There is increased interest to use NPs in agriculture to improve the characteristics of plant protection products and fertilizers. As a result of the production and use of NP containing materials, NPs are released into the environment. For future risk assessment it is, therefore, important to investigate whether NP negatively impact the environment and if they interact with plants and soil microorganisms.

In this thesis we addressed the effects of three of the ten most produced NPs on plants, their symbiotic microorganisms and on soil microbial communities. In a first experiment we exposed nitrogen fixing bacteria (*Rhizobium trifolii)* grown in liquid cultures to different titanium oxide (TiO_2) NPs, i.e. P25 and E171. The relative growth rate of *R. trifolii* was not affected by one TiO_2 NP (P25; 23 mg l^{-1}), while it decreased with the other TiO_2 (23 mg l^{-1} E171). To assess whether the decreased growth of *R. trifolii* influences nodulation on red clover roots, and whether red clover is affected by TiO_2 NPs, we exposed red clover and *R. trifolii* in a hydroponic system. TiO_2 NPs were suspended in the medium and plants were grown for four weeks. All of the tested TiO_2 treatments decreased shoot biomass (by 27-53%) and root length (by 30-44%) of red clover. Red clover took up less ^{15}N from the medium when treated with TiO_2 NPs. The formation of root nodules was observed in all treatments. However, on two red clover replications (n=6) treated with E171, no nodules were formed. P25 did not affect nodulation by *R. trifolii*.

Hydroponic systems are artificial, and exposure conditions are different than in soils, where heteroagglomeration of NPs with soil particles can occur. To test whether the effects on red clover and *R. trifolli* also occur in soils, we mixed TiO_2 NPs (10 to 1000 mg kg^{-1}) into an agricultural soil and grew red clover for three months. We also tested CeO_2 NPs and multiwalled carbon nanotubes (MWCNT). Red clover biomass was not affected by any of the

treatments. However, flower number decreased in plants grown in pots with MWCNTs (3 mg kg^{-1}) and biological nitrogen fixation increased by 8% when treated with MWCNT (3000 mg kg^{-1}). Root colonization by arbuscular mycorrhizal fungi (AMF) was not affected in any of the treatments. We also addressed a crop which is important for human nutrition, wheat. Wheat was exposed to different TiO_2 NPs in the same soil used for the red clover experiment. Wheat biomass and root colonization by AMF were not affected by any of the treatments. Microbial community structures were assessed using Illumina Miseq paired-end sequencing of ribosomal markers targeting prokaryotes and micro-eukaryotes ($16S_{V3-V4}$ and ITS2, respectively) for both experiments. The community structure of prokaryotic microorganisms changed when treated with TiO_2 NPs in the wheat experiment and MWCNT in the red clover experiment but was not affected by TiO_2 NPs in the red clover experiment. In both experiments, eukaryotic microorganism community structure was not affected by any of the tested NPs. In conclusion, this work indicates that red clover and its symbiont *R. trifolii* are more sensitive to TiO_2 NPs in hydroponic systems than in soils, and prokaryotic microorganisms were more sensitive to NPs than eukaryotic-microorganisms under the tested conditions. For further experiments it is recommended to directly test the plants and soil microorganisms in soil systems.

Dutch Summary - Samenvatting

Nanopartikels (NPs) zijn kleine deeltjes (kleiner dan 100 nanometer) die veel gebruikt worden in elektronica, verf, cosmetica en andere materialen. Er is steeds meer interesse om NPs ook in de landbouw te gebruiken, bijvoorbeeld om de effectiviteit van kunstmest en bestrijdingsmiddelen te verbeteren. Door de productie en het gebruik van NPs, komen ze in het milieu terecht. Om de risico's van NPs voor het milieu in te kunnen schatten, is het belangrijk te onderzoeken of NPs planten en bodemmicro-organismen beïnvloeden.

In dit proefschrift zijn de effecten van drie van de tien meest geproduceerde NPs op planten, plant-symbionten (stikstof fixerende bacteriën, mycorrhiza-schimmels) en bodemmicro-organismen onderzocht. In een eerste experiment werd de groei van stikstof fixerende bacteriën (*Rhizobium trifolii)* in vloeibaar medium onder invloed van verschillende titaanoxide (TiO_2) NPs (genaamd P25 and E171) bepaald. Het ene TiO_2 NP (P25; 23 mg l^{-1}) had geen invloed op de relatieve groeisnelheid van *R. trifolii* terwijl het andere TiO_2 (E171; 23 mg l^{-1}) een negatieve invloed op de relatieve groeisnelheid van *R. trifolii* had.

Om vervolgens te onderzoeken of de geremde groei van *R. trifolii* ook invloed heeft op de vorming van stikstof fixerende wortelknolletjes en of TiO_2 NPs de plant beïnvloeden, werd rode klaver met *R. trifolii* in een vloeibaar medium opgegroeid. De planten groeiden gedurende 4 weken onder invloed van TiO_2 NPs. De verschillende TiO_2 NPs hadden een negatief effect op de biomassa (een groeireductie van 27% tot 53%) en de wortellengte (een reductie van 30% tot 40%) van rode klaver. Rode klaver naam minder ^{15}N op onder invloed van TiO_2 NPs. Terwijl in alle behandelingen wortelknolletjes werden gevormd, werden in twee van de zes herhalingen met het TiO_2 NP E171 geen wortelknolletjes gevonden. Dit kan erop duiden dat E171 een negatief effect heeft op de vorming van deze voor de stikstoffixatie belangrijke wortelknolletjes.

De kweek van planten in systemen zonder substraat is kunstmatig en blootstelling van planten en micro-organismen aan NPs zal verschillen van die in de bodem waar NPs aggregaten kunnen vormen met bodemdeeltjes. Om te onderzoeken of de effecten van TiO_2 NPs op rode klaver en op *R. trifolli* ook in de bodem optreden, werden TiO_2 NPs (10 tot 1000 mg kg^{-1}) in potten met landbouwgrond gemengd waarin vervolgens rode klaver werd gegroeid. Naast TiO_2 NPs zijn in dit experiment ook effecten van twee andere soorten NPs, namelijk Cerium oxide (CeO_2 NPs) en koolstofbuisjes (MWCNT), onderzocht. De onderzochte NPs hadden geen effect op de groei en biomassa van rode klaver. Echter, het aantal bloemen nam af in potten met koolstofbuisjes (3 mg kg^{-1}) en de biologische stikstoffixatie nam met 8 procent toe wanneer koolstofbuisjes (behandeling met 3000 mg kg^{-}) door de bodem gemengd werden. De onderzocht NPs hadden geen invloed op de hoeveelheid mycorrhiza-schimmels die de wortel koloniseerde.

Naast klaver hebben we ook nog de effecten van NPs op tarwe onderzocht. Tarwe werd opgegroeid in grond waaraan verschillende hoeveelheden TiO_2 NPs werden toegevoegd. Zowel de biomassa als kolonisatie door mycorrhiza-schimmels van tarwe werd niet beïnvloed door de toevoeging van TiO_2 NPs. Om vervolgens te onderzoeken of NPs invloed hebben op microbiële gemeenschappen hebben we genetische fingerprinting methoden (Illumina sequencing) gebruikt waarbij we ons op prokaryoten (voornamelijk bacteriën) en eukaryoten (voornamelijk schimmels en enkele groepen protisten) gericht hebben. Behandeling met TiO_2 NP veranderde de structuur van de prokaryote microbiële gemeenschap van tarwe, maar niet die van rode klaver, terwijl er voor beide plantensoorten geen effect werd gevonden op de eukaryote gemeenschappen.

Dit onderzoek toont aan dat hoewel rode klaver en zijn wortelsymbiont *R. trifolii* door TiO_2 NPs worden beïnvloed bij kweek in vloeibaar medium, er nauwelijks effecten van de NPs in de bodem werden waargenomen. Verder bleek dat prokaryote micro-organismen sterker op NPs reageren dan eukaroyte micro-organismen. Vervolg onderzoek dient zich vooral te richten op de bodem en hoe NPs in de bodem planten en micro-organismen beïnvloeden.

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Curriculum Vitae

Janine Moll from Dulliken (SO), Switzerland, was born on September 16th 1983. After finishing high school at Kantonsschule in Olten, she studied environmental sciences at Swiss Federal Institute of Technology in Zurich. She got her MSc degree in 2010. Her thesis was entitled “Mating system of alpine *Arabidopsis thaliana* populations“, and was supervised by Prof. Alex Widmer. From September 2010 on Janine was employed as technical assistant at Uppsala University, Sweden, in the plant ecology group and was working for Prof. Sophie Karrenberg and Prof. Jon Ågren. Back in Switzerland, Janine started her PhD at Agroscope, Institute of Sustainable Sciences, in August 2011 and was supervised by Prof. Marcel G.A. van der Heijden and co-supervised by Dr. Franco Widmer. In 2016 she finished her thesis entitled “Effects of Engineered Nanoparticles on Crops, their Symbionts, and Soil Microbial Communities”.

Publications and Presentations

Janine Moll, Annette Okupnik, Alexander Gogos, Katja Knauer, Thomas D. Bucheli, Marcel GA van der Heijden and Franco Widmer (2016). Effects of titanium dioxide nanoparticles on red clover and its rhizobial symbiont. PLOS ONE, 11, 5, e0155111, DOI:10.1371/journal.pone.0155111.

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Gogos Alexander, Janine Moll, Florian Klingenfuss, Marcel GA van der Heijden, Fahmida Irin, Micah J. Green, Renato Zenobi and Thomas D. Bucheli (2016). Vertical transport and plant uptake of nanoparticles in a soil mesocosm experiment. Nanobiotechnology, 14, 1, DOI 10.1186/s12951-016-0191-z.

Presentations at scientific conferences

Oral presentation:

Janine Moll, Martin Hartmann, Marcel van der Heijden, Franco Widmer, Effects of different nanoparticles on soil microbial community structures and plant-microbe interactions, 6th Swiss Microbial Ecology Meeting 2015, Ascona, Switzerland.

Poster Presentations:

Janine Moll, Alexander Gogos, Annette Okupnik, Marcel van der Heijden, Katja Knauer, Thomas D. Bucheli, Franco Widmer, Effects of different TiO_2 nanoparticles on growth of *Rhizobium trifolii* in liquid culture. 6th SETAC World Congress 2012, Berlin, Germany.

Janine Moll, Alexander Gogos, Katja Knauer, Marcel van der Heijden, Thomas D. Bucheli, Franco Widmer. Effects of TiO_2 nanoparticles on rhizobia and their symbiosis with red clover, 23rd SETAC Europe Annual Meeting 2013, Glasgow, United Kingdom.

Janine Moll, Alexander Gogos, Katja Knauer, Thomas D. Bucheli, Marcel van der Heijden, Franco Widmer. Effects of TiO_2 nanoparticles on the symbiosis of red clover and *Rhizobium trifolii* in a hydroponic system, 24th SETAC Europe Annual Meeting 2014, Basel, Switzerland.

Presentations to the public

Janine Moll, guided tours for „ExpoNano – Mobile Ausstellung zur Nanotechnologie“. Aarau, Fribourg, Zug, Frauenfeld, Olten, Solothurn, Zürich and Chur, Switzerland, 2013-2016.

Janine Moll. Effekte von Nanopartikel auf Pflanzen und nützliche Bodenmikroorganismen, eawag PEAK Vertiefungskurs: Ökotoxikologische Effekte von Synthetischen Nanopartikel, Dübendorf, Switzerland, 2015/04/23.