Testing of TiO$_2$ Nanoparticles on Wheat and Microorganisms in a Soil Microcosm

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Abstract

Nanoparticles (NPs) are increasingly manufactured and implemented in various products. A new field of application of NPs is the use in fertilizer or plant protection products (PPP) in agriculture. The possible benefits for agricultural practices, however, might at the same time be a threat to organisms of the terrestrial environment. Despite the great potential of NP for agricultural use, only few studies analysed NP toxicity in complex environments such as soil.

In this study, a long term (84d) soil microcosm experiment was conducted using two types of TiO$_2$ NPs (P25 and E171) at spiked nominal concentrations of 1, 100 and 1000 mg/kg soil in order to assess the toxicity towards wheat and soil microorganisms. In addition, uptake of TiO$_2$ NPs into wheat plants and their mobility in soil were assessed.

Although no toxic effects were detected for wheat, indications for uptake of TiO$_2$ NPs into the plant root were demonstrated by transmission electron microscopy (TEM). Furthermore, raised Ti concentrations were observed in wheat grains, indicating a translocation of NPs from the soil to the grains. Root colonisation of arbuscular mycorrhizal fungi, soil microbial DNA and nitrous oxide emissions from soils did not differ between microcosms amended with TiO$_2$ NPs and the control treatment. This implies that the tested soil microbes were not affected by the presence of NPs. Elevated concentrations of Ti in microcosm leachates of soils spiked with TiO$_2$ NPs compared to the control treatment indicated possible mobilization of TiO$_2$ NPs. In conclusion, no toxicological effects were detected on the organisms tested. However, the findings raise new questions concerning the fate of TiO$_2$ NPs in the soil-plant system. These need to be addressed in order to provide robust information for a risk assessment of TiO$_2$ NPs in PPPs.
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1 Introduction

Particles with at least one dimension between 1 and 100 nm are referred to as nanoparticles (NPs) (ISO, 2010). Although also naturally occurring (Handy et al., 2008), NPs are increasingly manufactured and implemented in various products such as electronics, food and clothing (Project on Emerging Nanotechnologies, 2014). A new field of application of NPs is seen in the use as fertilizer or plant protection product (PPP) in agriculture (Gogos et al., 2012; Khot et al., 2012; Rai et al., 2012). This possible benefit for agricultural practice, however, is at the same time a possible threat to the terrestrial environment.

In 2010, the Swiss National Science Foundation launched a project called "Opportunities and Risks of Nanomaterials" (NRP 64), wherein a subproject aims to study the possible effects of NPs on beneficial soil microbes and crops (NanoMiCrops) (SNSF, 2014). With the present study being part of this subproject, the results of this, as well as the findings of accompanying work of NRP 64 in the fields of medicine, material sciences and environmental sciences provide a foundation for decision making of the handling of NPs.

In order to successfully assess the risk of NPs, ecotoxicological studies that examine specific questions are needed. The project NanoMiCrops aims at help filling the existing knowledge gap in both, the behaviour of TiO$_2$ NPs in soil as well as their effects towards important crops and soil microbes. TiO$_2$ NPs were chosen because they are among the most commonly mentioned type in the literature that aim at using NPs for agricultural purposes (Gogos et al., 2012). Furthermore, high input fluxes into agricultural fields are prospected for this material (Gogos et al., 2012). Despite this, little information about testing of TiO$_2$ NPs in soil can be found in the literature.

So far, toxicity testing of TiO$_2$ NPs has been conducted on different levels of ecological complexity. From the cellular (Xue et al., 2010), to the individual (McShane et al., 2012) and the community level (Ge et al., 2011). Furthermore, numerous organisms have been tested including bacteria, plants and animals (Du et al., 2011; Feizi et al., 2012; McShane et al., 2012). However, most studies using TiO$_2$ NPs have been performed in aqueous media with only few in soil.

Focussing on effects of TiO$_2$ NPs in soils, several organisms have been found to be affected. Changes in soil bacterial communities were found by Ge et al. (2012). Both, a reduction in total biomass and shifts in the community composition were shown (Ge et al., 2011; Ge et al., 2012). Structural diversity of soil microorganisms was also shown to be altered upon exposure to TiO$_2$ NPs by Nogueira et al. (2012). Besides this, functioning of bacteria was affected as indicated by changes in soil enzyme activities in a soil spiked with TiO$_2$ NPs at a concentration of approx. 90 mg/kg (Du et al., 2011). For earthworms it was shown that TiO$_2$ NPs affected their reproduction (Schlich et al., 2012) and induced avoidance behavior (McShane et al., 2012). Effects of TiO$_2$ NPs on plants differed depending on plant species. For wheat a reduction in biomass was found at concentrations of 90 mg/kg (Du et al., 2011).
Servin et al. (2013) has shown root to fruit translocation of TiO$_2$ in cucumber. In addition, catalase activity in leaves as well as chlorophyll content were increased in plants grown in soil with a concentration of 750 mg/kg TiO$_2$ NPs. Furthermore, Ti not in the nano scale was shown to either promote or suppress plant growth (Dumon et al., 1988).

In hydroponic systems, Larue et al. (2012) provides indication of uptake and distribution of TiO$_2$ NPs in wheat plants. However, no effects were observed. They reported a threshold diameter of 140 nm for root uptake and 36 nm for translocation to the shoot. Additionally, no influence of TiO$_2$ NPs on wheat biomass in a hydroponic system have been shown by Jacob et al. (2012). A promoting effect of low concentrations of TiO$_2$ NPs for seed germination of wheat was shown by Feizi et al. (2012). Accelerated germination, improved growth and chlorophyll formation of aged spinach was reported by Zheng et al. (2005). Also, on the gene level in plants effects have been found. Genotoxicity to *Allium cepa* and *Nicotiana tabacum* have been shown by Ghosh et al. (2010). For microorganisms, only a few studies were conducted in hydroponic systems. Fan et al. (2014) showed the symbiosis between Rhizobium bacteria and legumes to be disturbed by TiO$_2$ NPs. Delayed root nodule development and hence, altered nitrogen fixation, were detected.

The aim of the present study is to assess the potential toxicity of different TiO$_2$ NPs towards wheat and soil microbes in soil microcosms. More precisely, the research questions are whether different TiO$_2$ NPs affect (1) wheat growth, (2) root colonisation of arbuscular mycorrhizal fungi, (3) denitrification and (4) soil DNA content. For the analytical aspects the research questions are if (5) TiO$_2$ NPs leach and (6) if they are taken up from wheat. To answer these questions a pot experiment was conducted testing two different TiO$_2$ NPs (E171, P25). Wheat was chosen as the model plant because of its importance for human diet (FAO, 2013). Microbes, including arbuscular mycorrhizal fungi and soil bacteria, were selected because of their importance in ecosystem functioning (Altieri, 1999; Auge, 2001; Azcón-Aguilar et al., 1996; Estrada et al., 2013; Gianinazzi et al., 2010; Hildebrandt et al., 2007; van der Heijden, 2010; van der Heijden et al., 1998) Nano specific effects were tried to be detected by inclusion of TiO$_2$ bulk material in the experiment. In addition, the fate of TiO$_2$ NPs was assessed. The outcome of this study seeks to provide information on toxicological aspects of TiO$_2$ NPs, which can then be further used in assessing the risk.
Theoretical Background

2 Theoretical Background

2.1 Nanoparticles

NPs are particles of which at least one dimension is between 1 and 100 nm, which is the nano scale. It is generally thought that properties of particles in this size range differ from those of its bulk material. However, the upper limit of 100 nm has been suggested to be reduced to 30 nm (Auffan et al., 2009). NPs are by no means a sole human invention. Different NPs have even been found in 10’000 year old ice cores for example (Murr et al., 2004). They are formed geologically due to weathering, authigenesis/neoformation (i.e. nucleation and growth of dissolved species) and volcanic activity; hence, they naturally occur in soils and other environmental compartments. Also, biological mechanisms can lead to the formation of NPs (Handy et al., 2008). Despite this, it is the anthropogenic production and release of NPs into the environment that creates uncertainty among regulatory authorities and the scientific community.

In the recent decades, advances in nanotechnological engineering and the recognition of the advantages of nanotechnology led to increased application in various segments. Nanotechnology has found its way into product categories like appliances, electronics, food, health and fitness as well as goods for children (Project on Emerging Nanotechnologies, 2014). To satisfy this demand, production of NPs keeps up with the demand and reached an estimated amount of 10’000 t/a for nano-TiO$_2$, 1600 t/a for nano-ZnO, 380 t/a for carbon nano tubes (CNT), 30 t/a for nano-Ag and 20 t/a for fullerenes in the EU for 2012. However, TiO$_2$ bulk pigment production was orders of magnitudes higher (1’500’000 t/a) due to its wide application in paints, food and cosmetics (Römpp, 2013; Sun et al., 2014). This may constitute a hidden nano- TiO$_2$ production, since a substantial amount of pigments might be in the nano size range as well (Weir et al., 2012).

Alongside with product innovation in the consumer market are innovations in niche markets such as the agrochemical industry. A number of patents which intend to embed NPs in their formulations have come up recently and have been reviewed by Gogos et al. (2012); Khot et al. (2012); Rai and Ingle (2012).

Numerous NPs have been investigated for their potential application in agriculture including nano-silver (Ag), nano-silica (SiO$_2$), nano-aluminium (Al), nano-zinc oxide (ZnO), nano-copper (Cu), carbon nano tubes (CNT) and nano-titanium dioxide (TiO$_2$). Plant protection is the dominant purpose (75 %) with roughly half of the NP constituents being designed as active ingredient and the other half as additive. Other purposes include fertilization and UV-protection (Gogos et al., 2012).

Since TiO$_2$ is well known for its photocatalitic activity (Chen et al., 2007), TiO$_2$ -NPs have been proposed as an additive to PPP to reduce their half-lives (Gogos et al., 2012).

Another field of application is in the protection of photodegradation, which is essentially the opposite of the previously described application. This can be achieved by making use of the reduced
photocatalytical activity of NPs consisting of the rutile crystal form together with coating of different functional moieties. TiO$_2$ NPs can shield the active substance of a PPP from radiation, thus, prevent PPP sensitive to sunlight from photocatalytic degradation (Gogos et al., 2012). Both ways of applying TiO$_2$ NPs aim at a reduction of PPP needed in the field. Besides, TiO$_2$ has also been proposed as a photoprotective constituent shielding leaf surfaces from UV light, thereby reducing sunburn damage of leafs (Gogos et al., 2012). Other possible applications pose the use as dispersing agent and even fertilizer for spinach (Gogos et al., 2012).

This use of NPs in agriculture would pose an input into soils as has been shown by Gogos et al. (2012) (Table 1). Based on application rates of three publications from the scientific literature and patents, they calculated yearly fluxes of TiO$_2$ NPs into soil arising from the use of plant protection products (PPP) and fertilizers.

However, already today, without TiO$_2$ NP inputs arising from agricultural practice, there is an input of TiO$_2$ NPs into agricultural soils. The yearly nano-TiO$_2$ fluxes into soil treated with sludge in the EU was modelled to be around 1200 µg/kg*yr. (940 – 3600 µg/kg*yr.) (Sun et al., 2014). However, if no sludge is applied, as is the case in Switzerland due to banning, the figures are much lower with 0.57 µg/kg*yr. (0.39 – 1 µg/kg*yr.). This clearly shows sludge to be the major input when treated with sludge. If no sludge is applied, the main input of TiO$_2$ is airborne. Surprisingly, despite the 150 times higher production volume, TiO$_2$ bulk material concentration in soil is estimated to be only about one order of magnitude higher than its nano counterpart (Sun et al., 2014).

Comparing the deliberate inputs via the PPP-pathway with inputs of soils amended with sludge, the fluxes are of roughly the same extent or orders of magnitudes smaller. However, if TiO$_2$ NP containing PPP and fertilizers are applied on soils without sludge treatment, the inputs can be up to 18800 fold higher and pose a substantial input (Table 1).

### Table 1: Application rates and calculated fluxes of TiO$_2$ NPs from PPP and Fertilizers into soil. Flux ratio calculated by dividing the highest calculated flux into soil by the mode of sludge treated (EU) and non sludge treated soil (CH), respectively. Adapted from Gogos et al. (2012).

<table>
<thead>
<tr>
<th>Application rate [kg TiO$_2$ ha$^{-1}$]</th>
<th>Calculated flux into soil [µg TiO$_2$ kg$^{-1}$ soil y$^{-1}$]</th>
<th>Flux ratio with sludge</th>
<th>Flux ratio without sludge</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5–15</td>
<td>1607–5357</td>
<td>4.5</td>
<td>9400</td>
</tr>
<tr>
<td>0.0075</td>
<td>2.7</td>
<td>0.002</td>
<td>4.7</td>
</tr>
<tr>
<td>max 30</td>
<td>10714</td>
<td>8.9</td>
<td>18800</td>
</tr>
</tbody>
</table>

### 2.2 Wheat

Wheat (*Triticum ssp.*) is an annual grass that belongs to the Poaceae family. Domesticated forms of einkorn wheat (*T. monococcum*) and emmer wheat (*T. dicoccum*) already appeared about 9500 years ago in present-day southeastern Turkey (Lev-Yadun et al., 2000). In the following centuries, wheat cultivation spread, reaching Greece (8000 BP), Italy, France and Spain (7000 BP), and finally the UK and Scandinavia (about 5000 BP). China and Africa were reached by about 3000BP and introduction
Theoretical Background

to Mexico and Australia happened in 1529 and 1788, respectively (Shewry, 2009). Einkorn and emmer formed the early precursors of today’s wheat species (Winch, 2006, p. 143); hexaploid bread wheat (T. aesticum) and tetraploid hard or durum-type wheat (T. turgidum) (Gustafson et al., 2009, p. 8). The former - also known as common wheat – evolved from the hybridization of wild grass Triticum tauschii and cultivated emmer. Today, about 95% of the wheat grown is bread wheat, 5% durum wheat and a minority of other wheat species (Shewry, 2009).

Within the wheat (T. aesticum L.) species, cultivars are grouped into two types according to their growth habits. Winter wheat is usually planted in autumn and vernalization by low temperatures accelerates the transition from the vegetative to the reproductive stage. Contrarily to spring wheat, which is usually grown in spring, whose transition is not accelerated by vernalization (Yan, 2009, p. 57).

The Food and Agriculture Organization of the United Nations (2013) estimates wheat to be the cereal with the third highest production quantity worldwide in 2012. The 675 million tons (Mt) of wheat were only exceeded by the production of rice (718 Mt) and maize (875 Mt). Main producing areas in descending order were China, India, USA, France and Russian Federation (Food and Agriculture Organization of the United Nations, 2013). Wheat is grown in at least 124 countries (Food and Agriculture Organization of the United Nations, 2013), between the latitudes of 67°N, in Russia and Scandinavia, to 45°S, in Argentina and Chile (Gustafson et al., 2009, p. 6).

Wheat thrives and prospers best at a temperature of 25–27°C. An optimal soil for growth is a fertile soil with medium-heavy texture that is well drained (Winch, 2006, p. 146). For Swiss wheat varieties, nitrogen (N) fertilization of 110 – 150 kg/ha is recommended, depending on stand density, amongst others (IP-SUISSE, 2011).

Growth and development of wheat can be summarised by some basic developmental events. These are germination and emergence, leaf production, tillering, internode elongation, flowering, grain ripening and maturity. All shoots undergo these developmental stages. Therefore, developmental stage schemes could be established for the description of wheat phenology. Among the most widely used are the ones of Feekes and Zadoks (Table 2) (McMaster, 2009, p. 36f).
Theoretical Background

Generally, three major phases are differentiated; vegetative phase with initiation of leaves, reproductive phase with floret development and grain-filling phase with growth to final grain weight (Miralles & Slafer, 1999, p. 14).

Seed germination occurs by seed imbibition, which unleashes the metabolic activity of the seed. The shoot apex is the place where leaf initiation subsequently takes place. By emergence, five to seven leaf primordia (i.e. leaf tissue in its early stage) have formed. Shortly after emergence of the tip of the coleoptile through the soil, the leaf appears by pushing through the coleoptile (Miralles & Slafer, 1999, p. 15).

The appearance of the fourth leaf concurs with the first tiller, reflecting their close relation (Figure 1, Zadoks 21) (Miralles & Slafer, 1999, p. 16ff). The first culm is named main stem (MS), while subsequent calms are tillers (McMaster, 2009, p. 34). Axillary tiller buds are formed in each phytomer, with each bud potentially developing into a tiller. With resources becoming scarce, tillering slows down and stops until eventually, tillers may die in reverse order of appearance. This process generally coincides with the onset of stem elongation (Figure 1, Zadoks 31).

Furthermore, initiation of the terminal spikelet, and thus the reproductive phase, often sets in at that time. Thereafter, florets become initiated in the first spikelets. After termination of the initiation before

<table>
<thead>
<tr>
<th>Stage</th>
<th>Feekes</th>
<th>Zadoks</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination</td>
<td>No stage</td>
<td>01-07</td>
<td>Seed swells and coleoptile emerges</td>
</tr>
<tr>
<td>Emergence</td>
<td>No stage</td>
<td>09</td>
<td>First true leaf emerges through coleoptile (aboveground)</td>
</tr>
<tr>
<td>Tillering</td>
<td>1.0</td>
<td>21-29</td>
<td>First tiller is visible</td>
</tr>
<tr>
<td>Internode elongation</td>
<td>6</td>
<td>31</td>
<td>First node visible</td>
</tr>
<tr>
<td>Flag leaf</td>
<td>8-10</td>
<td>39</td>
<td>Last (i.e. flag) leaf appears</td>
</tr>
<tr>
<td>Booting</td>
<td>8-10</td>
<td>41-49</td>
<td>Boot swells, flag leaf sheath opens and awns become visible</td>
</tr>
<tr>
<td>Heading</td>
<td>10.1-10.5</td>
<td>50-58</td>
<td>Spikelet (of main stem) become visible until complete emergence of inflorescence from flag leaf</td>
</tr>
<tr>
<td>Anthesis</td>
<td>10.5.1-10.5.4</td>
<td>61-69</td>
<td>Begins with first anther (yellow) on inflorescence and ends with last one appearing</td>
</tr>
<tr>
<td>Physiological maturity</td>
<td>11.1-11.4</td>
<td>77-99</td>
<td>Ripening until ripe for cutting (when components of spike, internode tissue and leaf lost all green colour)</td>
</tr>
</tbody>
</table>

Generally, three major phases are differentiated; vegetative phase with initiation of leaves, reproductive phase with floret development and grain-filling phase with growth to final grain weight (Miralles & Slafer, 1999, p. 14).

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Furthermore, initiation of the terminal spikelet, and thus the reproductive phase, often sets in at that time. Thereafter, florets become initiated in the first spikelets. After termination of the initiation before
the flag leaf ligule appears, florets further develop. However, from booting to heading/anthesis, many induced florets abort with only a few (1-4) becoming fertile florets at anthesis (Figure 1, Zadoks 60) (Miralles & Slafer, 1999, p. 21f). Wheat is mainly a self-pollinator (Winch, 2006, p. 143). After fertilization of the developed florets, plants enter the grain-filling phase. This phase is marked by slow accumulation of dry matter in the beginning, subsequent development of endosperm cells and final grain growth. When grains exhibit maximum dry weight, physiological maturity is reached (Miralles & Slafer, 1999, p. 23).

The sequence of phenological development described above, however, varies among shoots in duration and timing. This is due to both, responses to genotypic differences and environmental conditions. The two most important factors are temperature and photoperiod. Other components, such as nutrition, water availability, plant density and radiation may have a small effect. Nevertheless, these differences fade away when shoots approach physiological maturity (McMaster, 2009, p. 32f, and references therein; Miralles & Slafer, 1999, p. 23f).

<table>
<thead>
<tr>
<th>Zadoks</th>
<th>10</th>
<th>21</th>
<th>26</th>
<th>30</th>
<th>31</th>
<th>32</th>
<th>37</th>
<th>39</th>
<th>45</th>
<th>50</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feekes</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>10.1</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Figure 1: Growing wheat plant and indication of its corresponding developmental stage according to the two common classifications of Feekes and Zadoks. Graphic by Jerry Downs. Adapted from: (Large, 1954)
2.3 Mycorrhiza

The symbiotic relationship between fungi and plant roots is commonly referred to as mycorrhiza. Colonisation of plant roots by mycorrhizal fungi can occur in two distinct ways. While ectomycorrhiza colonise roots extracellularly, arbuscular mycorrhizal fungi (AMF) extend parts of their hyphae into the plant cell (Parniske, 2008). Arbuscular mycorrhiza (AM) symbiosis are extremely ancient, which is made clear by their possible role in the colonization of land by plants (Smith et al., 2008, p. 13). The fungi from the order Glomeromycota developed into the most common mycorrhizal type nowadays.

The importance of AMF is because of both, the vast distribution and the ecological functions AMF perform. About 80% of the terrestrial plants of many phyla in most parts of the world form AM symbiosis. In contrast to this large number of potential hosts stands the low number of AM fungal species (~230) (Oehl et al., 2011; Smith & Read, 2008, p. 35). The ecological functions AMF perform are manifold. Besides influencing plant diversity and productivity, AMF have been shown to protect plants against biotic and abiotic stressors, such as plant pathogens, toxic heavy metals, high salt concentrations and water shortage (Auge, 2001; Azcón-Aguilar & Barea, 1996; Estrada et al., 2013; Gianinazzi et al., 2010; Hildebrandt et al., 2007; van der Heijden, 2010; van der Heijden et al., 1998). AM-fungi are obligate symbionts, which means that they depend on an organic C supply. This C is delivered by the photosynthetic partner, that transfers between 4 and 20% of net photosynthate to the fungus (Smith & Read, 2008, p. 143). Mycorrizhal fungi extend their hyphal network beyond the P depletion zone, that forms around plant roots (Karandashov et al., 2005). This way, the fungus gains access to P, which it provides the host with in return for the photosynthetic assimilates.

In fact, up to 100% of the plant P can be derived via the mycorrhizal pathway with a “downregulation” of the direct P uptake at the root/soil interface (Smith et al., 2003).

AMF possess some characteristical structures which are shown in Figure 2. The tree like shaped arbuscules poses the interface for nutrient exchange between fungus and plant (Figure 2, A). The other structure considered diagnostic for AM symbiosis are vesicles (Figure 2, C) (Smith & Read, 2008, p. 13). This ovoid to box-like component is most likely an important storage organ. Similar as for arbuscules, development of vesicles is strongly affected by the environmental conditions, such as reduced formation with increasing P availability (Smith & Read, 2008, p. 73). AMF ensure their long-term survival with the formation of spores, which are, in addition to root fragments and hyphae, a mean of root colonisation (Smith & Read, 2008, p. 42f). The hyphal network, reaching from inside the hosts root far into the soil, can be seen as the connecting bridge between the individual components (Figure 2, B).
Germination of some spores have been shown to be pH dependent. Germination is also temperature dependent with different species having different optimum. This may reflect the climatic conditions of the entophytes origin (Koltai & Kapulnik, 2010, p. 6). Inhibitory effects of Zn have been reported in respect to germination of *G. mosseae* and *G. caledonium* spores (Koltai & Kapulnik, 2010, p. 8). Colonisation of host roots by AMF is generally thought to be negatively correlated to soil phosphorus content (Treseder, 2004). This has also been demonstrated for wheat (Covacevich et al., 2007; Graham et al., 2000; Ryan et al., 2008).

The process of root colonisation by AM fungi is highly dynamical. While roots grow apically and lateral roots are initiated, the fungus infects and colonizes the root cortex. Not only is it the rate of infection unit formation and their growth that determine the degree of root system colonisation but also the rate of growth of the root system itself. Because increased branching of roots and hyphae increases the likelihood of infection of roots by infective hyphae. Therefore, factors influencing rate of root growth will inevitably affect its colonisation (Smith & Read, 2008, p. 82).

Root colonisation of AMF in winter wheat varies widely with no clear agreement in the literature. Rather low colonisations of 4 to 8% were found by Mäder et al. (2000). On the other hand, most studies show rates laying in the range of 16 to 50% (Al-Karaki et al., 2004; Hildermann et al., 2010;
The variation may be explained by the use of different cultivars, test systems (e.g. field or pot), test design (e.g. inoculation) and differing environmental variables such as soil type and temperature. Furthermore, root colonisation of wheat is also dependent on the growth and developmental stage of the wheat plant as was shown by Al-Karaki et al. (2004). They examined root colonisation of two wheat cultivars at different stages. Non-inoculated roots reached 18% colonisation at tillering, 31% at heading and decreased slightly to 25% at the grain filling stage.
3 Material and Methods

3.1 Material
Chemicals and all instruments used during the experiment are listed under Chemicals and Instruments in the Appendix.

3.2 Nanoparticle characterisation
The experimental design included the testing of two different types of TiO$_2$ NPs (CAS Number 13463-67-7), namely P25 and E171, which differ in crystalline structure. P25 consists of both anatase and rutile phases in a ratio of about to 4:1 to 3:1 (Bickley et al., 1991; Ohno et al., 2001). E171 NPs (Lot#: 10100387, Sachtleben Chemie GmbH, Duisburg, Germany) consist of the anatase phase only. P25 NPs (Lot#: MKBG9739V, Sigma-Aldrich, Buchs, Switzerland) have an average size of 28 nm and a specific surface area (SSA) of 35-65 m$^2$/g (BET) (manufacturer information). The size distribution ranges from 13 nm to 61 nm (Figure 3, P25). In contrast, E171 particles are larger, with an average size of 91 nm and a size distribution from 52 nm to 182 nm, containing particles exceeding the nano scale, (Figure 3, E171). No information on the SSA could be obtained for E171. Particles of the corresponding bulk TiO$_2$ (Lot#: MKBG3671V, Sigma-Aldrich, Buchs, Switzerland) are mostly greater than 100 nm, however, it also contains particles in the nano scale (Figure 3, Bulk TiO$_2$). Similarly to E171, the crystalline structure of the bulk material is anatase.
Material and Methods

3.3 Experimental design

Both NP types were tested at concentrations of 1000, 100 and 1 mg/kg soil (Table 3). 1000 mg/kg was chosen as a high exposure scenario, reflecting high application rates. Based on the estimation of yearly input, 100 mg/kg was chosen as a realistic concentration. The lowest, environmentally relevant concentration was chosen because a higher toxicity of low concentrations has been suggested due to

Figure 3: TEM pictures of TiO$_2$ NPs and Bulk TiO$_2$ used (left). Corresponding size distribution with counts per size class (right).
less aggregation of NPs and thus, more free NPs (K. Tiede et al., 2009).

In addition to the testing of TiO₂ in the nano-sized range, the material was included in the test design in its bulk form (see Chapter 3.2, Nanoparticle characterisation). Unspiked soil served as negative control and soil spiked with zinc sulphate served as positive control because effects on wheat have been demonstrated (Warne et al., 2008). Because NP testing was of main interest, both, bulk TiO₂ and zinc sulphate were only tested at the highest concentration of 1000 mg/kg. This concentration of zinc sulphate corresponds to approx. 400 mg/kg Zn. Each of the nine treatments was performed in seven replicates, leading to a total of 63 pots.

An overview of the most important events during the experiment is given (Figure 4). It includes sowing on day 1 and harvesting beginning on day 84 with some other events that will be outlined in detail below.

![Timeline with days after sowing showing the most important events during the experiment. The events will be outlined in the Chapters 3.4, Test preparations and 3.5, Course of Experiment.](image)

Table 3: Overview of the different treatments with the concentrations tested.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TiO₂ NP P25</th>
<th>TiO₂ NP E171</th>
<th>TiO₂ Bulk</th>
<th>Zinc sulphate (Zn)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration [mg/kg]</strong></td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000 (400)</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 4: Timeline with days after sowing showing the most important events during the experiment. The events will be outlined in the Chapters 3.4, Test preparations and 3.5, Course of Experiment.

### 3.4 Test preparations

#### 3.4.1 Spiking with Nanoparticles

A natural soil was obtained from an acre near the research centre of Agroscope, Zurich (N47° 25' 39.564" E8° 31' 20.04"). The soil was classified as brown earth with a sandy loamy to loamy fine fraction. The top layer (5 cm) of the soil was removed and approx. 0.9 m³ of the underlying 15 cm topsoil were sampled. The soil was then sieved through a mesh size of 5mm and stored in a container,
located in a dry place, until use. The soil was blended with quartz sand (50% v/v) in order to facilitate the handling with respect to the harvesting of the plants.

Mixing with the quartz sand and the test substances was done by dry mixing in two steps. In a first pre-mixing step, 300 g of a 50% (v/v) sand-soil mixture was weighed into a 500 mL Schott bottle and 0.03 g, 3 g or 30 g of test substance were added. Zinc sulphate was ground to powder in a mortar prior to addition in order to allow for homogenous mixing. Because the highest concentration resulted in a large volume of NPs, each of these treatments was prepared in two 300 g sand-soil mixtures. Sand, soil and test substance were then mixed in a powder mixer (Turbula® T 2 F, Willy A. Bachofen AG, Basel, Switzerland) for 30 min.

The pre-mixture was then put into a cement mixer. 30 kg of a sand-soil mixture (50% v/v) were added, to final test substance concentrations of 1, 100 or 1000 mg/kg, respectively. The mixing chamber was sealed with a plastic sheet to avoid dust formation and run for 6 hours in a slightly raised position. With the exception of the control treatment, the mass of the pre-mixture was subtracted from the total amount in order to reach exactly 30 kg. The soil was not dried and concentrations always refer to a slightly moist sand-soil mixture. This leads to higher actual concentrations of 1044, 104 and 1.04 mg/kg dry weight (dw).

### 3.4.2 Soil characterisation

The most important soil parameters were analysed by the soil analytical laboratory Agroscope Reckenholz, Switzerland and are illustrated in Table 4. The soil is slightly alkaline as indicated by the pH. This is not surprising, since the soil calcium carbonate (CaCO₃) pool is not depleted yet. Cation exchange capacity (CEC) is low but surface sites are occupied 100% with base cations. The background concentration of Ti was measured by Bachema AG, analytical laboratory, Schlieren, Switzerland. The concentration of Ti in the test soil is 783 mg/kg.

**Table 4:** Soil parameters including water holding capacity (WHC), pH (H₂O), humus content, calcium carbonate content (CaCO₃), cation exchange capacity (CEC), base saturation and the nutrients phosphorus (P), potassium (K), magnesium (Mg) and calcium (Ca).

<table>
<thead>
<tr>
<th>WHC</th>
<th>pH (H₂O)</th>
<th>Humus</th>
<th>CaCO₃</th>
<th>CEC</th>
<th>Base saturation</th>
<th>P</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>cmol'/kg</td>
<td>%</td>
<td>mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>7.7</td>
<td>0.55</td>
<td>2.6</td>
<td>6.0</td>
<td>100%</td>
<td>35.4</td>
<td>66.4</td>
<td>124.9</td>
<td>8330</td>
</tr>
</tbody>
</table>
3.4.3 Preparation of the pots

Pots consisted of a shortened PVC-sewer pipe glued on a plastic board (Figure 5). A ball valve, impermeably fitted in the middle of the plastic board, enabled the flow out of leachate. The valves were closed throughout the experiment except when capturing leachate at the end of the experiment. The pots were filled with 520 g of quartz sand as a separation layer between outlet and soil. A plastic mesh laid on top of the outlet prevented the overlaying sand from trickling through, and clogging the valve. Pots were filled in top of the quartz sand with 4 kg of mixed soil immediately after termination of the mixing process. The remaining soil (approx. 2 kg per treatment) was filled into a plastic bag and stored in a refrigeration room. After filling, pots were randomised, placed on trays (21 pots per tray) and then stored in the greenhouse until sowing.

3.4.4 Sowing

The experiment was performed in two blocks with a time lag of one week in order to reduce the workload during harvest. Shortly before sowing, all pots were watered with 100 ml of tap water. This was done to increase soil cohesion, which facilitated the sowing process. Wooden skewers were used to prepare small holes of approximately 1 cm depth (Figure 6). In a circular manner, three pairs of small holes with approx. 1 cm depth were created near the pot wall, approx. 3 cm apart from each other. Wheat seeds were placed in the holes and covered with soil. Damaged or abnormal looking seeds were sorted out. Two days after sowing, seedlings were watered with 50 ml tap water. In order to ensure similar heights among seedlings, three out of six seedlings per pot were removed after emergence of almost all of them (day 5). Removal was done by performing the following steps.
Material and Methods

1. indexing the emerged seedlings according to their height into three classes; 0-1.5 cm, 1.5-3 cm and >3 cm.
2. definition of the major height class.
3. determination of seedlings to be removed, which usually was the one of a minor height class.
4. seedlings decided to be removed were taken out of the soil with tweezers

The procedure was adjusted if the following special cases occurred. If none of the individual seedlings of a pair, however, was of the major height class, the one deviating most from the major class was removed. If no seedling of a pair emerged, another seedling from the same pot was carefully transferred to that place using tweezers. This, however, only occurred once. Also, at spots where no seedlings emerged, seeds were removed and it was documented, whether they germinated or not. Finally, seedlings were watered with 100ml tap water.

3.5 Course of Experiment

3.5.1 Greenhouse conditions

Wheat was grown in a greenhouse chamber under semi-controlled conditions. Light was set to a 16/8 hour day/night cycle and the chamber was heated to 25°C and 16°C, respectively.

3.5.2 Watering and fertilization

Plants were watered three times a week with tap water. Watering was done gravimetrically by refilling the soil water content to 60% of its water holding capacity (Table 4).

In order to obtain an estimate of the fertilizer demand, a sample of mixed control soil was sent for analysis of phosphorus (P), potassium (K) and magnesium (Mg) to the Labor für Boden- und Umweltanalytik, Steffisburg, Switzerland. P, K and Mg content was found to be sufficient, modest and sufficient, respectively, for plant growth. N demand of wheat lies between 110 and 150 kg N/ha for a normal stand density of 300-400 plants/m² (IP-SUISSE, 2011). However, because stand density was half of that (170 plants/m²), N application was reduced to 80 kg N/ha. An additional 50 kg N/ha was applied the week before termination of the experiment for nitrous oxide (N₂O) measurements as will be outlined under Chapter 3.5.4, N₂O measurement.

Fertilizer corresponding to 10 kg/ha was applied once in the first four weeks und every week thereafter. Hoagland solution was used as fertilizer. Macronutrients stock solution contained 6.07 g/l Potassium nitrate (Dr. Bender & Dr. Hobein Ag, Zürich, Switzerland), 9.45 g/l Calcium nitrate tetrahydrate (Merck, Darmstadt, Germany), 0.6 g/l Ammoniumnitrate (Merck, Darmstadt, Germany), 0.58 g/l Ammonium dihydrogenphosphate (Merck, Darmstadt, Germany) and 2.47 g/l Magnesium sulfate heptahydrate (Merck, Darmstadt, Germany). Micronutrients stock solution contained 2.78 g/l Calcium chloride (Sigma-Aldrich, Buchs, Switzerland), 1.55 g/l Boric acid (Sigma-Aldrich, Buchs, Switzerland), 0.34 g/l Manganese sulfate monohydrate (Merck, Darmstadt, Germany), 0.58 g/l Zinc sulfate heptahydrate (Sigma-Aldrich, Buchs, Switzerland), 0.13 g/l Copper(II) sulfate pentahydrate.
Material and Methods

(Sigma-Aldrich, Buchs, Switzerland) and 0.62 g/l Ammonium molybdate tetrahydrate (Sigma-Aldrich, Buchs, Switzerland). 10 ml of the micronutrient stock solution and 10 ml of a solution containing 7.34 g/l Ethylenediaminetetraacetic acid ferric sodium salt (Sigma-Aldrich, Buchs, Switzerland) were added to the macronutrients stock solution. Pots were fertilized with 7.9 ml of this working solution. Since soil P content was sufficient for plant growth and because AMF colonisation has been shown to be reduced by excess soil P availability (Treseder, 2004), its content of the solution, was reduced by 75%.

3.5.3 Plant height and chlorophyll content measurements
In the course of the experiment, plant height and chlorophyll content were measured. Plant height was documented by holding the leafs vertically and measuring the distance from the soil surface to the longest leaf using a folding yardstick. Chlorophyll content of leafs was estimated using a portable chlorophyll meter (SPAD-502, Konica Minolta Sensing, Osaka, Japan). For each round of measurements, the same leafs were measured three times per plant at different spots and the average was reported. This technique allowed to assess the plant condition by measuring so called SPAD values. These values are unitless and require calibrations between SPAD units and extracted chlorophyll values, which was not done. Therefore, they are only an estimate for chlorophyll content and hence, indicator for plant health. Both, plant height and chlorophyll content, were measured every 14 days and for each of the three plants per pot. In addition, plant height was also measured on day seven.

3.5.4 N₂O measurements
N₂O fluxes emitted from the soil were measured to estimate the presence of denitrifying bacteria and their ability to reduce oxidised forms of Nitrogen (N). These measurements were started six days before termination of the experiment (day 78). Due to the high time demand of the measurement, it was confined to the highest concentration of P25 and E171 plus the control.
Material and Methods

Pots were prepared by fertilization with 50 kg N/ha so as to provide a sufficient amount of N for denitrification and to represent a normal application of N for wheat in Swiss agricultural practice (IP-SUISSE, 2011). In principle, the fertilizer outlined in Chapter 3.5.2, Watering and fertilization, was used but the amount of Ammoniumnitrate was adjusted so as to reach the desired amount of N. The fertilizer was added together with watering as described below. Denitrification is largely performed by facultative anaerobic bacteria. Therefore, such an anaerobic environment was created by adjusting the water content of the pots to approx. 106% of the WHC. Within 2 days after watering, four flux measurements were performed 6, 20, 28 and 49 h after watering. A non-transparent cap was put over the plants to separate the system from the atmosphere. Great care was taken not to pull leaves off the plants. Due to the height of the plants, this resulted in a rather large headspace of 72 cm from the soil surface to the lid (Figure 7). The gas phase in the headspace was then pumped through a loop for 20 min, followed by a N₂O Analyser (TEI46c, Thermo Fisher Scientific, Waltham, USA). Prior to each measurement, pots were weighted in order to verify similar water content among pots. Because pots of block 1 were not entirely watertight, the water content could not be controlled. Therefore the measurements were confined to block 2.

3.5.5 Collection of leachate
Leaching of NPs out of the soil with percolating water was assessed by analysing the leachate for Ti. Leachate samples were collected from the pots during the preparations for the N₂O measurements and on the treatments tested there only (i.e. P25 and E171 1000 mg/kg plus control). Pots were watered to a water content of more than 100% of the WHC (see Chapter 3.5.4, N₂O measurements) and approx. 50 ml leachate was collected at the bottom of the pots. The leachate samples were analysed for Ti content within a day. Samples were not chemically digested. This was done to prevent the sample from being altered, which could manifest itself in the precipitation of NPs. However, small particles and living larvae were carefully removed with a clean tissue. Analysis was done with inductively coupled plasma optical emission spectrometry (ICP-OES) using a Spectro ICAL Ti standard (Arcos FHS 16, SPECTRO Analytical Instruments, Kleve, Germany).

3.5.6 Harvesting
Harvest begun on day 84. The throughput was 9 pots per day, one of each treatment in a randomised fashion. This led to a harvest of 3 days for the 27 plants of block 1 and 4 days for the 36 plants of block 2. Pictures of all plants were taken on day 84.
Material and Methods

The days before termination of the experiment, soil water content was adjusted so that the water content at harvest was about 25% of the WHC. The following list depicts the harvest procedure.

1. The strongest tiller of each plant in a pot was determined based on the largest flag leaf. If flag leaves were of the same size, then the longer tiller was chosen. Flag leaves were cut off the plant directly at the stem using a scissor. Their area was subsequently measured with a portable area meter (Li-3000A, LI-COR, Lincoln, USA), put in a paper bag and weighted.

2. Ears of the strongest tillers were cut off the plants directly below the lowest spikelet, put in a paper bag and weighted.

3. All other flag leaves and ears were cut off the plants. The parts were separately put in paper bags and weighted.

4. All residual leaves were cut away and put in paper bags.

5. The whole culms were cut about 1 cm above soil surface and separated into upper calm and lower calm by cutting below the first culm node. Lengths of both parts were recorded for strongest tillers only.

6. After plants were removed, two core samples of the soil were taken. The cores were separated into depth levels 0-5, 5-10 and >10 cm and put in plastic bags.

7. Plant roots were carefully removed from the soil matrix and thoroughly washed with water to remove soil particles. Root weight was recorded. Representative subsamples of the root were taken for quantification of the root colonization by AMF and for imaging with transmission electron microscopy (TEM).

   The sand used as drainage at the bottom of the pots was removed and the soil sieved through a 2 mm grid. Two times 10 g were weighted and put into a paper bag for soil dry weight determination. Two times 0.5 g were weighted and put into 1.5 ml Eppendorf tubes for DNA extraction, which contained about 0.5 g glass beads (0.1 mm).

8. After each pot, all material was thoroughly cleaned with water, dipped into an ethanol bath and flamed.

Plant samples were dried in an oven at 70°C and soil samples at 105°C, until constant weight was reached and then weighted to determine dry weight. Soil samples for enzyme analysis as well as core samples were stored at 5°C. As for the samples for DNA extraction, 1.4 ml DNA extraction buffer without dithiotreitol (DTT) according to Bürgmann et al. (2001) was added. Tubes were thoroughly shaken and stored at -20 °C.
Material and Methods

3.6 Post experiment analysis

3.6.1 Root colonization by mycorrhizal fungi

The presence of AMF spores in the test soil was proven prior to the experiment. Therefore, the ability of root colonisation by AMF was assumed to be given. Subsamples of the wheat roots were stained according to the method of Vierheilig et al. (1998) with slight modifications. For clearing the roots with potassium hydroxide (Sigma-Aldrich, Buchs, Switzerland), boiling time was set to 20 min. Staining was done by boiling the cleared roots in a ink-vinegar (Parker Quink, black) solution for 20 min. The roots were subsequently destained by rinsing with deionized water. Stained roots were stored in 50% Glycerol (Merck, Darmstadt, Germany) until further usage.

Roots were then mounted on a microscope slide and mycorrhizal colonization was examined under the microscope (DM 2000, Leica, Wetzlar, Germany) after McGonigle et al. (1990). Roots colonization by AMF was investigated for 5 replicates per treatment only.

3.6.2 Soil microbial DNA extraction and quantification

The DNA extraction basically followed the procedure of Bürgmann et al. (2001). However, no DTT was used in the extraction buffer. Extraction was done in a cell disrupter (FastPrep® FP120, Qbiogene, Illkirch, France) with speed adjusted to 5.5 m/s for 45 sec and centrifugation in a microliter centrifuge (Biofuge Pico, Thermo Fisher Scientific, Waltham, USA) for 1 min only. Extraction was repeated three times with transfer of the supernatant fluid to the same sterile tube each time. Sodium phosphate buffer (MP Biomedicals, Santa Ana, USA) was used as extraction buffer for the 2nd and 3rd extraction round. Final extraction was done by adding 2ml chloroform (Sigma-Aldrich, Buchs, Switzerland) to the supernatant and centrifugation (4K15C, SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany) at 16000 g for 5 min only. The aqueous phase was then incubated at 37 °C for 1 h. Centrifugation for DNA pellet creation was done for 15 min. After washing with 70% ethanol (-20 °C) and centrifugation at 16,000 g for 2 min, DNA was re-suspended in TE (life technologies, Zug, Switzerland).

After extracting the DNA, it was washed using a gDNA Clean-up kit (NucleoSpin®, Macherey-Nagel) with slight modifications. Mixing of the sample and DB (NucleoSpin®, Macherey-Nagel) during the binding step was done directly in the column. Final elution was done by adding 75 µl DE (NucleoSpin®, Macherey-Nagel), letting it stand for 5 min and subsequent centrifugation. This step was repeated once. In all steps, centrifugation lasted 1 min.

Samples were then diluted 10 times in ddH2O. Staining of DNA was done by adding 5 µl sample to 160 µl TE (life technologies, Zug, Switzerland) and 0.25 µl Quant-iT PicoGreen (Invitrogen, Carlsbad, USA). DNA was quantified with a fluorescence spectrometer (Cary Eclipse, Agilent Technologies, Santa Clara, USA).
3.6.3 Root fixation and thin-section preparation for TEM analysis

Fresh root samples were pre-fixed in 2.5% Glutaraldehyde (Sigma-Aldrich, Buchs, Switzerland) in phosphate buffered saline (PBS) directly on the day of the harvest and stored at 4°C until processing. Prior to the next steps of the fixation/embedding process samples were rinsed three times with PBS. Samples were then incubated with 1% Osmium tetroxide (Sigma-Aldrich, Buchs, Switzerland) at room temperature (RT) for 40 min and then rinsed with water three times. Subsequently, samples were incubated with 1% Uranyl acetate dihydrate (Sigma-Aldrich, Buchs, Switzerland) in water for 1 h and rinsed with water three times. Dehydration was performed with 50% (15 min), 70% (20 min), 90% (25 min), 100% (5 min) and 100% water free ethanol for 30 min and Propylene oxide (Sigma-Aldrich, Buchs, Switzerland) 100% for 30 min.

Epon stock solution was prepared by mixing 70.89 g Epon 812™ (Sigma-Aldrich, Buchs, Switzerland), 92.35 g Durcupan™ ACM (Sigma-Aldrich, Buchs, Switzerland) and 8.68 g Dibutylphthalat (Sigma-Aldrich, Buchs, Switzerland). Working solution was done by adding 5.85 g Epon stock solution to 5 g Epoxy embedding medium hardener DDSA (Sigma-Aldrich, Buchs, Switzerland) and 310 mg Accelerator DMP 30 (Sigma-Aldrich, Buchs, Switzerland).

Samples were incubated in 50% Epon working solution and 2 times 100% Epon working solution for 1 h respectively. The samples were placed in an oven at 60°C overnight for polymerization.

Root thin sections were prepared by pre-trimming the Epon blocks with a razor blade to lay open the root. They were then mounted into ultramicrotome (Ultracut E, Leica, Wetzlar, Germany) and specimen surface was flattened perfectly with a glass knife.

A diamond knife, filled with water, was then used to cut sections at 70 nm. Root thin sections were collected from water surface with an eyelash, transferred to a formvar/carbon coated copper TEM grid and dried at RT. A TEM (Tecnai G2 Spirit, FEI, Hillsboro, USA) was used for imaging, coupled with an energy-dispersive X-ray (EDX) spectroscope (X-Max, 80mm², Oxford Instruments, Abingdon, UK) for elemental analysis.

3.6.4 Nutrient and Titanium content of wheat grains

In order to analyse N, P and Ti content in wheat grains, they had to be separated from the ears. To ensure analysis of ripe grains only, abnormal looking grains and grains smaller than half the size of the largest were sorted out. Seeds of the ears from the strongest tillers were counted. Together with the grains of the residual ears, they were ground in a ball mill (MM 400, Retsch, Haan, Germany) at a frequency of 25 s⁻¹ for 4 min. Grains of all three plants per pot were pooled.

For the digestion for P and Ti analysis, approximately 200 mg of ground grains per sample were digested in Teflon tubes. 15 mL 70% HNO₃ (Sigma-Aldrich, Buchs, Switzerland) was added and heated at 120°C for 90 min in a digestion block (DigiPREP, SCP Science, Quebec, Canada). After cooling down, 3 mL 30% H₂O₂ (Sigma-Aldrich, Buchs, Switzerland) were added and the digestion
block was again heated to 120°C for 90 min. The solution cooled down and was filled up with Milli-Q water to 50 ml. Measurements were conducted on an ICP-OES using an IPE 198 standard. For N analysis, approx. 4 mg of ground grains were weighted into tin cartridges and subsequently analyzed for N and C content in a CHN-Analyzer (EURO EA, Eurovector SpA, Milano, Italy).

3.7 Statistics

Statistical analysis was done with Rstudio (R Core Team, 2013). Data was tested for homoscedasticity and normality using the Barlett’s test and Shapiro–Wilk test, respectively. In case of homoscedastic and normally distributed data, analysis of variance was conducted with a one-way ANOVA and Dunnet’s test was performed for comparison of treatments with the control. If data was not homoscedastic and / or normally distributed, Mann-Whitney test was performed for comparison. In any case, level of significance was 5%.

If the blocks showed significant differences between each other, generalized linear model of gaussian family was fitted to include the error of the block.

Data was nominated and tested for correlation among variables. If correlations with Pearson's r greater than |0.8| were found, one variable was removed before performing a principal component analysis. Removed were the variables N, residual ears, residual flag leafs, shoot to root ratio and number of grains (Appendix, Correlations of Variables). A principal component analysis was conducted with the remaining data.
4 Results

The first three subchapters of the results primarily deal with the fate of NPs assessed in the present study. The next subchapters, beginning with wheat physiology, capture the findings on the effect side.

4.1 Ti concentration in leachate

The Ti concentrations in the leachates were similarly low for the control and the P25 1000 mg/kg treatment (Figure 8). However, concentrations in the E171 1000 mg/kg treatment were significantly higher compared to both, the control and P25 treatment.

![Figure 8: Concentrations of Ti in mg/l in leachates captured at the end of the experiment. Leachates were captured for the control, P25 NPs 1000 mg/kg and E171 NPs 1000 mg/kg only (N=7). The box represents the 1st and 3rd quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively. Asterisks indicate significant difference to the control treatment at a 5% significance level.](image)

4.2 Ti concentration in grains

Ti concentrations in grains were significantly lower for the smallest concentration of the treatments as well as the positive control (i.e. Zn 400 mg/kg) compared to the control (Figure 9). Although not significantly different, some plants of the P25 100, E171 100 and 1000 mg/kg and the TiO₂ bulk treatments showed concentrations up to seven times larger than the background level of the control. For both types, highest concentrations of Ti were found for the concentration of 100 mg/kg. Peaks were smaller for the 1000 mg/kg treatment and no Ti was found for the 1 mg/kg treatments. Recovery of Ti was calculated by dividing the amount of Ti recovered by the amount of Ti in a reference
material multiplied with 100. This is a measure of effectiveness of the extraction method used. Recovery of Ti was 31%.

4.3 TEM analysis of root thin sections

Treatments of mostly 1000 mg/kg and some 100 mg/kg of both P25 and E171 were screened for NPs. One to two sections were examined per sample. Neither in treatments with P25 nor with E171 were NPs found. However, conspicuous particle and agglomerate like structures were found in one section of the bulk treatment (Figure 10). Spectral analysis revealed that they contain Ti. The first particle appeared to be an agglomerate of smaller particles leading to a size of 320 nm. Similarly, the second one possessed a rather large size of 210 nm. The third one, however, may have been a single particle with a size of 70 nm. The fourth spectrum of the background did not contain Ti. These structures were located inside the plant root.
Figure 10: Overview of a root thin section from the bulk treatment 4800 × magnified with black frame indicating enlarged view (top). The cell was fully surrounded by the plant cell wall (A). However, Epon was damaged within the cell (B). Enlarged view 23000 × magnified with particles (2) and agglomerates (1 & 3) containing Ti (lower left) and a structure not containing Ti (4). Spectra in keV corresponding to the objects (lower right).
4.4 Wheat physiology

4.4.1 Wheat height and chlorophyll content

The plant heights measured at the end of the experiment (day 75) indicated similar heights within block 1 and 2. Block 1 was sown one week before block 2. This similarity within block 2 is illustrated in Figure 11.

![Figure 11](image)

Figure 11: Pictures of plants of block 2 at the end of the experiment (day 84 – 88) of the treatments control, P25 and E171 TiO$_2$ NPs at concentrations of 1, 100 and 1000 mg/kg, respectively, as well as bulk TiO$_2$ and Zn at concentrations of 1000 and 400 mg/kg, respectively.

Plant height between the two blocks differed significantly from each other with 8.3 cm (+/- 2.1 cm) (Figure 12). If data from the two blocks were combined (data not shown), variation of the data was substantially increased. As for plant height, the difference of the blocks was detected at day 7, 45, 60 and 75. With the exception of treatment E171 100 mg/kg at day 60, treatments did not differ significantly from the control.
Results

Chlorophyll content of the leaves was the second continuously recorded endpoint. All but the Zn treatment had similar values (Figure 13). Chlorophyll content of the Zn treatment was significantly smaller to the control already at day 45 and 60 (data not shown). Similarly to the plant height, chlorophyll content was significantly different between the two blocks. Chlorophyll content of plants of block 2 were on average 2.2 (+/- 2.4) SPAD units smaller than from block 1.

Figure 12: Plant heights in cm at day 75 of the experiment for block 1 (left) and block 2 (right). Treatments are the control, P25 and E171 TiO\textsubscript{2} NPs at a concentration of 1, 100 and 1000 mg/kg, respectively as well as the TiO\textsubscript{2} bulk treatment with a concentration of 1000 mg/kg TiO\textsubscript{2} and Zn treatment with 400 mg/kg Zn (N=7). Blocks differ significantly at a 5% significance level. The box represents the 1\textsuperscript{st} and 3\textsuperscript{rd} quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively. Black dots are outliers.
Results

Above- and belowground biomass

Dry weight (dw) of all the aboveground plant parts as well as the root are illustrated in Figure 14. All three concentrations of the E171 treatments had significantly higher total aboveground biomass than the control. This was only the case for concentrations of 1 and 1000 mg/kg of the P25 treatments. The Zn treatment resulted in a aboveground weight significantly higher than that of the control. The blocks differed significantly from each other with aboveground biomass of block 1 $1.5\,\text{g} (\pm 0.4\,\text{g})$ smaller compared to block 2.

In contrast, blocks did not differ from each other for the root biomass. The Zn treatment was the only one having significantly increased root biomass compared to the control treatment.

When looking at the dry weight of the individual plant parts, no differences in dry weight were seen for the ears of the strongest tillers, flag leaves and the lower culms. Significantly heavier, however, were the flag leaves for the Zn treatment, the upper culm for the Zn and P25 1 mg/kg treatments. Furthermore, the residual ears were heavier for the Zn and E171 100 mg/kg treatments.

Despite the partly increased biomass, no statistical differences were found in the shoot to root ratio. Shoot dry weight was about five times higher than root dry weight.

In addition, neither the area of the flag leaves, nor for the lengths of the upper and lower culms were significantly different to the control treatment. Furthermore, no differences in number of tillers and ears per plant were detected.

Figure 13: Chlorophyll content of leaves in SPAD units at day 75 of the experiment. Treatments are the control, P25 and E171 TiO$_2$ NPs at a concentration of 1, 100 and 1000 mg/kg, respectively as well as the TiO$_2$ bulk treatment with a concentration of 1000 mg/kg TiO$_2$ and Zn treatment with 400 mg/kg Zn (N=7). Blocks differ significantly at a 5% significance level. The box represents the 1st and 3rd quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively. Black dots are outliers and asterisk delimit statistical differences at a 5% significance level.

4.4.2 Above- and belowground biomass

Dry weight (dw) of all the aboveground plant parts as well as the root are illustrated in Figure 14. All three concentrations of the E171 treatments had significantly higher total aboveground biomass than the control. This was only the case for concentrations of 1 and 1000 mg/kg of the P25 treatments. The Zn treatment resulted in a aboveground weight significantly higher than that of the control. The blocks differed significantly from each other with aboveground biomass of block 1 $1.5\,\text{g} (\pm 0.4\,\text{g})$ smaller compared to block 2.

In contrast, blocks did not differ from each other for the root biomass. The Zn treatment was the only one having significantly increased root biomass compared to the control treatment.

When looking at the dry weight of the individual plant parts, no differences in dry weight were seen for the ears of the strongest tillers, flag leaves and the lower culms. Significantly heavier, however, were the flag leaves for the Zn treatment, the upper culm for the Zn and P25 1 mg/kg treatments. Furthermore, the residual ears were heavier for the Zn and E171 100 mg/kg treatments.

Despite the partly increased biomass, no statistical differences were found in the shoot to root ratio. Shoot dry weight was about five times higher than root dry weight.

In addition, neither the area of the flag leaves, nor for the lengths of the upper and lower culms were significantly different to the control treatment. Furthermore, no differences in number of tillers and ears per plant were detected.
Figure 14: Dry weight in g of aboveground and belowground biomass of the wheat plants. Treatments are the control, P25 and E171 TiO$_2$ NPs at a concentration of 1, 100 and 1000 mg/kg, respectively as well as the TiO$_2$ bulk treatment with a concentration of 1000 mg/kg TiO$_2$ and Zn treatment with 400 mg/kg Zn (N=7). Aboveground parts consist of the three ears of the strongest tiller (s.t.), the residual ears, all flag leafs, the residual leafs as well as the lower and upper culm. Belowground biomass consists of the roots. Error bars indicate errors for total aboveground and belowground biomass, respectively. Asterisk indicate significant difference of total aboveground or belowground biomass compared to the control treatment at a 5% significance level.
4.4.3 Wheat grains

The plants produced an average number of 35 grains per ear. No differences between the control and the treatments were detected. Furthermore, the comparison of carbon (C) and N content of the grains did not reveal any significant differences of treatments compared to the control (Figure 15).

Figure 15: C to N ratio of grains (dimensionless). Treatments are the control, P25 and E171 TiO$_2$ NPs at a concentration of 1, 100 and 1000 mg/kg, respectively as well as the TiO$_2$ bulk treatment with a concentration of 1000 mg/kg TiO$_2$ and Zn treatment with 400 mg/kg Zn (N=7). The box represents the 1$^{st}$ and 3$^{rd}$ quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively. Black dots are outliers.
Results

P content of grains were similar among most treatments (Figure 16). Contents of the treatments P25 1 mg/kg and E171 100 mg/kg were greater than the control. However, they were not significantly different from the control. Furthermore, variation was larger for these two treatments than for all others. Recovery of P in samples was high with 97%.

![Figure 16: P concentration measured in the wheat grains in mg/kg. Treatments are the control, P25 and E171 TiO\(_2\) NPs at a concentration of 1, 100 and 1000 mg/kg, respectively as well as the TiO\(_2\) bulk treatment with a concentration of 1000 mg/kg TiO\(_2\) and Zn treatment with 400 mg/kg Zn (N=7). The box represents the 1\(^{st}\) and 3\(^{rd}\) quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively. Black dots are outliers.](image)

4.5 Soil Microorganisms

4.5.1 AMF colonisation

All root samples investigated were colonised by AMF (Figure 17). Total colonisation ranged from 33 to 75%. No significant differences between the treatments and the control were detected. Likewise, neither arbuscular nor vesicular colonisation indicated abnormalities in mycorrhizal colonisation (data not shown).
Results

Figure 17: Total colonisation of plant roots by AMF in %. Treatments are the control, P25 and E171 TiO$_2$ NPs at a concentration of 1, 100 and 1000 mg/kg, respectively as well as the TiO$_2$ bulk treatment with a concentration of 1000 mg/kg TiO$_2$ and Zn treatment with 400 mg/kg Zn (N=5). The box represents the 1$^{st}$ and 3$^{rd}$ quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively. Black dots are outliers.
4.5.2 \( \text{N}_2\text{O} \) emission

The \( \text{N}_2\text{O} \) emissions measured at different times after watering and fertilization are shown in Figure 18. While there were only small emissions 6 and 49 h after watering, elevated emissions were measured at the time points 20 and 28 h. Treatments did not differ within any time point.

![Figure 18: \( \text{N}_2\text{O} \) fluxes from soil in ng/m\(^2\)/s for the control and the highest concentration of the two NP treatments P25 and E171 6, 20, 28 and 49 hours after watering and fertilization with 50 kg N/ha (N=4). Measurements were started on day 78 of the experiment. The box represents the 1\(^{st}\) and 3\(^{rd}\) quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively. Black dots are outliers.](image-url)
4.5.3 Soil microbial DNA

The experiment caused a treatment independent reduction in the amount of DNA extracted from the soil as indicated by the agarose gel electrophoresis in Figure 19. The addition of sand to the test soil caused a strong decrease of the abundance of microorganisms (Figure 19, 1 to 2). A further reduction in microorganisms was caused if the sand-soil mixture underwent the treatment of roughly three months in plastic pots (Figure 19, 2 to 3). Generally, the amount of DNA and RNA in the soils tested here was rather low.

![Figure 19: Agarose gel after electrophoresis indicating different amounts of microbial DNA and RNA (arrow) in soils tested. DNA/RNA of three replicates were extracted and applied on the gel respectively. Bands of the soil used in the experiment without sand (1), the soil with sand not used in the experiment (2), the soil with sand used in the experiment (3) and earthworm cast as control (4) are shown. Control DNA ladder are shown on the far left and right respectively. The soil and soil-sand mixtures that were not used in the experiment were stored at a dry place during the experiment.](image)
Results

Quantification of the DNA in the treatments confirmed the low amount in the test soil (Figure 20). The amount of DNA extracted was similar among treatments. Only the E171 1000 mg/kg treatment contained slightly more DNA but without being statistically significant.

![Box plot showing DNA amount in different treatments](image)

Figure 20: Amount of DNA extracted from the soil at termination of the experiment (day 84 – 88) for the control, treatments P25, E171 and TiO$_2$ bulk for 1000 mg/kg and Zn for a concentration of 400 mg/kg (N=7). The box represents the 1$^{st}$ and 3$^{rd}$ quartile and includes the median (thick line). Whiskers delimit the smallest and largest non-outlier, respectively.
5 Discussion

5.1 Fate of TiO$_2$ NPs in the soil microcosms

5.1.1 Ti concentration in leachate

The fate of the NPs was examined by collecting the leachate from the pots and measuring the total Ti concentrations. This analysis could provide indication for NP mobility and translocation in the soil profile. The significantly higher concentration found for the E171 1000 mg/kg treatment compared to both, the control and P25 treatment is an indication for such a movement of TiO$_2$ NPs. The absolute amount of Ti measured in 50 ml of the leachate from the E171 treatment was 3.3 µg. This figure might have risen if more water percolated through the soil column and if those concentrations in the leachate were similarly high, or even higher. Despite this, it stands in contrast to the roughly 2400 mg of Ti per pot, added in the form of TiO$_2$ NPs. From this point of view, only a minor part (0.0000014%) of Ti was washed out. Furthermore, if the Ti measured is assumed to be TiO$_2$ NPs only, the concentration measured in the leachate was roughly three times smaller than concentrations estimated for sewage treatment plant effluents in the EU today (Sun et al., 2014). However, it has to be kept in mind that the element Ti was measured. This neither gives indication for speciation nor size of the Ti measured. Therefore, it could arise from weathering and subsequent leaching of soil minerals containing Ti instead of the added TiO$_2$ NPs. Nevertheless, if the increased concentrations for E171 stem from weathered Ti minerals, the same amount of weathered Ti should have leached out of the control and P25 treatment as well. Furthermore, Ti weathering and mobility is generally very low as indicated by its use as chemically conservative element for soil mass balance weathering rate studies (Cornu et al., 1999). Thus, the Ti measured in the leachate of the E171 treatment was likely to be either in the form of TiO$_2$ NPs or NP aggregates.

As for the latter, Fang et al. (2009) have suggested bigger aggregates to be more difficult to pass through soil columns. Hence, conditions favouring aggregation of NPs could lead to a retention of NPs in the soil matrix.

Generally, pH, ionic strength and dissolved organic carbon (DOC) have been reported to have a major influence on TiO$_2$ NP aggregation (Domingos et al., 2009; Fang et al., 2009; French et al., 2009). TiO$_2$ NPs rapidly aggregate at ionic strengths typical for most soils as the suspension pH approaches the pH point of zero charge (pH$_{pzc}$) of TiO$_2$ NPs (6.3 – 6.8) (Domingos et al., 2009; French et al., 2009). Furthermore, increasing ionic strength leads to increased aggregation. Another aggregating effect could arise from the concentration of TiO$_2$ NPs at which they were tested, because this has been shown to affect colloidal stability. NPs aggregated to a greater extent at high than low concentrations (Domingos et al., 2009). Therefore, mobility of lower concentrations should be tested as well.
On the other hand, a counterbalancing effect to this aggregation might arise from humic and fulvic acids (i.e. DOC), which are naturally found in soils (Domingos et al., 2009). These are thought to stabilize TiO\textsubscript{2} NPs and hence, prevent them from aggregation. At high pH, however, this stabilizing effect has been shown to be limited (Domingos et al., 2009). Since the pH of the soil used in this experiment was rather high with 7.7, the stabilizing effect of DOC can be expected to be small. Furthermore, aggregation of NPs was likely to occur since the pH did not deviate much from the pH\textsubscript{pzc} of TiO\textsubscript{2} NPs. It can be hypothesised that these soil properties affect P25 and E171 differently, favouring E171 to be more mobile. The most apparent differences between P25 and E171 were the crystalline structure and the size. The smaller P25 contained a mixture of anatase and rutile while E171 consisted of the anatase form only. Differences in the behaviour coming from the two forms have been demonstrated already. Sorption capacity and affinity towards lead have been shown to be greater for pure anatase NPs than for anatase-rutile mixtures (Giammar et al., 2007).

Fang et al. (2009) have shown leaching of significant relative concentrations of TiO\textsubscript{2} after adding TiO\textsubscript{2} NPs with anatase structure (E171-like). The soils have been of high sand content, similar to the one used in this experiment. However, no anatase-rutile (P25-like) particle have been tested.

It remains difficult to draw conclusion about the possible leaching of E171 NPs upon verification of the occurrence of TiO\textsubscript{2} in the nano scale in the leachate media. To determine this, imaging of the solution using electron microscopy techniques might be used. This could verify if TiO\textsubscript{2} NPs have leached and how they were present. Also, alterations of the structure and aggregation could be compared to non-aged NPs. This might help to understand how the exposure of organisms to NPs has changed in the course of the experiment.

5.1.2 TEM analysis of root thin sections

The uptake of NPs via plant roots was examined visually using TEM. In spite of advances in NP analytics, it remains difficult to assess the possible uptake of NPs by plants (Hassellöv et al., 2008). If roots are digested and analysed for Ti by ICP-OES, not only the Ti within the plants but also the one on the surface of the roots will be measured. Therefore, optical investigation with TEM was chosen. Here, indications for TiO\textsubscript{2} NPs and aggregates in thin sections of roots from the bulk treatment were found but have to be verified (Figure 10).

Firstly, the spectra of the particles found clearly showed peaks in the Ti range and agglomerates 1, 2 and 3, indicating local abundance of Ti (Figure 10). Furthermore, no Ti was found in other structures of the cell (Figure 10, e.g. 4). This illustrates that the high Ti abundance was indeed confined to the particle like structures. In addition, visual comparison of the particles found in the root thin section (Figure 10), with bulk particles (Figure 3) supported the assumption that NPs were found in the plant cell. With the smallest particle measuring approx. 70 nm, the particle was well within the nano scale. It was also shown that the TiO\textsubscript{2} bulk material contained particles of this size (Figure 3). Taken together, high Ti abundance and apparent morphological similarity are strong indicators that the
Discussion

particles found were TiO\(_2\) bulk particles. Nevertheless, it is unclear if the particles were taken up by the plant root in this experiment. They could be artefacts from the embedding and cutting process. Although the surrounding cell wall did not seem to be damaged, holes in the Epon matrix were observed. It has been shown that TiO\(_2\) NPs can form aggregates at wheat root surfaces (Wild et al., 2009). Therefore, particles from the root surface could have been introduced into the root via these holes during cutting for example.

Possible routes of entry of NPs into the plant are via the rhizodermis/cortex, wounding, lateral root junctions or root tips (Dietz et al., 2011). Although no effects were reported, Larue et al. (2012) have provided indication of uptake and distribution of TiO\(_2\) in wheat plants in a hydroponic system. They have reported a threshold diameter of 140 nm for root uptake. Hydroponic systems are highly artificial and different from soil. Therefore, comparison of the two systems with respect to uptake of NPs is difficult.

Another route might be the uptake of Ti in a soluble form and subsequent precipitation in the roots.

With the release of exudates from roots, plants influence the pH in the rhizosphere to a large degree (El-Shatnawi et al., 2001). This can cause pH rise or decline. These changes in pH, then affect the solubility of metals (El-Shatnawi & Makhadmeh, 2001). Although Ti is known to be largely insoluble (Cornu et al., 1999), natural anatase and rutile TiO\(_2\) may become available to plants under some conditions (Dumon & Ernst, 1988). Such a natural uptake by wheat was reflected by the Ti levels found in the grains for the control treatment (Figure 9). Conditions favouring Ti uptake might be a low pH because Ti has been shown to be soluble in a very acidic soil with pH 3.1 (Dumon & Ernst, 1988). In fact, Ti concentrations in leaves of Corynephorus canescens and Rumex acetosella were 60 and 40 times higher, compared to a similar soil with pH 4.9, respectively. Release of Ti ions from TiO\(_2\) NPs by altered rhizosphere pH has been suggested (Jacob et al., 2012). Therefore, Ti may be taken up in a soluble form due to a low pH in the immediate vicinity of the wheat roots, which is caused by plant exudates.

However, because of the morphological similarity of bulk TiO\(_2\) particles and the structures found in the plant root, it seems more likely, that Ti was taken up in form of particles rather than precipitation after uptake of soluble Ti (Figure 10).

Because no root specimen was fully intact, it was difficult to identify the rhizodermis and investigate for TiO\(_2\) NPs and agglomerates adhered to the root surface. Moreover, this was the reason why the plant region where the particles were found could not be specified. The section where the particles were found also contained a lot of undefined structures, possibly artefacts of either the embedding or the cutting process. Other specimen also contained similar structures, however, the structures were not found in all specimen. Improving the embedding and subsequent cutting process would probably lead to less disturbed specimen. This might simplify the visual observation and increases throughput of this rather time intensive analysis. Also, root thin sections of the control treatment need to be analysed to
Discussion

see whether similar structures than the ones found for the bulk treatment are found there. Because the examination of the roots thin slices was time consuming, only a few specimen could be analysed during the present project. Further analysis are needed to verify if TiO\textsubscript{2} NPs have been taken up by wheat in the experiment.

5.1.3 Ti concentration in grains

After entering the plant, long distance transport of NPs to the shoot could happen via the xylem (Dietz & Herth, 2011). Such translocation of TiO\textsubscript{2} NPs from the soil into aboveground plant tissues was investigated. The wheat grains were of particular interest, since they are the final product used for consumption and because translocation of TiO\textsubscript{2} NPs to the grains would likely have substantial implications for food safety.

As already mentioned for the determination of Ti concentration in the leachate, the element Ti was measured, which does not give any indications for speciation or size of Ti. However, Ti levels of the treatments P25 100, E171 100 and 1000 mg/kg as well as the bulk treatment were markedly higher than the background Ti level of the control. In fact, values were up to 15 times higher for the E171 100 mg/kg treatment.

Surface contamination of the grains due to TiO\textsubscript{2} containing dust from spiked soils during the experiment can almost certainly be excluded, because control treatments would also have been contaminated since pots were arranged on trays in a randomized fashion.

No TiO\textsubscript{2} NPs were found in the roots of most of these samples investigated with TEM, with bulk as possible exception as discussed in Chapter 5.1.2, TEM analysis of root thin sections. Only limited time was available for examination of the roots for NPs, thus, not ensuring representative and robust results. However, the higher Ti concentration in the grains is an indication for a translocation of TiO\textsubscript{2} NPs into the shoot and the grains. For verifying this, Ti concentrations in shoot and root should also be measured.

Assuming TiO\textsubscript{2} NPs were actually translocated to the grains, it can be speculated why highest concentrations of Ti in grains were found for the concentration of 100 mg/kg for both NP treatments. Domingos et al. (2009) have assumed that higher concentrations of NPs lead to more aggregation. Thus, more small particles could have been taken up in the case of 100 mg/kg treatments. The formation of larger aggregates at the higher concentration of 1000 mg/kg, not being able to enter the plant, might have led to a reduced uptake. As for the smallest concentration, the amount of Ti might not have been high enough for resulting in a measureable difference to the control.

Furthermore, the large variation, especially for the E171 100 mg/kg treatment indicates large differences in Ti concentrations in grains within some treatments. Since grains of all three plants per pot were pooled before analysis, it could mean that Ti concentrations were substantially higher in grains of some individual plants.
Additionally, due to the low recovery of only 31% of Ti from the matrix, concentrations may have been underestimated. A similar digestion of clover tissue using hydrofluoric acid resulted in an even lower recovery of 13% (Moll et al., unpublished).

Further analysis need to be conducted, in order to verify if it actually was TiO$_2$ NPs present in the wheat grains. However, as already mentioned, the analytics of NPs in complex matrices such as wheat grains is difficult and there is a lack of methodologies for NP analysis (Tiede et al., 2008). Also, an adaption of the digestion method is needed to achieve a better result.

5.1.4 Exposure

NPs were homogenously incorporated into the soil matrix by dry spiking. This technique is favoured over the wet spiking method and was shown to produce adequate spiking homogeneity (Hund-Rinke et al., 2012).

The verification of the exposure of the organisms tested to TiO$_2$ NPs during the experiment was not feasible. The reason is that NP extraction and determination in soil is challenging (Hassellöv et al., 2008). In fact, this is a general problem that is faced when toxicity studies with NPs are conducted (Handy et al., 2012). Apart from the technological gap (Handy et al., 2012), especially for Ti, the analytical challenge arises from the great abundance of natural Ti in soils (von der Kammer et al., 2012). The soil used in this experiment contained a natural background concentration of Ti 783 mg/kg, which constitutes 78% of the Ti that was added in the form of TiO$_2$ NPs with the highest concentration of 1000 mg/kg. High background levels of Ti make the distinction of natural from anthropogenic as well as non-nano and nano particles difficult (von der Kammer et al., 2012). Identification has been done using EM techniques with Ti detection. Smooth homogeneous morphologies together with high Ti abundance are seen as evidence for anthropogenic origin of TiO$_2$ NPs (von der Kammer et al., 2012). However, with this technique being prone to artefacts, the state of the NPs, if they are present as single particles or aggregates, could not be verified during the exposure.

5.2 Toxicity of TiO$_2$ NPs towards wheat and soil microbes

5.2.1 Plant health

Growth and health of the wheat plants were assessed with a number of plant physiological and morphological parameters. Plant height and chlorophyll content were parameters recorded throughout the experiment. Plant height did not differ at any given time point. However, plant height of the blocks differed significantly from each other. This difference between the two blocks represents a major problem that was faced in the statistical analysis.

Despite the lower chlorophyll content in the leaves, the Zn treatment resulted in more shoot weight than the control. Most treatments were significantly heavier than the control. The principal component analysis did not reveal any grouping of the treatments (see Appendix, Principal Component Analysis).
Discussion

This is not in line with what has been shown by Jacob et al. (2012). They did not observe differences in wheat biomass upon exposure to TiO$_2$ NPs in a hydroponic system. On the other hand, Du et al. (2011) have reported reduced wheat biomass at a concentration of approx. 90 mg/kg TiO$_2$ NPs. However, differences in the test soil parameters (e.g. sand content) as well as the NPs used might have led to this discrepancy.

Taken together growth and health of wheat did not seem to be negatively affected in this experiment. The higher biomass for the Zn treatment was unexpected, since it was the positive control. The amount of Zn added might has not been high enough to cause toxic effects. However, a negative effect was found with the reduced chlorophyll content.

5.2.2 Soil Microbes

5.2.2.1 Mycorrhiza

AMF have been shown to be resistant to different metals, which is also reflected by their role in phytoremediation (Gianinazzi et al., 2010). Despite this and their important ecological role (Chapter 2.3, Mycorrhiza), information about the influence of metal based NPs on AMF is scarce. In a pilot study, Dubchak et al. (2010) have shown negative effects on colonisation of sunflower *Helianthus annuus* by AMF due to exposure to TiO$_2$ NPs and silver (Ag) NPs in pot trials. However, lack of information about the TiO$_2$ NPs used and the use of only three replicates of a different model plant make comparison difficult. On the other hand, positive colonisation responses of clover grown in a sand perlite mixture have been documented for Ag NPs and iron oxide nanoparticles (FeO NPs) already at low concentrations (Feng et al., 2013). In contrast, the result presented here do not indicate colonisation of wheat roots to be disturbed by TiO$_2$ NPs. Neither the total, nor the arbuscular and vesicular colonisation differed significantly for soils spiked with TiO$_2$ NPs compared to the control. Colonisations were well within what has been reported for wheat (see Chapter 2.3, Mycorrhiza). AMF are known to influence P uptake by plants (Smith & Read, 2008, p. 120f). Although high P concentrations in some treatments were measured (Figure 16), these findings were not reflected by a higher colonisation of AMF in this experiment.

5.2.2.2 $N_2O$ emissions

After elevated emissions of $N_2O$ in all treatments at the time points of 20 and 28 h, they declined again 49 h after watering and fertilization. Water content at the last time point (approx. 80%) was still favourable for denitrification. Therefore, the decline in emissions might be due to the exhausted N sources for denitrification. The decline shows, that the measurements were conducted during the emission phase. No significant differences were detected among treatments. Therefore, the data did not indicate an impaired functioning of the denitrification processes caused by TiO$_2$ NPs. One reason could be the variation within treatments. These were rather large with standard deviations of 54, 20 and 85 ng/m$^2$/s for the control, P25 1000 mg/kg and E171 1000 mg/kg treatment, respectively (Figure
This variation was due to shifts in the emission curve of individual pots. The onset of emissions can be delayed or the course of the emission curve can be different. Other studies measuring N$_2$O fluxes from soils have also reported large variation (Bender et al., 2013). In addition, temporal resolution of measurements with only four time points was too low to integrate the emissions. More time points need to be analysed to gain a better resolution of the course of the emissions. Due to the lack of control of the water content for block 1, only measurements of block 2 could be taken into account (see Chapter 3.5.4, N$_2$O measurements). Therefore, statistical power is rather low with only four replicates measured per treatment. Nevertheless the findings of this experiment can be of use to adapt the measurement time points and methods for further experiments.

A study using slurry and silver NPs on soil showed temporal differences in N$_2$O emissions (Colman et al., 2013). Emissions were significantly higher with AgNP treatment 8 days after spiking but converged with the control on day 50 (Colman et al., 2013). Further analysis are needed to verify if such observations do not occur with TiO$_2$ NPs.

### 5.2.2.3 Soil microbial DNA

The amount of DNA extracted from the soil was similar among treatments. This finding is in contrast to what Ge et al. (2011) have shown. They have quantified extractable soil DNA for a soil spiked with TiO$_2$ NP at concentrations similar to this experiment (500, 1000 and 2000 mg/kg). A strong negative dose – response relationship 15 and 60 d after spiking has been found. Furthermore, the reduction in DNA has been stronger at day 60, with a reduction of approx. 50%. An influence of the NPs on the extraction efficiency could be excluded in their experiment (Ge et al., 2011).

Conditions of the microcosms in the experiment of Ge et al. (2011), however, have been set to maximize microbial activity. This has been done by lowering water content to 18% of the WHC and not diluting the soil with sand. Contrastingly, the experimental system used in the present experiment was established for optimal plant and fungal growth. Hence, it caused a decline in the abundance of microorganism as can be seen from Figure 19. Sensitive microorganisms are likely to be reduced due to the pressure (e.g. changed microhabitat, temp., etc.) arising from the system. This might result in a selection for more resistant microorganisms. This rather resistant community then might better withstand the possible pressure of the TiO$_2$ NPs. This could be an explanation why no differences in the amount of DNA extracted between treatments and the control were detected at the end of the experiment.

In addition, the amount of DNA extracted from a soil is only a bulk parameter. Similar amount of DNA does not necessarily mean no effects on microbial communities. The community composition might be altered due to shifts either caused by direct toxicity of TiO$_2$ or indirect effects as demonstrated by Ge et al. (2012). Therefore community profiling based on large scale molecular gene sequence analysis is necessary to gain more detailed information on this rather sensitive and ecologically important endpoint.


6 Conclusion

In conclusion, no adverse effects of TiO$_2$ NPs (1, 100 and 1000 mg/kg soil) were detected on wheat and soil microbes with the experimental design presented in this study. This, however, must not lead to the wrong assumption that TiO$_2$ NPs can be safely used in PPP. Other studies detected effects on the same organisms that were tested here. Therefore, this study fosters the assumption that ecotoxicity of TiO$_2$ NPs in soil microcosms is likely to be strongly dependent on the test parameters. There is a great need for advances in NP analytics because methodological and technical problems have been faced in the verification if and how TiO$_2$ NPs were present in complex media, namely wheat roots, grains and the soil matrix. This has to be addressed in order to provide clear and doubtless information for assessing the fate, and hence the risk of TiO$_2$ NPs in the terrestrial environment.
7 References


References


References


## Appendix

### Chemicals and Instruments

List of chemicals used in the experiment including name, formula and manufacturer.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Formula</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accelerator DMP 30</td>
<td>[(CH₃)₂NCH₂]C₆H₅OH</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ammonium dihydrogenphosphate</td>
<td>(NH₄)H₂PO₄</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Ammonium molybdate tetrahydrate</td>
<td>(NH₄)₆Mo₇O₂₄ x 4H₂O</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>NH₄NO₃</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Boric acid</td>
<td>H₃BO₃</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl₂</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Calcium nitrate tetrahydrate</td>
<td>Ca(NO₃)₂ x 4H₂O</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Chloroform</td>
<td>CHCl₃</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Copper(II) sulfate pentahydrate</td>
<td>CuSO₄ x 5H₂O</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Dibutyl phthalate</td>
<td>C₆H₄-1,2-</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Durcupan™ ACM</td>
<td>-</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Epoxy embedding medium (Epon™ 812)</td>
<td>-</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Epoxy embedding medium, hardener DDSA</td>
<td>C₁₂H₂₀O₃</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Ethylenediaminetetraacetic acid ferric sodium salt</td>
<td>Fe(Na)EDTA</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Glutaraldehyde</td>
<td>OHC(CH₂)₉CHO</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Glycerol 85%</td>
<td>C₃H₆O₃</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₃</td>
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</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>MgSO₄ x 7H₂O</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Manganese sulfate monohydrate</td>
<td>MnSO₄ x H₂O</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Nitric acid</td>
<td>HNO₃</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Osmium tetroxide</td>
<td>OsO₄</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
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<tr>
<td>Potassium hydroxide</td>
<td>KOH</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>KNO₃</td>
<td>Dr. Bender &amp; Dr. Hobein Ag, Zürich, Switzerland</td>
</tr>
<tr>
<td>Propylene oxide</td>
<td>C₃H₆O</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Uranyl acetate dihydrate</td>
<td>UO₂(OCOCH₃)₂ 2H₂O</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
<tr>
<td>Zinc sulfate heptahydrate</td>
<td>ZnSO₄ x 7H₂O</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
</tr>
</tbody>
</table>
List of instruments used in the experiment including type in brackets and manufacturer.

<table>
<thead>
<tr>
<th>Instrument (type)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automated \textit{N}_2\textit{O} analyzer (TEI46c)</td>
<td>Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>Ball Mill (MM 400)</td>
<td>Retsch, Haan, Germany</td>
</tr>
<tr>
<td>Cell disrupter (FastPrep® FP120)</td>
<td>Qbiogene, Illkirch, France</td>
</tr>
<tr>
<td>Centrifuge (4K15C)</td>
<td>SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany</td>
</tr>
<tr>
<td>Chlorophyll Meter (SPAD-502)</td>
<td>Konica Minolta Sensing, Osaka, Japan</td>
</tr>
<tr>
<td>CHN-Analyzer (EURO EA)</td>
<td>Eurovector SpA, Milano, Italy</td>
</tr>
<tr>
<td>Digestion block (DigiPREP)</td>
<td>SCP Scienc, Quebec, Canada</td>
</tr>
<tr>
<td>EDX-spectroscope (X-Max, 80mm$^2$)</td>
<td>Oxford Instruments, Abingdon, UK</td>
</tr>
<tr>
<td>Fluorescence Spectrometer (Cary Eclipse)</td>
<td>Agilent Technologies, Santa Clara, USA</td>
</tr>
<tr>
<td>ICP-OES spectrometer (Arcos FHS 16)</td>
<td>SPECTRO Analytical Instruments, Kleve, Germany</td>
</tr>
<tr>
<td>Microlitre Centrifuge (Biofuge Pico)</td>
<td>Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>Microscope (DM 2000)</td>
<td>Leica, Wetzlar, Germany</td>
</tr>
<tr>
<td>Portable Area Meter (Li-3000A)</td>
<td>LI-COR, Lincoln, USA</td>
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<tr>
<td>Soil mixer (Turbula® T 2 F)</td>
<td>Willy A. Bachofen AG, Basel, Switzerland</td>
</tr>
<tr>
<td>Transmission Electron Microscope (Tecnai G2 Spirit)</td>
<td>FEI, Hillsboro, USA</td>
</tr>
<tr>
<td>Ultramicrotome (Ultracut E)</td>
<td>Leica, Wetzlar, Germany</td>
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</tbody>
</table>
## Correlations of Variables

Table of correlations between variables used for principle component analysis. If variables had Pearson’s P greater than 0.8, one variable was deleted from the dataset.

<table>
<thead>
<tr>
<th></th>
<th>N content grain</th>
<th>C/N ratio grain</th>
<th>upper culm length</th>
<th>lower culm length</th>
<th>plant chlorophyll content 75d</th>
<th>ears s.t. weight</th>
<th>flag leaves s.t. weight</th>
<th>root weight</th>
<th>flag leaves s.t. weight</th>
<th>root weight</th>
<th>resididual ears weight</th>
<th>resididual flag leaves</th>
<th>shoot weight</th>
<th>shoot/root ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>N content grain</td>
<td>1.000</td>
<td>0.925</td>
<td>0.029</td>
<td>0.024</td>
<td>0.070</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>0.041</td>
<td>0.195</td>
<td>-0.038</td>
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<tr>
<td>C/N ratio grain</td>
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<td>1.000</td>
<td>0.127</td>
<td>0.051</td>
<td>0.341</td>
<td>-0.293</td>
<td>0.364</td>
<td>0.042</td>
<td>0.343</td>
<td>0.384</td>
<td>0.186</td>
<td>0.034</td>
<td>0.385</td>
<td>0.259</td>
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<td>upper culm length</td>
<td>-0.029</td>
<td>0.127</td>
<td>1.000</td>
<td>0.760</td>
<td>0.223</td>
<td>-0.041</td>
<td>0.185</td>
<td>0.032</td>
<td>0.242</td>
<td>0.106</td>
<td>0.110</td>
<td>0.016</td>
<td>0.222</td>
<td>0.224</td>
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<tr>
<td>lower culm length</td>
<td>0.024</td>
<td>0.051</td>
<td>0.760</td>
<td>1.000</td>
<td>0.148</td>
<td>0.052</td>
<td>0.285</td>
<td>0.161</td>
<td>0.000</td>
<td>0.303</td>
<td>0.042</td>
<td>0.048</td>
<td>0.025</td>
<td>0.060</td>
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<td>plant height 75d</td>
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<td>0.341</td>
<td>0.223</td>
<td>0.148</td>
<td>1.000</td>
<td>0.615</td>
<td>0.314</td>
<td>0.029</td>
<td>0.723</td>
<td>0.381</td>
<td>0.363</td>
<td>0.014</td>
<td>0.783</td>
<td>0.738</td>
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<tr>
<td>plant chlorophyll II content 75d</td>
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<td>0.293</td>
<td>0.041</td>
<td>0.052</td>
<td>0.615</td>
<td>1.000</td>
<td>0.171</td>
<td>0.104</td>
<td>0.580</td>
<td>0.334</td>
<td>-0.435</td>
<td>0.380</td>
<td>0.419</td>
<td>-0.549</td>
</tr>
<tr>
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<td>0.364</td>
<td>0.185</td>
<td>0.285</td>
<td>0.314</td>
<td>-0.171</td>
<td>1.000</td>
<td>0.046</td>
<td>0.183</td>
<td>0.112</td>
<td>-0.093</td>
<td>0.089</td>
<td>0.345</td>
<td>-0.020</td>
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<tr>
<td>flag leaves s.t. weight</td>
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<td>0.042</td>
<td>0.032</td>
<td>0.161</td>
<td>0.029</td>
<td>-0.104</td>
<td>0.046</td>
<td>1.000</td>
<td>0.059</td>
<td>0.557</td>
<td>0.281</td>
<td>0.209</td>
<td>0.122</td>
<td>-0.035</td>
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<tr>
<td>upper culm weight</td>
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<td>0.343</td>
<td>0.242</td>
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<td>0.723</td>
<td>-0.580</td>
<td>0.183</td>
<td>0.059</td>
<td>1.000</td>
<td>0.352</td>
<td>0.592</td>
<td>0.052</td>
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<td>lower culm weight</td>
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<td>0.303</td>
<td>0.381</td>
<td>-0.334</td>
<td>0.112</td>
<td>0.557</td>
<td>0.352</td>
<td>1.000</td>
<td>0.527</td>
<td>0.289</td>
<td>0.125</td>
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<tr>
<td>residual ears weight</td>
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<td>0.186</td>
<td>0.110</td>
<td>0.042</td>
<td>0.363</td>
<td>-0.435</td>
<td>0.093</td>
<td>0.281</td>
<td>0.592</td>
<td>0.527</td>
<td>1.000</td>
<td>0.010</td>
<td>0.643</td>
<td>0.838</td>
</tr>
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<td>root weight</td>
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<td>0.034</td>
<td>0.016</td>
<td>0.048</td>
<td>0.014</td>
<td>-0.380</td>
<td>0.089</td>
<td>0.209</td>
<td>0.052</td>
<td>0.289</td>
<td>0.100</td>
<td>1.000</td>
<td>0.001</td>
<td>-0.030</td>
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<tr>
<td>flag leaves s.t. weight</td>
<td>-0.195</td>
<td>0.385</td>
<td>0.222</td>
<td>0.025</td>
<td>0.783</td>
<td>-0.419</td>
<td>0.345</td>
<td>0.122</td>
<td>0.600</td>
<td>0.125</td>
<td>-0.019</td>
<td>0.001</td>
<td>1.000</td>
<td>0.575</td>
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<tr>
<td>residual flag leaves</td>
<td>0.036</td>
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<td>0.224</td>
<td>0.060</td>
<td>0.718</td>
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<td>0.020</td>
<td>0.035</td>
<td>0.867</td>
<td>0.310</td>
<td>0.643</td>
<td>0.030</td>
<td>0.575</td>
<td>0.100</td>
</tr>
<tr>
<td>shoot weight</td>
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<td>0.401</td>
<td>0.217</td>
<td>0.202</td>
<td>0.601</td>
<td>-0.547</td>
<td>0.405</td>
<td>0.376</td>
<td>0.742</td>
<td>0.656</td>
<td>0.838</td>
<td>0.167</td>
<td>0.303</td>
<td>0.665</td>
</tr>
<tr>
<td>shoot/root ratio</td>
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<td>0.077</td>
<td>0.129</td>
<td>0.094</td>
<td>0.241</td>
<td>0.149</td>
<td>0.098</td>
<td>0.106</td>
<td>0.213</td>
<td>0.059</td>
<td>0.225</td>
<td>0.873</td>
<td>0.094</td>
<td>0.290</td>
</tr>
</tbody>
</table>
Principal Component Analysis including various variables. Coloured dots represent one pot of a specific treatment. P25 1 mg/kg – blue, P25 100 mg/kg – lightblue, P25 1000 mg/kg – cadetblue, E171 1 mg/kg – darkviolet, E171 100 mg/kg – violet, E171 1000 mg/kg – blueviolet, Bulk 1000 mg/kg – orange, Zn 400 mg/kg – red, Control – black.