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# Effects of titanium dioxide nanoparticles on soil microbial communities and wheat biomass

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ABSTRACT

Titanium dioxide nanoparticles ($TiO_2$ NPs) are the most produced NPs worldwide and have great potential to be utilized in agriculture as additives for plant protection products. However, concerns have been raised that some NPs may negatively affect crops and soil microbial communities, including beneficial microbes such as arbuscular mycorrhizal fungi. Here we tested two different $TiO_2$ NPs (P25, E171) and a bulk $TiO_2$ (particle size >100 nm) for their effects on the diversity and community composition of soil microorganisms. In addition we tested whether increasing concentrations of $TiO_2$ NPs had effects on wheat growth and yield. Microbial diversity was analyzed using Illumina Miseq paired-end sequencing of ribosomal markers (prokaryotic $16S_{V3V4}$ and fungal ITS2 of the ribosomal RNA operon). Application of $TiO_2$ NPs altered the detected prokaryotic but not fungal community structure. Prokaryotic community structure differed significantly between the three NP treatments and the control treatment without NP, although differences were smaller compared to those between the positive and the negative control. Specific microbial taxa responded positively or negatively to particular $TiO_2$ NP treatments and, thus, may be used as bio-indicators for $TiO_2$ NPs. No negative effects on wheat growth and on arbuscular mycorrhizal root colonization were detected, and no evidence for a dose-response relationship between wheat performance and $TiO_2$ NP concentration was found. Overall, these results reveal that prokaryotes are more sensitive than fungi to the $TiO_2$ NP treatments.



## 1. Introduction

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

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**Abbreviations**

| | |
|---|---|
| AMF | Arbuscular mycorrhizal fungi |
| ANOSIM | analysis of similarity |
| CAP | Canonical analysis of principal coordinates |
| NPs | Nanoparticles |
| PCO | Principal coordinate analysis |
| PERMANOVA | Permutational analysis of variance |
| PERMDISP | analysis of multivariate dispersion |
| $TiO_2$ | titanium dioxide |

soil organisms differently than the natural occurring $TiO_2$ in soils, and potentially affect ecosystem functioning at various trophic levels (Gardea-Torresdey et al., 2014). For instance, $TiO_2$ NP might cover the root or soil particle surface and inhibit growth and functioning of some micro-organisms or impair root colonization by beneficial soil microbes (e.g. mycorrhizal fungi or nitrogen fixing bacteria). Hence, there is a need to investigate potential non-target effects of $TiO_2$ NPs on soil organisms, plants, and plant-associated organisms.

Soil microorganisms conduct important ecosystem functions. For example they are important for soil carbon cycling, nitrogen fixation, and nutrient acquisition for plants (Carney and Matson, 2005; Hättenschwiler et al., 2005; van der Heijden et al., 2008). A key group of soil organisms that associate with two thirds of all terrestrial plants are arbuscular mycorrhizal fungi (AMF) of the phylum Glomeromycota (Smith and Read, 2008; Smith and Smith, 2011; van der Heijden et al., 2015). AMF acquire limiting nutrients, especially immobile nutrients, such as phosphorus, for plants and can enhance plant growth. Wheat, which is of particular importance for human nutrition, is one of the plant species that can benefit from a symbiosis with AMF (Pellegrino et al., 2015). Even though soil microorganisms play a crucial role in cropping systems, there are only a few studies that investigate the effects of $TiO_2$ NPs on soil microbial community structure, plants and the symbiosis between AMF and plants (Du et al., 2011; Ge et al., 2011; Song et al., 2013; Burke et al., 2014; Simonin and Richaume, 2015). For instance, soils planted with maize and soybean had been exposed for six weeks to 200 mg $TiO_2$ NPs $kg^{-1}$ soil (Burke et al., 2014). While plant biomass and soil bacterial community structure were not affected by $TiO_2$ NPs, AMF communities were altered (Burke et al., 2014). In another study assessing AMF communities on soybean roots, no effects were found (Burke et al., 2015). Even less studies used high-throughput sequencing tools to investigate the effects of $TiO_2$ NPs on soil microbial communities. In one study using bar-coded pyrosequencing, Ge et al. (2012) observed that soil bacterial community structure was altered when treated with 0.5–2 mg $TiO_2$ NPs $g^{-1}$ soil. However, that study only focused on bacteria and so far there is no study that simultaneously investigated effects of different $TiO_2$ NPs on bacteria, fungi, wheat and AMF root colonization in one experiment.

The current study was conducted to evaluate whether different concentrations and qualities (primary particle size and crystal structures) of $TiO_2$ NPs in agricultural soil affect (1) the diversity of soil prokaryotic and fungal communities, (2) root colonization by AMF and phosphorus uptake of wheat, and (3) the performance (yield) of wheat. For this purpose, we used industrially relevant $TiO_2$ NPs, i.e., P25 (anatase/rutile) and E171 (anatase), and a bulk anatase control (>100 nm) for E171. We assumed that the smallest NP, i.e. P25, would reveal the strongest effects on microorganism composition and plant growth, and that the effect size decreases with increasing particle size (E171 < bulk $TiO_2$). Different photoreactivity (ROS production) due to the different crystal structures (anatase and rutile) were assumed to have low influence, because in soils dark conditions prevail. Concentrations were chosen to represent soils with application of sewage sludge (Sun et al., 2014), application of NP containing agrochemicals (Gogos et al., 2012) and accidental spill.

## 2. Material and methods

### 2.1. Nanoparticles used

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

### 2.2. Soil substrate

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

### 2.3. Experimental design and NPs addition to the substrate

Wheat (*Triticum* ssp. var. Fiorina, spring wheat, 3 seedlings per pot) was grown in soil exposed to three $TiO_2$ NPs, P25 and E171 in three concentrations (1, 100, and 1000 mg $kg^{-1}$ soil) as well as bulk $TiO_2$ (1000 mg $kg^{-1}$ soil). A control treatment without NP addition and a positive control with $ZnSO_4 \cdot 7H_2O$ (1000 mg $kg^{-1}$ soil, Sigma-Aldrich, Art. No., Z0251) addition was also included. We used $ZnSO_4 \cdot 7H_2O$ because it has been shown to affect wheat growth as well as soil microbial community structures (Frostegård et al., 1993, 1996; Warne et al., 2008; Rousk et al., 2012). These nine treatments were replicated 7 times, resulting in a total of 63 pots.

Three different amounts (0.03, 3, and 30 g) of each $TiO_2$ NP (E171, P25) and 30 g of bulk $TiO_2$ were added to 300 g soil substrate (50% v/v sand and soil) in a 500 ml Schott bottle and shaken in a powder mixer (Turbula T2F, Switzerland) for 30 min. In order to prepare the highest concentration of 30 g $TiO_2$ NPs, two bottles with 15 g NPs and 300 g substrate each were mixed. These pre-mixed soil-particle mixtures were then diluted in 30 kg sand-soil substrate in a cement mixer for 6 h. This was done separately for each concentration 1, 100, and 1000 mg $kg^{-1}$ for $TiO_2$ NPs E171, P25, and of bulk $TiO_2$. Control substrate was treated as the spiked substrate but without adding NPs. Pots (15 cm diameter, 20 cm high, Fig. S1) were filled with a drainage layer of sand (520 g) at the bottom, and then covered with 3.3 kg spiked substrate per pot. The total titanium concentration in the soils was determined by X-ray fluorescence spectroscopy at the end of the experiment to verify the exposure concentrations as described by Gogos et al. (2016). Titanium concentrations of the control soil was on average

1024 ± 284 mg Ti $kg^{-1}$ (n = 16), while soils spiked with 1000 mg $kg^{-1}$ $TiO_2$ contained significantly ($p \leq 0.001$) more elemental Ti with values averaging 1720 ± 280 (n = 18), 1659 ± 347 (n = 18), and 2064 ± 71 (n = 17) mg $kg^{-1}$ for P25, E171, and bulk $TiO_2$, respectively (Gogos et al., 2016). The treatments with the lower $TiO_2$ NP concentrations (1 and 100 mg $kg^{-1}$) did not differ significantly from the high natural background concentration of titanium in the soil.

The pots with wheat were grown in the greenhouse (16 h 25 °C 300 W m2, 8 h 16 °C dark) and had fully randomized positions. The experiment was performed with two temporary shifted blocks (one week between) harvesting one block within one week. Plants were watered three times a week and the moisture content was kept between 50 and 60% by weighing. Weeds were removed regularly. Wheat was fertilized every week, starting after week 3, with 7.9 ml of ($KNO_3$ 60 mM, $Ca(NO_3)_2 \cdot 4H_2O$ 40 mM, $NH_3NH_4$ 7.5 mM, $NH_4H_2PO_4$ 5 mM, $MgSO_4 \cdot 7H_2O$ 10 mM, in 990 ml water with addition of 10 ml micro nutrient solution (KCl 37 μM, $H_3BO_3$ 25 μM, $MnSO_4 \cdot H_2O$ 2 μM, $ZnSO_4 \cdot 7H_2O$ 2 μM, $CuSO_4 \cdot 5H_2O$ 0.5 μM, $(NH_4)_6Mo_7O_{27} \cdot 4H_2O$ 0.5 μM, Fe(III) EDTA 20 μM)). This corresponds to a total of 23 kg N $ha^{-1}$ $year^{-1}$ and 1.6 kg P $ha^{-1}$ $y^{-1}$. In the last week before harvest, 50 kg N $ha^{-1}$ were added for measuring $N_2O$ production (data not shown here). During plant growth, the chlorophyll content of wheat was measured by a chlorophyll meter (SPAD-502, Konica Minolta, Japan) after 14, 30, 45, 60, and 75 days.

### 2.4. Harvest

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

### 2.5. Root colonization by arbuscular mycorrhizal fungi

Root samples that were stored in 50% ethanol, were stained to determine AMF colonization according to Vierheilig et al. (1998). The preparation of the samples (McGonigle et al., 1990) and the counting of colonization is explained in the supplementary Information (Text S1).

### 2.6. DNA extraction, PCR and DNA sequencing

DNA of the soil samples treated with 1000 mg $kg^{-1}$ $TiO_2$ NPs, bulk $TiO_2$, and controls were extracted as described by Bürgmann et al. (2001) with some adjustments as outlined in the Supplementary Information (Text S1).

The V3-V4 region of the prokaryotic 16S rRNA gene (bacteria and archaea) was amplified with variants of primers 341F (CCTAYGGGDBGCWSCAG) and 806R (GGACTACNVGGGTHTCTAAT) recently published by Frey et al. (2016), while the ITS2 region of the eukaryotic ribosomal operon (fungi and some protists) was amplified with degenerate versions of primers ITS3 (CAHCGATGAAGAACGYRG) and ITS4 (TCCTSCGCTTATTGATATGC) recently published by Tedersoo et al. (2014). The 16S rRNA primers, initially designed for targeting bacteria, were modified in order to maximize detection of archaeal sequences without compromising detection of bacterial sequences; however, due to the low coverage in the databases, archaeal sequences might still be underrepresented. The ITS2 primers, initially designed for fungi, also targets some, but not the majority of, protist groups (Tedersoo et al., 2016) which is why we refer here to fungi. The 5' ends of the primers were tagged with the CS1 (forward primers) and CS2 (reverse primers) adapters required for multiplexing samples using the Fluidigm Access Array™ System (Fluidigm, South San Francisco, CA, USA). The PCR conditions to amplify the 16S rRNA gene fragments consisted of an initial denaturation at 95 °C for 10 min, 36 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s and elongation at 72 °C for 1 min followed by a final elongation at 72 °C for 10 min. The PCR conditions to amplify the ITS2 fragments consisted of an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation (95 °C for 40 s), annealing (58 °C for 40 s) and elongation (72 °C for 1 min) steps with a final elongation step at 72 °C for 10 min. PCR was performed on a C1000 Touch Thermal Cycler (BIO-RAD, USA) in a volume of 50 μl. First, 3.5 μl ddH2O, 1.5 μl BSA and 10 μl DNA (2 ng μl) were incubated for 5 min at 90 °C to bind PCR-inhibiting substances. Subsequently, 23.1 μl ddH2O, 5 μl 10× Buffer (15 mM $MgCl_2$, Quiagen, Germany), 2 μl $MgCl_2$ (25 mM), 1 μl dNTP-Mix, 1 μl primer forward (μM), 1 μl primer reverse (10 μM), 1.5 μl BSA and 0.4 μl Quiagen HotStarTaqPlus (5U $\mu l^{-1}$, Quiagen, Germany) were added to the solution. The quality of the PCR product was confirmed by electrophoresis in 1.4% (v/w) agarose gels using ethidium bromide for staining. Each PCR was repeated four times and technical replicates were pooled for sequencing. Amplicon pools were sent to the Génome Québec Innovation Center at McGill University (Montréal, Canada) for barcoding using the Fluidigm Access Array technology (Fluidigm, South San Francisco, CA, USA) and paired-end sequencing on the Illumina MiSeq v3 platform (Illumina Inc., San Diego, CA, USA).

### 2.7. Bioinformatics

Quality filtering and clustering into operational taxonomic units (OTUs) was performed using a customized pipeline (Frey et al., 2016) based on USEARCH v.8 (Edgar, 2010, 2013) and other tools. In brief, paired-end reads were merged using USEARCH (Edgar and Flyvbjerg, 2015) and primers were trimmed using Cutadapt (Martin, 2011) allowing for one mismatch. Reads not matching the primers or with read lengths below 300 ($16S_{V3V4}$) or 200 bp (ITS2) were discarded. Trimmed reads were quality-filtered in USEARCH using a maximum expected error threshold of one. After strict dereplication, singleton reads were removed and the remaining sequences were clustered into OTUs at 97% sequence identity using the USEARCH *cluster_otu* function including an "on-the-fly" chimera detection (Edgar, 2013; Edgar and Flyvbjerg, 2015). OTU centroid sequences, i.e. seed sequences being representative of each OTU, were subjected to an additional round of chimera filtering by running UCHIME (Edgar et al., 2011) against customized versions of the GREENGENES (DeSantis et al., 2006) and UNITE

(Nilsson et al., 2015) database, respectively. The remaining centroid sequences were tested for having prokaryotic or eukaryotic ribosomal signatures using V-Xtractor (Hartmann et al., 2010) or ITSx (Bengtsson-Palme et al., 2013), and centroid sequences with no ribosomal signatures were discarded. All quality filtered reads that remained after the filtering step were mapped to the final centroid sequences using the *usearch_global* algorithm (*maxrejects 0*, *maxaccepts 0*, *top_hit_only*) in USEARCH. Centroid sequences were queried against selected reference databases for taxonomic assignment using the naïve Bayesian classifier (Wang et al., 2007) implemented in MOTHUR (Schloss et al., 2009) and a minimum bootstrap support of 60%. Prokaryotic $16S_{V3V4}$ sequences were queried against GREENGENES (DeSantis et al., 2006; McDonald et al., 2012), whereas eukaryotic ITS2 sequences were first queried against a custom-made ITS2 reference database retrieved from NCBI GenBank (Benson et al., 2005) and sequences assigned to fungi were subsequently queried against UNITE (Abarenkov et al., 2010). The 16S rRNA primers potentially amplify ribosomal DNA from eukaryotic organelles (chloroplast, mitochondria), whereas the ITS2 primers potentially amplify ribosomal DNA from plants (Viridiplantae) and soil animals (Metazoa). OTUs assigned to these taxonomic groups as well as OTUs not classified beyond the eukaryotic superkingdom level were removed from further analysis. Raw sequences have been deposited in the European Nucleotide Archive (ENA: PRJEB13134).

### 2.8. Statistics

Between-treatment variation in prokaryotic and fungal community structure (β-diversity) were measured by Bray-Curtis similarities calculated from OTU abundances using a 1000-fold iterative subsampling approach implemented in MOTHUR (Frey et al., 2016). As recommended by Anderson and Willis (2003), unconstrained as well as constrained multivariate statistical tests were applied to measure differences in community structure (Anderson et al., 2011). More precisely, as outlined in previous studies (Hartmann et al., 2014, 2015), principal coordinate analysis (PCO), canonical analysis of principal coordinates (CAP), permutational analysis of variance (PERMANOVA), analysis of multivariate dispersion (PERMDISP), and analysis of similarity (ANOSIM) were applied with $10^5$ permutations using the homonymous routines in Primer7 (Clarke and Gorley, 2015). P values were adjusted for multiple testing using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) using the R function *p. adjust* (R Core Team, 2014). Effects on phylum level were assessed on the sum of relative abundances per phylum per pot using PERMANOVA. Correlation-based indicator species analysis was performed with the R package *indicspecies* (De Cáceres and Legendre, 2009) with $10^5$ permutations and 5 orders of group combinations (De Cáceres et al., 2010). Indicator status for rare OTUs is difficult to assess. Therefore OTUs with an abundance >10 were used for the *indicspecies* test. P values were not adjusted for multiple testing, but a significance level of $p < 0.01$ was chosen. Estimates of alpha diversity (within treatment diversity) was assessed by observed richness $S_{obs}$ and Smith-Wilson evenness $E_{var}$. These values were calculated from the OTU abundance table using a 1000-fold iterative subsampling approach implemented in MOTHUR. Univariate PERMANOVA was performed for both richness and evenness based on Euclidean distances using Primer7.

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

## 3. Results

### 3.1. Effects of NPs on variation of community structure and α-diversity

In total 581,576 $16S_{V3V4}$ and 2,363,759 ITS2 high-quality sequences were obtained. These sequences clustered into 3603 prokaryotic OTUs as well as 1295 fungal OTUs (raw data ENA No. PRJEB13134). Average number of sequences (±standard deviation, $n = 35$) per sample were 16,616 ± 6232 and 67,536 ± 24,702 for the prokaryotic and fungal datasets, respectively. Average number of OTUs was 1919 ± 268 and 560 ± 46, respectively. Application of $TiO_2$ NPs did not affect the prokaryotic and fungal α-diversity (i.e. richness and evenness) when compared to the control treatment (Table S1). Only the $ZnSO_4$ treatment (positive control) decreased the prokaryotic richness when compared to the control (Table S1). In contrast to these minor effects on α-diversity, $TiO_2$ NP application significantly altered the community structure of prokaryotes ($p < 0.001$) but not of fungi ($p = 0.61$, Fig. 1 and Fig S2, Tables S2 and S3). The largest shift compared to the control treatment was observed for the positive control ($ZnSO_4$), but treatments E171, P25 and bulk $TiO_2$ revealed significantly different community structures as assessed by ANOSIM and PERMANOVA (Tables S2 and S3).

Among the 3603 prokaryotic and 1295 fungal OTUs, a total of 3563 and 1253 OTUs, respectively, were assigned at the phylum level. For prokaryotes 3495 (97%), 3005 (83%), 1843 (51%), 667 (19%) and 63 (2%) OTUs could be assigned at the taxonomic levels of class, order, family, genus and species, respectively. 40 (1%) OTUs remained unclassified. For ITS2, these values were 1150 (89%), 1068 (82%), 983 (76%), 900 (69%) and 774 (60%). 42 OTUs (3%) remained unclassified. The abundances of these OTUs were tested for correlation with the treatments. A total of 153 prokaryotic and 28 fungal OTUs could be associated to a treatment or a group of treatments. Visual inspection suggests that members of Actinobacteria were more frequently affected than those of other phyla. Sensitive to NPs (P25 and E171) were 25 OTUs (Table 1). Treatment effects at phylum level were assessed for the ten most abundant prokaryotic phyla (Fig. 2). Actinobacteria and Chloroflexi revealed a shift in relative abundances from the controls for P25 and bulk $TiO_2$. Verrucomicrobia were sensitive to bulk $TiO_2$ and $ZnSO_4$. For the fungal phyla, no differences to the control could be detected (Fig. S3). Focusing on the phylum Glomeromycota, which contains the ecological important group of AMF, 74 OTUs including 13,549 sequences were found (0.6% of the total sequences). One of these OTUs was associated with P25 and was of the family Diversisporaceae and contained 255 sequences.

### 3.2. Symbiosis with AMF

Focusing on one group of microorganisms associated with plants, the wheat root colonization of AMF was determined as well as phosphorus content of wheat grains as a measure of nutrient acquisition by plant roots and AMF. The application of $TiO_2$ NPs did not influence the ability of wheat to form a symbiosis with AMF. Total root colonization did not differ between plant roots of the control treatments and those in soils treated with $TiO_2$ NPs (Fig. 3). The phosphorus content of the wheat grains treated with $TiO_2$ NPs and $ZnSO_4$ were also not significantly different from the control treatment (Fig. 3).

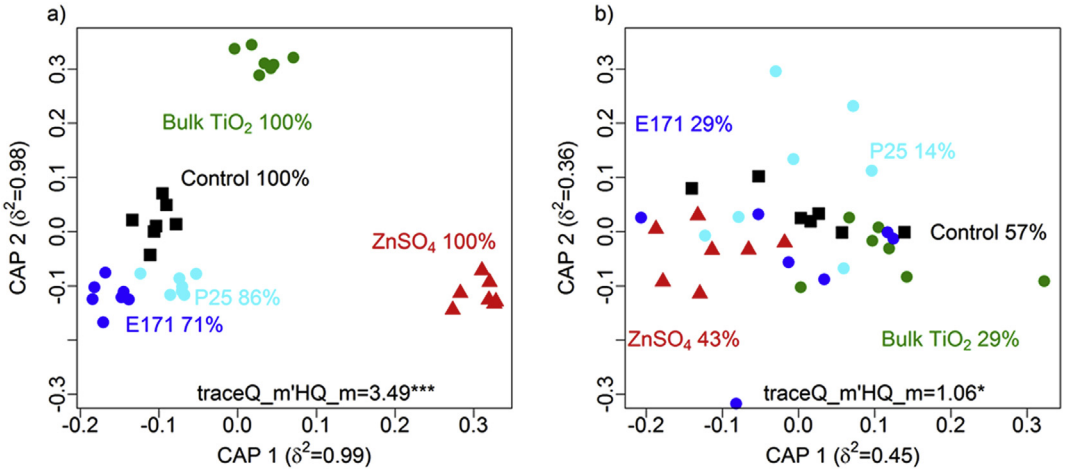


**Fig. 1. Effects of $TiO_2$ NPs on microbial community structure.** Effects of NP application at the highest concentration (1000 mg NPs $kg^{-1}$) on prokaryotic (a) and fungal (b) community structure. *CAP* ordinations based on Bray-Curtis similarities calculated from OTU abundances maximize discrimination among the different NP treatments. The canonical correlation ($\delta^2$) of each *CAP* axis is given in parentheses. The *CAP* reclassification rates (%) for each treatment are provided next to the treatment label. The traceQ_m'HQ_m statistic (sum of canonical eigenvalues) given in the plots tests the null hypothesis of no significant differences in multivariate location among NP treatments and represents an overall test of rejecting the null hypothesis. Significant p-values are indicated by asterisks (*≤0.05 and ***≤0.001). Treatments include the negative control (black squares), the positive control $ZnSO_4$ (red triangles), the bulk $TiO_2$ control (green circles), as well as the two $TiO_2$ NPs P25 (turquoise circles) and E171 (blue circles). The circles stand for $TiO_2$ treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.3. Biomass and plant health

Root and shoot dry weight of wheat were unaffected by the different $TiO_2$ NPs (Fig. 4, Table S4). The positive control with 1000 mg $kg^{-1}$ $ZnSO_4$*$7H_2O$ increased the shoot dry weight significantly ($p < 0.001$) by 14%. Average chlorophyll contents of wheat leaves of E171 at 1 and 100 mg $kg^{-1}$ and P25 at 1 and 1000 mg $kg^{-1}$ were significantly ($p < 0.001$) increased after 30 d by 6%; however, after 45 days, chlorophyll contents of plants grown in $TiO_2$ NP treated pots, did not differ anymore from control pots (Fig. S4). The number of ears and their dry weight were not affected by any of the $TiO_2$ NP treatments (Table S4).

## 4. Discussion

This study demonstrates that the addition of nanoparticles to the soil changed the community structure of prokaryotes but not fungi. This suggests that prokaryotes are more sensitive to $TiO_2$ NPs and $ZnSO_4$ than fungi, at least during the three months of exposure. NPs have been reported to interact with bacterial surfaces (Neal, 2008). The difference in susceptibility of bacteria and fungi might potentially be attributed to different interactions of NPs with their surfaces.

The observed effects on prokaryotes are in agreement to other studies testing $TiO_2$ NPs (Ge et al., 2011, 2012; Shah et al., 2014). However, so far, only one study (Ge et al., 2014) used high throughput sequencing tools to analyze microbial communities and that study focused only on bacteria and not on fungi, wheat and AMF root colonization in one experiment such as in our work. An earlier study using TRFLP observed effects of $TiO_2$ NPs (40–60 nm) on AMF community structure (Burke et al., 2014), in contrast to our study where no such effects on fungi and AMF were found. In these studies other crops were used, i.e., soybean and maize, which might influence soil microorganisms and interactions with NPs due to their different root exudates. For bacteria communities, it has been reported that they were differently affected by NPs when exposed with or without soybeans growing in the soil (Ge et al., 2014). However, the mechanisms of how plant exudates, NPs and bacteria interact with each other are not known yet.

$TiO_2$ NPs had a negative effect on the abundance of four prokaryotic OTUs and increased the abundance of 15 prokaryotic and six fungal OTUs. Three of these fungal OTUs belonged to the phylum Ascomycota. However, when treatment effects were investigated at phylum level, Ascomycota showed no differences between the NP treatments and the control. For prokaryotes, 14 phyla were affected by the treatments (Table 1, Fig. 2). The causes for decline or increase of these taxa are unknown; it might be related to the abiotic environment (see below) or because of biotic effects such as altered root exudation patterns. Long-term exposure experiments are required to draw more solid conclusions regarding the susceptibility of microorganisms to increased NP loads in soil. In this respect it is important to mention that other studies demonstrated that duration of exposure influences the effects on bacterial community structure, with higher effects after 60 days compared to 15 days of exposure (Ge et al., 2011, 2012). Notably, our observation that 25 microbial taxa were significantly affected by $TiO_2$ NP applications suggest that such taxa could be used as “bio-indicators” for $TiO_2$ NP applications if further studies confirm that they are indeed sensitive to $TiO_2$ NP applications. Future studies also need to investigate the ecological role of such microbes and how they function in microbial networks (van der Heijden and Hartmann, 2016). Note that the precise identity of several taxa is unknown because the databases lack proper references. As a consequence similarities to higher order taxa (e.g. family or order) are shown. Also, in some cases, the sequences are too short, or the target region for DNA amplification is too variable, to specifically identify taxa and further technological advances such as longer sequences (e.g. see Schlaeppi et al., 2016 for AMF) will facilitate the taxa identification process.

Factors such as primary particle size and crystal structure of the particles potentially drive the effects of NPs on microbial communities. Assessing the influence of particle size and crystal structure using multivariate statistical techniques in our study revealed that the treatment response of the prokaryotic communities was more similar for E171 and P25 compared to bulk $TiO_2$ (Fig. 1). Bulk $TiO_2$ particles have a larger diameter than E171 and P25, which might be one reason why prokaryotes responded differently. Moreover, these particles differ in their crystal structure, with E171 and bulk $TiO_2$

**Table 1**
OTUs significantly associated with NPs E171 and P25.

| OTU | phylum | class | order | family | genus | species | Treatment Association | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | bulk $TiO_2$ | control | E171 | P25 | $ZnSO_4$ | stat | p value |
| OTUp_0641 | Planctomycetes | Phycisphaerae | WD2101 | | | | 0 | 0 | 1 | 0 | 0 | 0.5749 | 0.0039 |
| OTUp_2939 | | | | | | | 0 | 0 | 1 | 0 | 0 | 0.5629 | 0.0058 |
| OTUp_1560 | Acidobacteria | Sva0725 | Sva0725 | | | | 0 | 0 | 0 | 1 | 0 | 0.5778 | 0.0053 |
| OTUp_0216 | Actinobacteria | Actinobacteria | Actinomycetales | Nocardioidaceae | | | 0 | 0 | 0 | 1 | 0 | 0.6143 | 0.0004 |
| OTUp_1101 | Euryarchaeota | Thermoplasmata | E2 | | | | 0 | 0 | 0 | 1 | 0 | 0.5396 | 0.0081 |
| OTUp_1508 | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadales | Ellin5301 | | | 0 | 0 | 0 | 1 | 0 | 0.5533 | 0.0079 |
| OTUp_1335 | OD1 | ZB2 | | | | | 0 | 0 | 0 | 1 | 0 | 0.5831 | 0.0079 |
| OTUp_0503 | | | | | | | 0 | 0 | 0 | 1 | 0 | 0.6223 | 0.0012 |
| OTUp_1471 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | AKIW874 | | | 1 | 1 | 0 | 0 | 0 | 0.6840 | 0.0000 |
| OTUp_0356 | Actinobacteria | Actinobacteria | Actinomycetales | Streptomycetaceae | Streptomyces | | 0 | 0 | 1 | 1 | 0 | 0.8614 | 0.0000 |
| OTUp_0066 | Firmicutes | Bacilli | Bacillales | | | | 0 | 0 | 1 | 1 | 0 | 0.7117 | 0.0000 |
| OTUp_2001 | OD1 | ZB2 | | | | | 0 | 0 | 1 | 1 | 0 | 0.5898 | 0.0046 |
| OTUp_1142 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | | | 0 | 0 | 1 | 1 | 0 | 0.5500 | 0.0085 |
| OTUp_0068 | Chloroflexi | Ellin6529 | | | | | 1 | 1 | 0 | 0 | 1 | 0.5712 | 0.0038 |
| OTUp_1263 | Chloroflexi | TK10 | AKYG885 | Dolo_23 | | | 1 | 1 | 0 | 0 | 1 | 0.5622 | 0.0063 |
| OTUp_0688 | Proteobacteria | Deltaproteobacteria | Myxococcales | Myxococcaceae | Anaeromyxobacter | | 1 | 1 | 0 | 0 | 1 | 0.5625 | 0.0049 |
| OTUp_1232 | OD1 | ZB2 | | | | | 1 | 0 | 1 | 1 | 0 | 0.7221 | 0.0000 |
| OTUp_0771 | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Sinobacteraceae | | | 1 | 0 | 1 | 1 | 0 | 0.5885 | 0.0042 |
| OTUp_1334 | Proteobacteria | Alphaproteobacteria | | | | | 1 | 0 | 1 | 1 | 0 | 0.5685 | 0.0051 |
| OTUp_1347 | Proteobacteria | Deltaproteobacteria | Myxococcales | Polyangiaceae | | | 1 | 0 | 1 | 1 | 0 | 0.5521 | 0.0069 |
| OTUp_1047 | Tenericutes | Mollicutes | Anaeroplasmatales | Anaeroplasmataceae | Asteroleplasma | | 1 | 0 | 1 | 1 | 0 | 0.5719 | 0.0048 |
| OTUp_0928 | Verrucomicrobia | Pedosphaerae | Pedosphaerales | | | | 1 | 0 | 1 | 1 | 0 | 0.5618 | 0.0067 |
| OTUp_0017 | Actinobacteria | Actinobacteria | Actinomycetales | Glycomycetaceae | Glycomyces | harbinensis | 0 | 1 | 1 | 1 | 1 | 0.6963 | 0.0002 |
| OTUp_0015 | TM7 | TM7-3 | | | | | 0 | 1 | 1 | 1 | 1 | 0.7155 | 0.0000 |

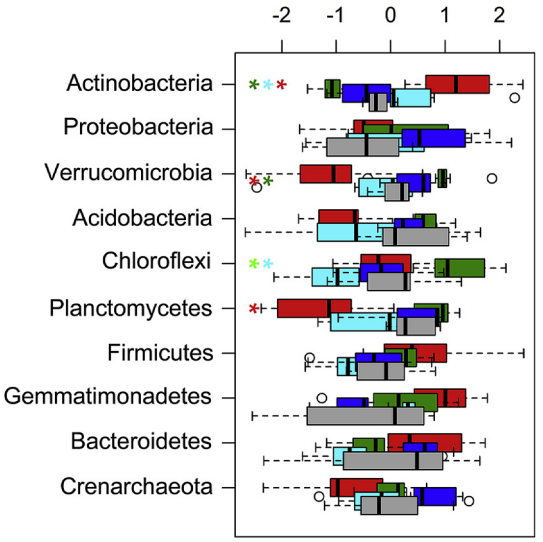


**Fig. 2. Treatment effects on the relative abundance of major prokaryotic groups at phylum level.** Relative abundances were z-transformed and analyzed per phylum using PERMANOVA. Asterisks indicate statistically significant ($p < 0.05$) differences, compared with the control, in the color of the treatment which is different. Controls are shown in grey, P25 in turquoise, E171 in blue, bulk $TiO_2$ in green and $ZnSO_4$ in red. Empty circles indicate outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

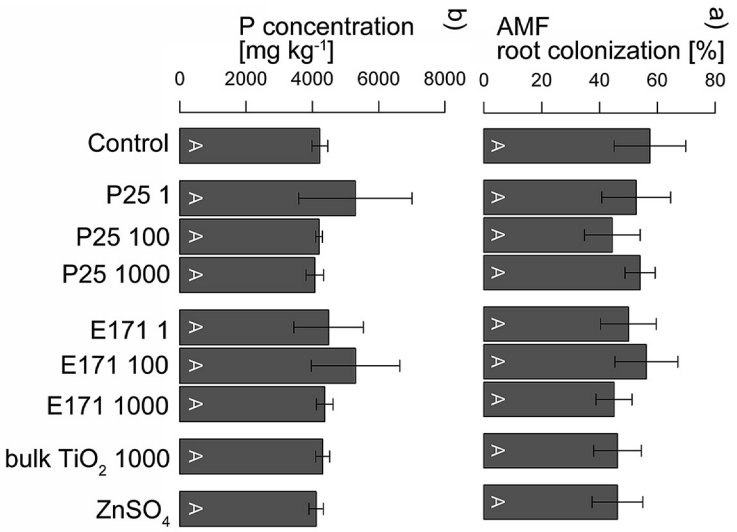


**Fig. 3. Total root colonization by arbuscular mycorrhizal fungi and phosphorus content of wheat.** a) Total root colonization (%) by arbuscular mycorrhizal fungi ($n = 5$), and b) phosphorus content of wheat grains ($n = 7$) at the end of the three month exposure for the control, P25 and E171 in three concentrations (1, 100 and 1000 mg $kg^{-1}$ soil), Bulk $TiO_2$ and positive control $ZnSO_4*7H_2O$ at 1000 mg $kg^{-1}$. Error bars show standard deviations and significant differences are shown with letters ($p < 0.05$).

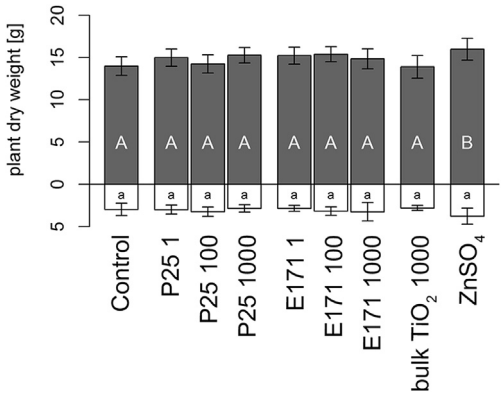


**Fig. 4. Wheat plant dry weight.** The weight is divided in shoot (grey), and root (white) at the end of the three month exposure. Results are shown for control, P25 and E171 in three concentrations, i.e., 1, 100 and 1000 mg $kg^{-1}$ soil, Bulk $TiO_2$ and the positive control $ZnSO_4$*$7H_2O$ at 1000 mg $kg^{-1}$. Error bars show the standard deviations (n = 7). Capital letters show significant differences for shoots, and small letters for roots compared to the control ($p < 0.05$).

consisting of 100% anatase and P25 being a mixture of 21% rutile and 79% anatase. *PERMANOVA* (Table S2) revealed that the similarity of prokaryotic communities between P25 and bulk $TiO_2$ was smaller than the one of E171 and bulk $TiO_2$, which suggests a potentially different effect of the crystal structure. However, the similarity between bulk $TiO_2$ and E171 was the same as between E171 and P25. Therefore our findings suggest that both primary particle size and crystal structure might trigger the community shift with primary particle size being the more important factor (Fig. 1, Tables S2 and S3). Shah et al. (2014) found that the different crystal structures of $TiO_2$ NPs, i.e. anatase and rutile, affected bacterial community structure assessed by pyrosequencing. Rutile (55 nm) revealed stronger effects compared to the control than the smaller anatase particles (5–10 nm) (Shah et al., 2014). Physical interactions between NPs and other surfaces, such as heteroaggregation with soil particles, are also potentially important factors determining the impact on the microbial community. The NPs can potentially interact with the surrounding surfaces and, thus, differences in soil texture could also explain the different findings compared to Shah et al. (2014). For example, it has been reported that soil types determine the effect size of $TiO_2$ NPs on microbial abundance as assessed by quantitative PCR (Simonin et al., 2015). In loamy soils, bacterial abundance decreased, while it remained unaffected in sandy loam and silty clay soils. Additionally to different soil types, also farming systems approaches with, e.g., different fertilization levels, have been reported to change microbial communities (Alguacil et al., 2008; Lumini et al., 2010; Verbruggen et al., 2010; Leff et al., 2015) and could potentially also affect NP interactions with microorganisms.

In addition to the fungal community structures, we specifically assessed the effects of $TiO_2$ NPs on one important group of fungi, i.e. AMF, in more detail by counting root colonization (Fig. 3). No effects of $TiO_2$ NPs on the colonization of roots were observed ($p = 0.56$). These results suggest that AMF were not affected by the $TiO_2$ NPs. In agreement to our study Burke et al. (2015) found no effects of $TiO_2$ NPs on AMF communities colonizing roots of soybeans. Note that the primers we have used to characterize the fungal community are not very specific for AMF and further studies with primers that specifically target AMF are required (e.g. Schlaeppi et al., 2016).

Shoot biomass of the wheat plants was not affected by $TiO_2$ NPs (Fig. 4). In contrast, Du et al. showed that wheat shoot biomass decreased by 13% after $TiO_2$ NP treatment (90 mg $kg^{-1}$, 20 ± 5 nm) for six months (Du et al., 2011). Several reasons may account for the deviating results in the two studies, such as the use of aged $TiO_2$ NPs (e.g. properties of NP can change with time), different soil properties, different wheat varieties and extended exposure time in the study of Du et al. (2011). The $ZnSO_4$ treatment increased the wheat biomass. It is known, that with increasing pH, the solubility of Zn in soils decreases (Lindsay, 1972), which might be the reason why in our experiment with a high soil pH of 7.7 the 1000 mg $kg^{-1}$ $ZnSO_4$ treatment acted as a fertilizer for wheat rather than a toxin. However, for prokaryotes the $ZnSO_4$ treatment worked as positive control and community structure was significantly different from the negative control.

Applied NP concentrations in our experiment (Gogos et al., 2016) were relatively high compared to expected environmental concentrations (Sun et al., 2014). However, in our experiment vertical transportation of the NPs in the pots was not statistical significant (Gogos et al., 2016) and thus it is probable that the $TiO_2$ NPs accumulate over time. Therefore, it will be important to investigate more long-term effects on the plant-microbiome system before we can statistically draw more solid conclusions on the impact of $TiO_2$ NPs on the environment.

For future experiments, it is important to go into depth and investigate how NPs interact with bacteria and fungi in soils, and how environmental factors, such as plant exudates, interact with NPs. Also the ecological role of microbial species which are affected by NPs and their function in the microbial networks (van der Heijden and Hartmann, 2016), are important to assess in more detail. Our experiments were conducted under controlled green house conditions. Future studies should be performed under field conditions (e.g. in experimental agricultural fields). This also allows to investigate effects on soil food webs.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.03.019.

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