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# Compositional alterations in soil bacterial communities exposed to TiO<sub>2</sub> nanoparticles are not reflected in functional impacts

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## ABSTRACT

Titanium dioxide nanoparticles (TiO<sub>2</sub>NP) are increasingly released in soil ecosystems, while there is limited understanding of the impacts of TiO<sub>2</sub>NP on soil bacterial communities. Here we investigated the effects of TiO<sub>2</sub>NP on the taxonomic composition and functional profile of a soil bacterial community over a 60-day exposure period. In short-term exposure (1-day), contradictory effects on the taxonomic composition of soil bacterial communities were found after exposure to a low realistic environmental concentration of TiO<sub>2</sub>NP at 1 mg/kg as compared to the effects induced by medium and high concentrations of TiO<sub>2</sub>NP at 500 and 2000 mg/kg. After long-term exposure (60-day), the negative effects of TiO<sub>2</sub>NP at the low concentration disappeared, and the inhibition by TiO<sub>2</sub>NP of the abundance of core taxa was enhanced along with increasing exposure concentrations. However, although significant alterations were observed in the taxonomic composition over time and exposure concentrations, no significant change was observed in the community functional profile as well as enzyme activity after 60-day exposure, indicating that functional redundancy likely contributed to the bacterial community tolerance after the exposure to TiO<sub>2</sub>NP. Our study highlighted the importance of assessing bacterial community compositional and functional responses in assessing the environmental risk of nanoparticles on soil ecosystems.

## 1. Introduction

Titanium-dioxide nanoparticles (TiO<sub>2</sub>NP) are among the metal NPs that are produced in the highest volumes. They are, amongst others, nowadays applied in agriculture as biosolids, nano-agrochemicals, and additives for crops (Liu et al., 2019). In addition to agricultural applications, TiO<sub>2</sub>NP may also enter the environment as waste e.g. landfills (Tan et al., 2018). The release of TiO<sub>2</sub>NP into soil as induced by anthropogenic activities could result in elevated concentrations. This raises concerns about their potential impact on the soil bacterial community as well as on the soil ecosystems (McKee and Filser, 2016).

Previous studies on single bacterial cultures have shown that TiO<sub>2</sub>NP can have negative effects on bacteria (Heinlaan et al., 2008; Sohni et al., 2015) including membrane damage, surface coating

related photocatalytic oxidation and reactive oxygen species (ROS) production (Dizaj et al., 2014). When TiO<sub>2</sub>NP enter the environment, the natural aging processes such as aggregation/agglomeration and sorption could change the effect of TiO<sub>2</sub>NP on natural bacterial communities, resulting in different toxicity from what has observed on single cultures (Fang et al., 2009; Hotze et al., 2010). The toxic effects of TiO<sub>2</sub>NPs on microbial communities are highly dependent on the type of soil, exposure concentration, incubation time, microbial endpoints etc. (Simonin and Richaume, 2015). Inconclusive findings have been reported regarding impacts of TiO<sub>2</sub>NP on soil bacteria community. Simonin et al. found no TiO<sub>2</sub>NP toxicity on the C-mineralization and microbial community abundance except for soils with a high organic matter content (Simonin et al., 2015), while Ge et al. observed a decline in microbial biomass and community diversity in forest soil exposed to

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TiO<sub>2</sub>NP (Ge et al., 2011). This highlighted the importance to further investigate the impact of TiO<sub>2</sub>NP on soil microbial community abundance and diversity. It is suggested that within a highly diverse community, the response pattern to TiO<sub>2</sub>NP varies among taxa due to bacterial compensation and competition as well as NPs behaviour (e.g. aging, aggregation and agglomeration in soil) at different time scales (Ge et al., 2012; Simonin et al., 2017). This raises the question if and to what extent soil bacteria respond to TiO<sub>2</sub>NP as function of concentration and exposure time at a community-level.

Moreover, studies of TiO<sub>2</sub>NP-exposed soil bacterial communities have been performed with high concentrations of TiO<sub>2</sub>NPs (ranging from 500 to 2000 g TiO<sub>2</sub>NP/kg soil) (Ge et al., 2011, 2012). The impact of exposure at environmentally relevant concentration of TiO<sub>2</sub>NP on soil bacterial community remain largely unexploited. A recent study found that the growth of ammonia-oxidizing bacteria cultured from sediments were significantly inhibited by TiO<sub>2</sub>NP at predicted environmental concentrations ( $\leq 2$  mg/L in cultural medium) (Luo et al., 2015). However, it is difficult to extrapolate these conclusions to complex microbial communities. Recently a lack of dose-response relationship was observed on the toxicity of environmental concentrations of TiO<sub>2</sub>NP on soil nitrification (Simonin et al., 2017), which highlights the importance to include the environmentally relevant concentration in assessing the overall impacts of TiO<sub>2</sub>NP on soil bacterial community.

Given that a soil bacterial community is essential in maintaining soil biogeochemical processes, it is important to further investigate the impact of TiO<sub>2</sub>NP on the soil bacterial associated functioning (Nannipieri et al., 2003). It could be possible that part of soil processes after exposure to TiO<sub>2</sub>NP are not ultimately fixed by adaptation of the bacterial community, resulting in functional dissimilarities (Strickland et al., 2009). For instance, significant disruption induced by TiO<sub>2</sub>NP has been observed in various functional endpoints including soil nitrification (Simonin et al., 2017), carbon mineralization (Simonin and Richaume, 2015), enzyme activity (Simonin et al., 2016b), and soil respiration (Ge et al., 2013). However, a broad array of soil microorganisms with highly diverse species could also compensate the disruption of partial soil process regulated by certain bacteria, and functional redundancy/equivalence became dominant in maintaining the overall community functioning (Rosenfeld, 2002). This raises the second research question of whether genus specific responses to TiO<sub>2</sub>NPs exposure would produce functional significance.

The aim of the present study is to investigate the effect of TiO<sub>2</sub>NP on both taxonomic composition and functional response of a soil bacterial community as function of exposure concentration and time. The TiO<sub>2</sub>NP concentrations were selected to be 1 mg/kg in order to represent a low realistic environmental concentration (Sun et al., 2014), and 500 and 2000 mg/kg to represent medium and high concentrations (Ge et al., 2011; Simonin et al., 2016b) that are used more often in ecotoxicological studies. Soil samples were collected after 1, 15 and 60 days of exposure to different concentrations of TiO<sub>2</sub>NP based on the previous studies (Table S1). We hypothesized that 1) the impact of TiO<sub>2</sub>NP on the soil bacterial community depended on exposure concentration and time; and 2) the community compositional shift induced by different TiO<sub>2</sub>NP treatments could result in functional disruption. The effects of TiO<sub>2</sub>NP on the soil bacterial community were determined by targeting the total bacterial abundance, enzyme activity, community structure, composition and functional profile.

## 2. Materials and methods

### 2.1. Nanoparticles

TiO<sub>2</sub>NP (mixture of anatase (80%) and rutile (20%) crystal structure) with 99.5% purity were purchased from Sigma-Aldrich. According to the manufacturer information, TiO<sub>2</sub>NP presented a spherical shape with a mean particle size of 25 nm and a specific surface area of

35–65 m<sup>2</sup>/g in powder. Transmission electron microscopy (TEM) (JEOL 1010, IEOL Ltd., Japan) and dynamic light scattering (DLS) (Malvern, Instruments Ltd., UK) were used to characterize the morphology and hydrodynamic size distribution of TiO<sub>2</sub>NP.

### 2.2. Soil collection

Three sandy soils of 2 kg each were randomly collected from the top 15 cm of a site dominated by non-polluted deciduous trees (52°07'06.7"N 5°11'23.1"E, Bilthoven, The Netherlands) and thoroughly mixed. Details of the soil characteristics were provided previously (Zhai et al., 2016). In brief, the soil was sandy-loam with pH at 6.2, and containing  $4 \pm 0.6$  mg/kg dissolved organic matter. The collected soils were sieved to 2 mm, stored at 4 °C with soil moisture at 18% of the dry soil weight (Zhai et al., 2017). For determining the characterizations of TiO<sub>2</sub>NP in soil extract, 1 g soil sample per replicate were mixed with 10 mL BIS-TRIS buffer (Sigma-Aldrich B9754, 2.09 g/L, pH = 7) and then centrifuged at 1500 rpm for 10 min. The supernatant was then diluted 5 times using the same buffer to obtain the soil extract (Rutgers et al., 2016).

### 2.3. Experimental design

Soils were pre-incubated at 20 °C for one week before the experiment. Each soil microcosm consisted of 60 g of soil (50 g dry weight equivalent) with exposure concentrations of 1, 500, and 2000 mg TiO<sub>2</sub>NP/kg dry soil, representing a low, elevated, and contaminated exposure scenarios (Ge et al., 2011; Sun et al., 2014) respectively, and a control with no TiO<sub>2</sub>NP spiked. Three sets of nano-TiO<sub>2</sub> stock dispersions (0.025, 12.5, and 50 mg/mL) were prepared following the Risk Assessment of Engineered Nanoparticles (ENPRA) protocol by sonicating the suspensions at 4 °C at  $38 \pm 10$  KHz for 16 min (Jacobsen et al., 2010). After sonication, the stock suspensions were continuously stirred to maintain homogeneity. TiO<sub>2</sub>NP stock suspensions were amended drop by drop using a pipet to each microcosm allowing to achieve the concentration of each exposure scenario (Ge et al., 2011). The same amount of sterilized water without TiO<sub>2</sub>NP were added to the control. The suspensions and the soil were intensively mixed for 5 min (Sillen et al., 2015). Four treatments (control and three concentrations of TiO<sub>2</sub>NP) with 3 replicates (in total 12 microcosms) were prepared, and the soil microcosms were incubated at 20 °C in the dark for 60 days and were weekly aerated for atmosphere renewal (Simonin et al., 2016b). During the incubation, the soil water content was maintained at 24% by replenishing the water lost with sterile water. Sub-samples of the different TiO<sub>2</sub>NP treatments and the control were collected for further analysis at day 1, 15 and 60.

### 2.4. DNA extraction and Illumina Miseq sequencing

The DNA was extracted from the soil samples with/without TiO<sub>2</sub>NP exposure using a Qiagen DNeasy PowerSoil Kit (Hilden, Germany). Negative controls were measured with TiO<sub>2</sub>NP dissolved in DNA-free water, and the details of DNA extractions of the samples are listed in Table S2. This is a pre-quality control step, the downstream sequencing further confirmed that the DNA samples passed the quality control checks. A universal bacterial primer set (515F: 5'-GTGCCAGCMGCCG CGGTA-3' and 909R: 5'-CCCCTCAATTCMTTTRAGT-3') targeting the variable V4–V5 regions of bacterial 16S rRNA genes was used for PCR amplification (Liu et al., 2017). Paired-end sequencing was done by BaseClear (Leiden, the Netherlands) using 2 × 300 bp Illumina Miseq platform (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's instructions. The sequences have been deposited into the NCBI database (Project number: PRJNA491925) with sample information provided in Table S3.

## 2.5. Quantification of bacterial abundance

The total bacterial abundance was quantified using the QX200™ Droplet Digital™ PCR System (Bio-Rad Supermix, Bio-Rad, Hercules, CA, USA). The reaction mixture contained of: 11  $\mu$ L evagreen, 1  $\mu$ L forward primer, 1  $\mu$ L reverse primer, 2  $\mu$ L template DNA (diluted  $\times 10,000$ ), 7  $\mu$ L Milli-Q per sample. Every PCR column contained a negative control instead of a DNA template to check for possible contamination. The negative control for control sample was filled with 2  $\mu$ L DNA-free water, and the negative control for treated samples was filled with TiO<sub>2</sub>NP dissolved in 2  $\mu$ L DNA-free water. This mixture was emulsified with Bio-Rad droplet generation oil and partitioned into 15,000–20,000 droplets using the Bio-Rad QX-100 droplet generator (Bio-Rad). Each replicate was loaded onto a semi skirted 96-well plate, sealed, and processed with the GeneAmp 9700 thermocycler (Life Technologies, Inc. Gaithersburg, MD). The PCR conditions were 5 min at 95 °C, 30 s at 95 °C, followed by 1 min at 56 °C. Steps 2 and 3 were repeated 39 times (to have a total of 40 cycles). This was followed by 5 min at 4 °C, 5 min at 90 °C and holding the replicate at 4 °C. After the PCR program was finished, the 96-well plate was transferred to the Bio-Rad QX-200 Droplet Reader (Bio-Rad). Each droplet was checked for fluorescence as a result of DNA amplification. The example for the PCR plate reading is shown in Fig. S1.

## 2.6. Pre-processing of high-throughput sequencing data

Data obtained through Illumina sequencing were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME version 1.8.0) pipeline (<http://qiime.sourceforge.net>). After initial trimming and screening, all failed sequence reads and low quality sequence ends were removed. Chimeric sequences and singletons were removed, chloroplasts, mitochondria, archaea and eukaryotes were filtered. Rarefaction was performed to remove sampling depth heterogeneity. Qualified sequences were processed for operational taxonomic unit (OTU) assignment at a 97% sequence similarity level (Liu et al., 2018). The taxonomic assignment was conducted using the UCLUST consensus taxonomy classifier. Sequences were aligned with Python Nearest Alignment Space Termination (PyNAST) (Liu et al., 2014). To predict functional responses to the different TiO<sub>2</sub>NP treatments, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) (Langille et al., 2013) was used to translate the 16S rRNA gene amplicon data sets into predicted metagenomes to predict the functional capabilities of bacterial communities (PICRUSt v1.0.0 pipeline in QIIME). Prior to metagenomes prediction, the OTUs of 16S rRNA gene sequencing were normalized by dividing each OTU by the known/predicted 16S copy number abundance. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to annotate the predicted metagenomes (Ballou et al., 2016).

## 2.7. Enzyme activity

In addition to the functional prediction, the effect of TiO<sub>2</sub>NP on the soil bacterial community functioning was also evaluated by the dehydrogenase activity as an endpoint for expressing the functional impact. Dehydrogenase activity were measured according to the 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) assay (Von Mersi and Schinner, 1991). For each replicate in each treatment, soil samples was mixed with Tris Buffer (Tris(hydroxymethyl)aminomethane, 1M, Sigma-Aldrich) and substrate solution (2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (iodonitrotetrazolium chloride (INT), 10 mM, Sigma-Aldrich). Soil-INT mixture were incubated for 2 h at 40 °C in the dark for idonitrotetrazolium formazan (INTF) development. Afterwards extraction solution (N,N-dimethylformamide/ethanol in a 1:1 ratio) was added, and incubated for 30 min at 20 °C in the dark to extract the developed INTF. The developed INTF was measured by using spectrophotometry at 464 nm (UV-

1800, Shimadzu, Kyoto, Japan). Negative controls were measured with TiO<sub>2</sub>NP dissolved in sterilized Milli-Q water. For the measurement of INTF absorbance (A<sub>464</sub>), the absorbance of control soils was corrected by the cuvette filled with INTF extraction from sterilized soils without TiO<sub>2</sub>NP, and the absorbance of treated soils were corrected by the cuvette filled with INTF extraction from sterilized soils with the same amount of TiO<sub>2</sub>NP as the treatments. The details of INTF extractions of the samples are listed in Table S2.

## 2.8. Statistical analysis

The analysis of the bacterial communities consisted of quality checking, community diversity assessment, and significance testing. Both unweighted and weighted UniFrac distance matrices were constructed from the phylogenetic tree (built by a FastTree algorithm) and used to evaluate bacterial community similarity between samples (Liu et al., 2014). The community dissimilarities were illustrated through principal coordinates analysis (PCoA) based on weighted UniFrac distance matrices. The significance of community compositional dissimilarity between each treatment was further tested by QIIME (beta-significance.py). The category of metabolism in KEGG pathways was selected for further analysis. Pairwise one-way ANOSIM (9999 permutations, Euclidean distance) was also conducted to test the significance of functional community dissimilarity between each treatment. The linear discriminant analysis (LDA) effect size (LEfSe) was performed to determine the abundant features (OTUs and KEGG functions) that most likely explains the differences between each TiO<sub>2</sub>NP treatment and control samples at each exposure time (Segata et al., 2011). The cut-off logarithmic LDA score was 2.0 (Crusell et al., 2018). By using LEfSe, statistical significance, biological consistency and effect relevance were taken into account to identify biomarkers for each sample (Segata et al., 2011). One-way analysis of variance (ANOVA) was performed to test the effect of exposure concentration on the total bacterial abundance (16S bacterial gene copy), enzyme activity and alpha diversity at each sampling time. A Tukey test was performed to test the significance between each treatment where the global effect was significant ( $p < 0.05$ ). Analyses were conducted using R v3.3.2 (IBM SPSS Statistics 23) and Paleontological Statistics (PAST, v3.14).

## 3. Results

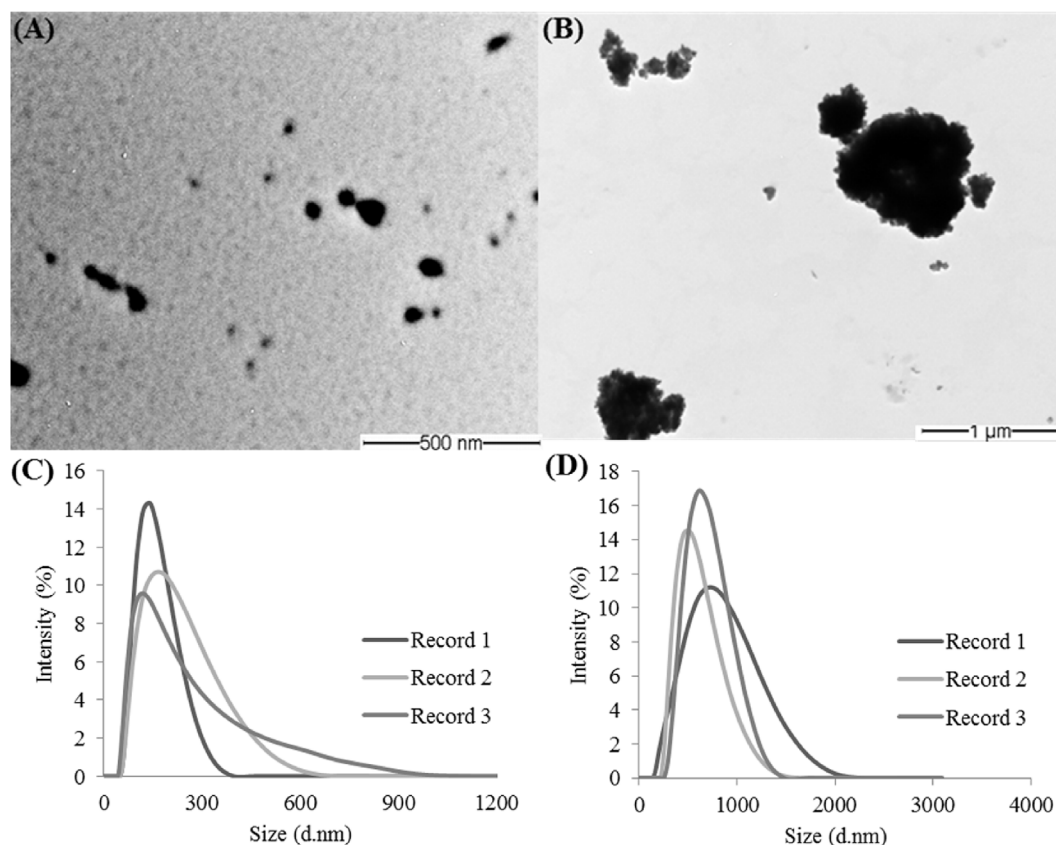
### 3.1. TiO<sub>2</sub>NP characteristics

TEM pictures revealed that the pristine shape of the 25 nm TiO<sub>2</sub>NP was spherical (Fig. 1A). The results of DLS measurements showed that the TiO<sub>2</sub>NP formed aggregates immediately after being dispersed in Milli-Q water (Fig. 1C), with an average hydrodynamic diameter of  $162 \pm 35$  nm. When dispersed in a soil extract, the TiO<sub>2</sub>NP aggregated together with soil particles and the hydrodynamic diameter of these aggregates increased to  $527 \pm 124$  nm (Fig. 1B and D).

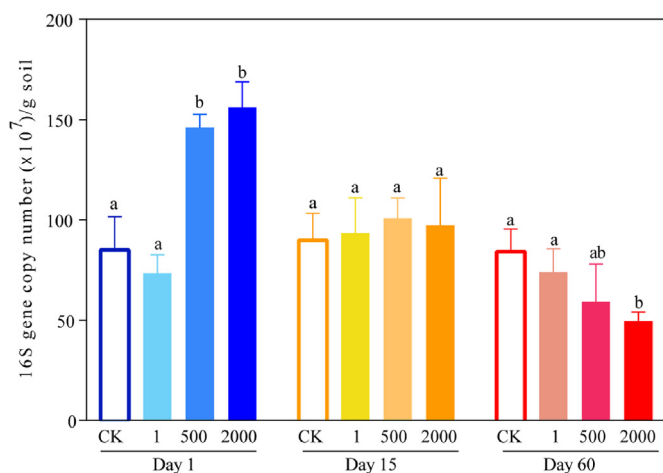
Effect of TiO<sub>2</sub>NP on total bacterial abundance, enzyme activity and community structure.

The total bacterial abundance (16S rRNA gene copy number) was measured during the exposure time at different treatments (Fig. 2). On the first day of exposure, the treatment of 1 mg/kg TiO<sub>2</sub>NP slightly lowered the bacterial abundance, while significant increases in bacterial abundance were found in the 500 and 2000 mg/kg treatments. No significant changes were found between any of the treatment after 15 days of exposure compared to the control. After 60 days, there was no significance difference found between the control and the 1 and 500 mg/kg treatments, while the bacterial abundance in the 2000 mg/kg treatment was significantly lowered compared with the control. The enzyme activity (dehydrogenase activity) was measured during the exposure time at different treatments (Fig. S2). No statistically significant change in the dehydrogenase activity was observed in the soil bacterial community in any treatment.





**Fig. 1.** Characteristics of (suspensions of) TiO<sub>2</sub>NPs. (A) TEM of TiO<sub>2</sub>NPs dispersed in milli-Q water. (B) TEM picture of TiO<sub>2</sub>NPs dispersed in soil extract. (C) Size distribution of TiO<sub>2</sub>NPs dispersed in milli-Q water. (D) Size distribution of TiO<sub>2</sub>NPs dispersed in soil extract.



**Fig. 2.** Total bacterial abundance of soil bacterial community in soil samples exposed to different TiO<sub>2</sub>NPs concentrations over time. Different letters represent significant changes in abundance. CK: Control soil without TiO<sub>2</sub>NPs. n = 3.

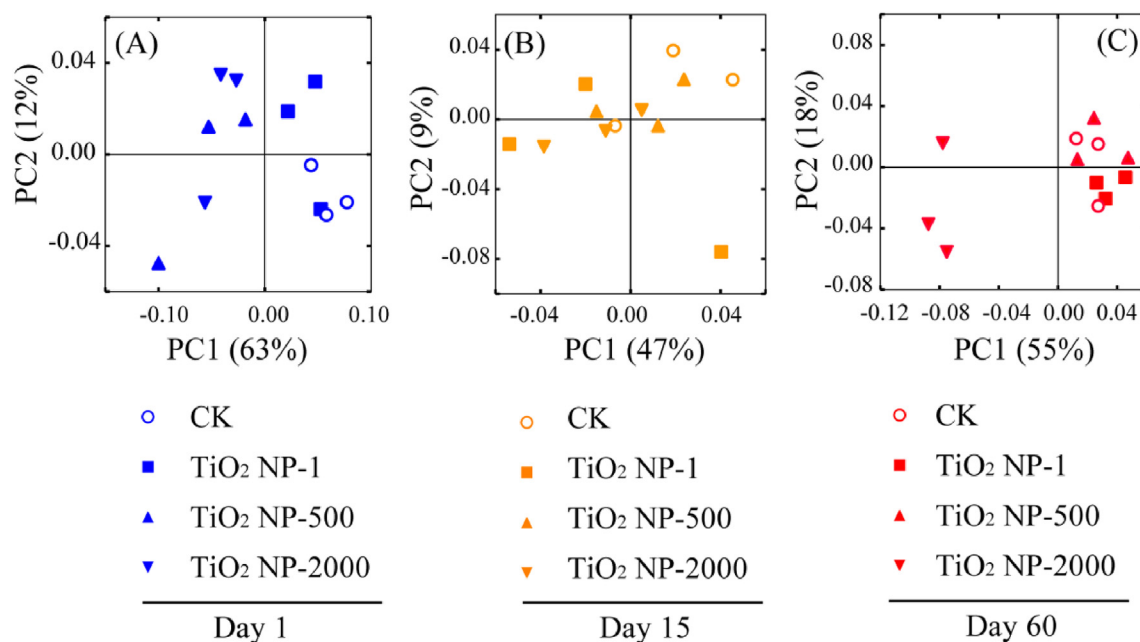
The effects of TiO<sub>2</sub>NP on the community structure were further evaluated based on the sequencing data. An overview of the alpha diversity in each treatment is given in Table S4. The dissimilarities between bacterial communities in different TiO<sub>2</sub>NP treatments were further investigated. The results of significance testing of dissimilarity of soil bacterial communities are summarized in Table S5. The shifts in bacterial communities in response to TiO<sub>2</sub>NP exposure are shown in Fig. 3. On the first day of exposure, the samples from the treatments of 500 and 2000 mg/kg TiO<sub>2</sub>NP significantly separated from the 1 mg/kg treatment and the control samples, suggesting that increased

concentrations of TiO<sub>2</sub>NP induced a shift of the soil bacterial community during acute exposure (Fig. 3A). However, as the incubation time increased to 15 days, all samples clustered more closely to each other. This convergence of the communities suggests the insignificant alterations in community structure before and after TiO<sub>2</sub>NP application (Fig. 3B). After 60-days of exposure, the community in the control group was clustered with those of the 1 and 500 mg/kg TiO<sub>2</sub>NP treatments. The community treated with 2000 mg/kg TiO<sub>2</sub>NP was significantly separated from the control group, indicating that a high TiO<sub>2</sub>NP application altered the bacterial community of the samples after a longer exposure period (Fig. 3C).

### 3.2. Effect of TiO<sub>2</sub>NP on taxonomic composition

To further identify the changes in the bacterial community in the presence of TiO<sub>2</sub>NP, the relative abundance of the phylotypes was summarized. Twelve predominant bacterial phyla represented > 90% of the total bacterial counts in all the TiO<sub>2</sub>NP treated and control samples (Fig. S3). The OTUs with average relative abundance > 0.1% were selected as abundant OTUs. LDA values > 2.0 was used to select the significantly changed abundant OTUs (core OTUs) in each treatment. The LDA values of the core OTUs are shown in Fig. S4. The relative abundance of each significantly changed core OTU at each treatment is shown in Fig. S5. The detailed taxonomic information of the significantly changed core OTUs (which contains the OTU IDs of Fig. 4) is provided in Table S5.

The numbers of significantly changed core OTUs and the distribution of these core OTUs in each treatment are shown in Fig. 4. On the first day of exposure, even an environmentally relevant concentration of 1 mg/kg TiO<sub>2</sub>NP induced a significant change in the relative abundance of OTUs, with 6 promoted and 11 reduced core OTUs observed in the bacterial community. The number of significantly changed core



**Fig. 3.** Principal coordinates analysis (PCoA) of the bacterial community in soil samples exposed to different  $\text{TiO}_2\text{NP}$  concentrations based on taxonomic composition. (A) Incubation for 1 day. (B) Incubation for 15 days. (C) Incubation for 60 days. The percentages in parentheses indicate the proportion of variation explained by each ordination axis. CK: Control soil without  $\text{TiO}_2\text{NPs}$ .  $n = 3$ .

OTUs increased with increasing exposure concentrations of  $\text{TiO}_2\text{NP}$ , and more promoted core OTUs were observed at the highest exposure concentration (24 promoted core OTUs at 2000 mg/kg  $\text{TiO}_2\text{NP}$ ) compared with the number of promoted core OTUs at the 1 mg/kg treatment (6 promoted core OTUs). This suggests a short-term stimulation of the bacterial community by a high concentration of  $\text{TiO}_2\text{NP}$ . After 15 days of incubation, the number of significantly changed core OTUs declined, with only 6–15 significantly changed core OTUs found in all treatments. After 60 days the treatment of 1 mg/kg  $\text{TiO}_2\text{NP}$  induced more promoted core OTUs, compared to the number of promoted core OTUs in the same treatment on days 1 and 15. However, the number of reduced core OTUs increased with increasing exposure concentrations of  $\text{TiO}_2\text{NP}$ , with more reduced core OTUs observed in the high treatment (20 reduced and 16 promoted core OTUs at 2000 mg/kg  $\text{TiO}_2\text{NP}$ ). This indicated that the effect of  $\text{TiO}_2\text{NP}$  on the community composition was enhanced after long-term exposure.

Linear regression of the 127 core OTUs was performed based on the relative abundance and exposure concentrations at each sampling time, and the slopes and intercepts for each OTU are summarized in Table S6. The slopes of the core OTUs at each sampling time to indicated whether the OTUs were promoted (slope above 0) or suppressed (slope below 0) as the exposure concentration increased (Fig. S6). The representative OTUs with slope either  $> 0.1$  or  $< -0.01$  were selected. The correlation of relative abundance and exposure concentrations (with log transformation) are given in Fig. S7. Promoted OTUs were only observed on the short-term exposure. The number of suppressed OTUs declined after 15-day exposure but increased after 60-day exposure. These results further confirmed that the low treatment affected the community composition in the short-term, whereas the effect of high  $\text{TiO}_2\text{NP}$  concentration on the bacterial community was enhanced after long-term exposure.

### 3.3. Effect of $\text{TiO}_2\text{NP}$ on functional profile

The predicted functional profile making use of PICRUSt analysis based on observed taxonomic composition shifts is shown in Fig. 5. The results of significance testing on the functional dissimilarity of the bacterial community are given in Table S5. On the first day of exposure,

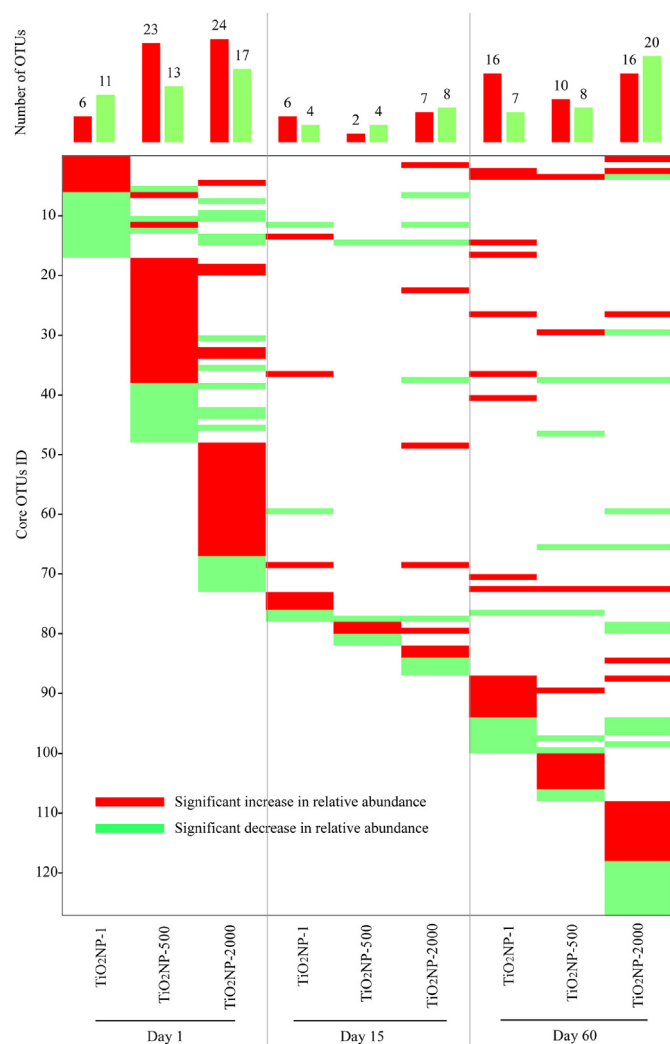
although not significantly, the samples from the treatments of 500 and 2000 mg/kg separated from the control samples (Fig. 5A). After 15–60 days of exposure, all the samples clustered together (Fig. 5B and C), which revealed the functional resilience of the bacterial community in the presence of  $\text{TiO}_2\text{NP}$ .

Level 3 of the KEGG pathway was used to assess the effect of  $\text{TiO}_2\text{NP}$  exposure on the community-wide genetic potential of the main environmentally relevant catabolic processes (further referred to as KEGG functions). The significantly changed KEGG functions compared to the control were selected based on the LDA analysis, and the LDA values are shown in Fig. S8. Significantly changed KEGG functions were only observed after the first day of exposure. The distribution of the significantly changed functions with different treatments and the classification of each significantly changed function are shown in Fig. 6. There was no significantly changed function in the 1 mg/kg  $\text{TiO}_2\text{NP}$  treatment, while 14 significantly changed functions were observed in the treatment of 500 mg/kg  $\text{TiO}_2\text{NP}$ , with 12 functions significantly promoted and 2 functions significantly reduced. For the treatment of 2000 mg/kg  $\text{TiO}_2\text{NP}$ , although the number of significantly changed functions decreased to 7, the treatment induced more reduced functions compared to the number of promoted functions (3 promoted and 4 reduced functions). However, there was no significantly changed function in the 15-day and 60-day samples at any exposure concentrations compared to the control, indicating a declined effect of  $\text{TiO}_2\text{NP}$  on the bacterial functional profile after long-term exposure.

## 4. Discussion & conclusions

### 4.1. Response of soil bacterial community over exposure time and concentration

Toxic effects of  $\text{TiO}_2\text{NP}$  on microbes have been detected even in the absence of light (e.g. soils and sediments), due to the attachment to the cell membrane by electrostatic force, causing damage on cell membrane integrity, leading to higher cell permeability (Hou et al., 2019; Simonin et al., 2016a; Sohm et al., 2015). In our results, short-term exposure (1-day) to an environmentally relevant concentration of  $\text{TiO}_2\text{NP}$  of 1 mg/kg induced more reduced core OTUs compared to the control, while



**Fig. 4.** Heatmap of the significantly changed core OTU after exposure to 1, 500 or 2000 mg/kg TiO<sub>2</sub>NP for 1, 15 and 60 days. Red OTUs are significantly up-regulated, and green OTUs are significantly down-regulated compare to the control. OTU IDs are listed in Table S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

exposure to higher concentrations (500 and 2000 mg/kg) resulted in more promoted core OTUs (Fig. 4). This apparent inconsistency between exposure concentration and adverse effect was also observed in the abundance of ammonia oxidizing bacteria and nitrite-oxidizing bacteria in a soil exposed to concentrations ranging from 0.05 to 500 mg/kg TiO<sub>2</sub>NP (Simonin et al., 2017). A low concentration of TiO<sub>2</sub>NP could result in lower aggregation and higher bioavailability, which subsequently causes a toxic effect (Simonin et al., 2017). In contrast, the application of 500 and 2000 mg/kg TiO<sub>2</sub>NP induced more promoted core OTUs, compared with the number of reduced core OTUs. The possible reason underlying this observation might be related to TiO<sub>2</sub>NP particles collisions at a higher concentration, which induced a decrease in the surface area of the particles as well as the reactive sites (Hotze et al., 2010). Moreover, the promoted OTUs observed in the high concentration treatment, e.g. *Planomicrobium*, *Exiguobacterium* and *Cattellatospora* are genus of *Firmicutes* and *Acidobacteria* that could survive in contaminated conditions by high yield of extracellular slime and siderophores (Schmidt et al., 2005), bacterial aggregates formation (Tang et al., 2018), and slow metabolic rates under stressful conditions (Ward et al., 2009). The resistant taxa therefore were competitive to survive in the presence of TiO<sub>2</sub>NP and selective growth promoted their

relative abundance in the bacterial community (Tang et al., 2018).

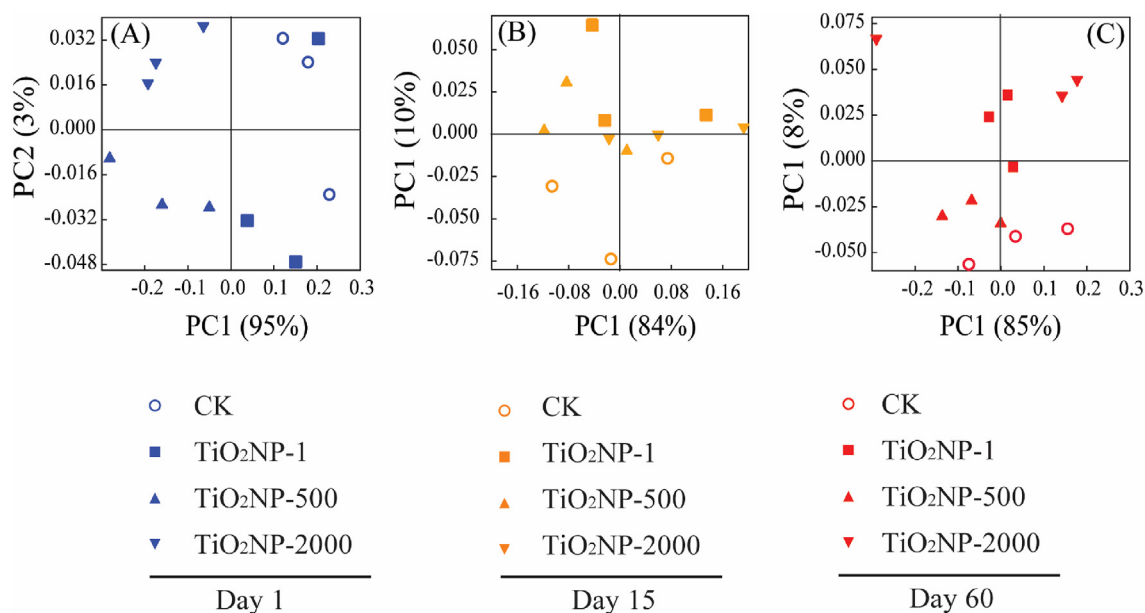
After 15 days of exposure, the number of significantly changed OTUs declined dramatically compared with the number observed in the day-1 exposure, indicating that the relative abundance of the OTUs did not really differ between the TiO<sub>2</sub>NP exposed samples and the control (Fig. 4). Moreover, there was no significant difference in bacterial abundance (16S total bacterial gene copy) in any treatment (Fig. 2). These indicated a re-convergence of bacterial community upon increasing exposure duration. It is suggested that aging and soil organic matter could affect the fate of TiO<sub>2</sub>NP in soil, which would limit the bioavailability and adverse effects (Fang et al., 2009; Tan et al., 2018). In addition, the high diversity, widespread dispersal, horizontal gene transfer, rapid growth rates and evolutionary adaptation could allow a sensitive community to be resilient and to recover to its initial state (Allison and Martiny, 2008).

When the incubation time increased to 60 days, the impact of TiO<sub>2</sub>NP at the environmentally relevant concentration (1 mg/kg) disappeared, and the number of significantly promoted core OTUs increased compared to that in the samples of 1-day exposure (Fig. 4). This indicated that although the growth of bacterial cells was inhibited after 1-day exposure to the environmental TiO<sub>2</sub>NP concentration, adaptive rebound turned to enhanced growth after long-term exposure (Tang et al., 2018). However, the number of significantly reduced core OTUs increased with the increasing exposure concentration after 60-day exposure, and more reduced than promoted core OTUs were found in the treatment of 2000 mg/kg TiO<sub>2</sub>NP. The observed decrease in the total bacterial abundance in the treatment of 2000 mg/kg TiO<sub>2</sub>NP further revealed the reduction of actual abundance (Fig. 2). The significantly reduced core OTUs e.g. *Methylosinus*, *Burkholderia* and *Gemmatimonas* play important roles in methane metabolism (Abujabbah et al., 2018), organic pollutants degradation (Sandrin and Maier, 2003) and poly-phosphate accumulation (Abujabbah et al., 2018), respectively. The long-term accumulation of high concentrations of TiO<sub>2</sub>NP due to the persistent nature and low mobility (Tourinho et al., 2012) might cause a chronic effect on the bacterial composition, which would inhibit the susceptible taxa and in turn their associated soil biogeochemical processes, warranting consideration of a cascading effect on soil ecosystems.

Overall, addressing the research question that how soil bacterial respond to TiO<sub>2</sub>NP at a community-level, the impact of different concentrations of TiO<sub>2</sub>NP on the soil bacterial community composition was observed to be time-dependent. Within a complex community, the highly diverse bacterial species with distinct tolerance might respond differently to stressors at different time scales. The compensatory effect as well as competition between sensitive and resilient taxa potentially play roles in shaping bacterial communities at different time scale (Clements and Rohr, 2009; Loreau and de Mazancourt, 2013). Moreover, TiO<sub>2</sub>NP can undergo complex physicochemical transformations along with time (e.g. homo- and hetero-aggregation, interaction with organic matter etc.) in soil, which could also influence the bioavailability of TiO<sub>2</sub>NP (Tourinho et al., 2012). The dose-dependency effect of TiO<sub>2</sub>NP observed on single bacterial cultures thus might not well characterize for community-level response (Simonin et al., 2017). Longitudinal samplings are suggested to be taken into consideration, to better capture the time-dependent response of soil bacterial communities to TiO<sub>2</sub>NP.

#### 4.2. Bacterial community functional redundancy compensates genus specific response

In addition to the response of specific bacterial populations to different TiO<sub>2</sub>NP treatments, the effect on functional capabilities of bacterial communities was further investigated based on metabolic capacity measurement and metagenome prediction. We observed significant changes in microbial biomass along with exposure time and exposure concentrations (Fig. 2). However, these fluctuation in biomass was not



**Fig. 5.** Principal coordinates analysis (PCoA) of the bacterial community in soil samples exposed to different  $\text{TiO}_2\text{NP}$  concentrations based on functional composition. (A) Incubation for 1 day. (B) Incubation for 15 days. (C) Incubation for 60 days. The percentages in parentheses indicate the proportion of variation explained by each ordination axis. CK: Control soil without  $\text{TiO}_2\text{NP}$ .  $n = 3$ .

reflected in community functioning (i.e. dehydrogenase activity, Fig. S2). Within a diverse and complicated natural microbial community, although bacterial biomass was lost, the highly diverse species performing similar metabolic capacity within the community could compensate for the partially missing activity performed by the suppressed taxa, reflecting no significant change in overall community metabolic functioning (Yin et al., 2000).

Based on the predicted metagenome, although the taxonomic composition on day 1 and 60 was significantly altered by different treatments of  $\text{TiO}_2\text{NP}$ , no significant change in the community functional profile over time was found following exposure to  $\text{TiO}_2\text{NP}$  (Fig. 5). We further investigated the specific response of each KEGG function to  $\text{TiO}_2\text{NP}$ . Although no significant dissimilarity of the bacterial communities was found among the different treatments (Fig. 5), we did observe functional disruptions on the 1-day samples (Fig. 6), and the exposure to  $\text{TiO}_2\text{NP}$  induced both stimulation and suppression of specific functions. The promoted KEGG functions were often compensated by the suppression of reduced KEGG functions, which could explain why we observed no significant change in the overall community functional profile in any treatment. As exposure time went by, there was no significantly changed KEGG function in the 15-day and 60-day samples at any exposure concentrations compared to the control. This indicates that the effects of  $\text{TiO}_2\text{NP}$  on soil bacterial functional composition are short-lived, and the bacterial functioning recovered and turned to be robust as the incubation time increased. The early divergent behaviour and convergence after 60-day exposure suggested that functional redundancy within natural communities can mitigate the impacts of  $\text{TiO}_2\text{NP}$  on microbial communities (Moore et al., 2016).

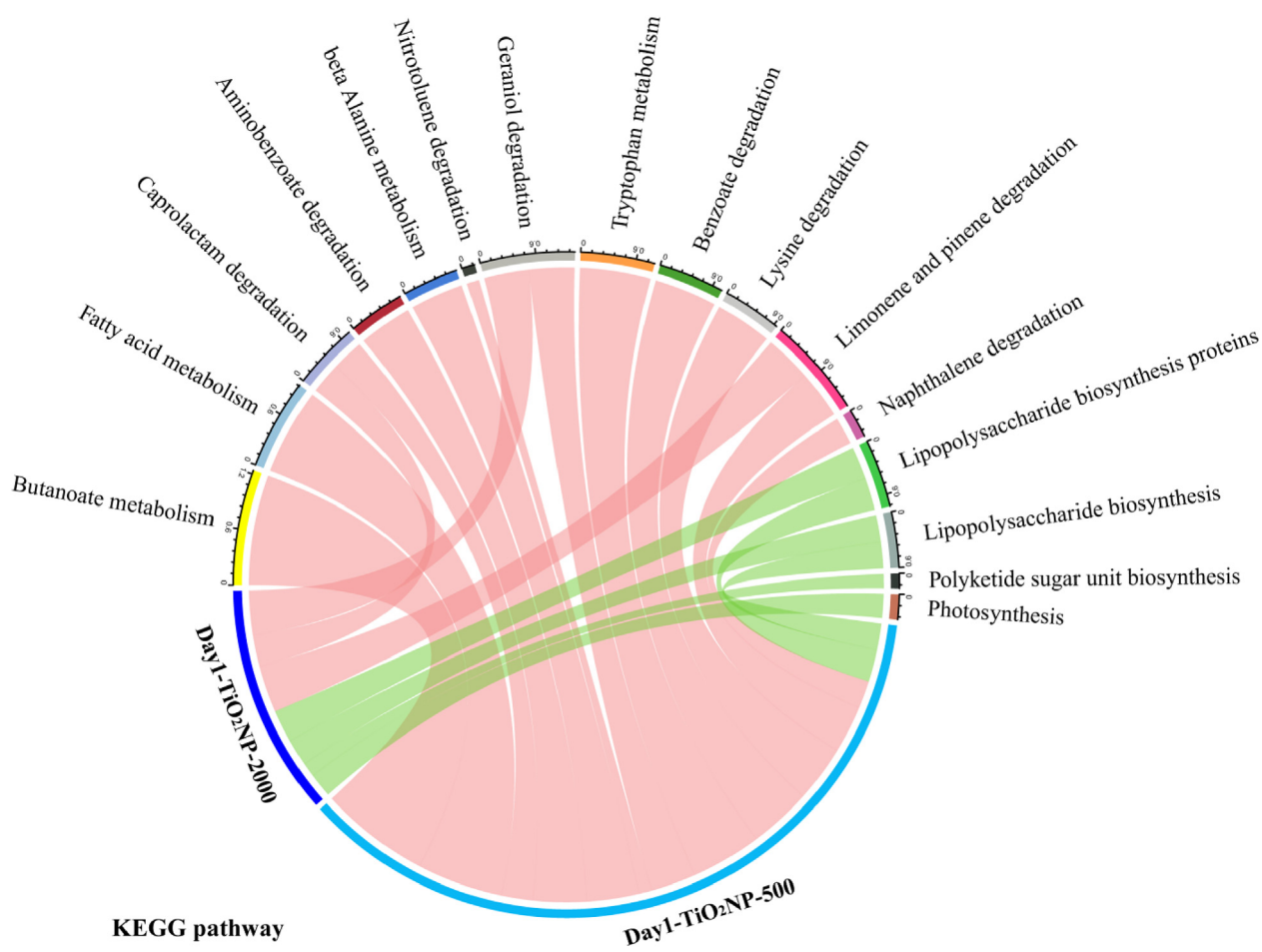
It is more often seen that bacterial communities in complicated ecosystems are functionally redundant (Briones and Raskin, 2003; Sheng et al., 2015), whereby the highly diverse species performing similar functions within the soil bacterial community could compensate the partial disruption of soil processes regulated by certain taxa (Strickland et al., 2009). Recently a few studies have proposed that functional redundancy plays an important role in bacterial communities in stream water and sediments exposed to silver and copper nanoparticles (Colman et al., 2012; Moore et al., 2016; Sheng et al., 2015). In a diverse bacterial community, different taxa carrying the same functional gene can perform similar functions, and the loss of

suppressed taxa is compensated for by the promotion of others (Rosenfeld, 2002). Although our results showed that  $\text{TiO}_2\text{NP}$  induced a shift in taxonomic composition, the functionally redundant community might recover to the original community process rates after long-term incubation (Allison and Martiny, 2008), reflecting no significant effect of  $\text{TiO}_2\text{NP}$  on the community functional profile. Overall, addressing the research question on the functional response of soil bacterial community to  $\text{TiO}_2\text{NPs}$  exposure, our results indicated that compositional alterations occur but they do not yet necessarily reflect a biological significant impact on the overall functional profile of the soil bacterial community. However, it should be noted that although the functional redundancy likely contributed to the  $\text{TiO}_2\text{NPs}$  tolerance of soil bacterial community, the significant alteration in the taxonomic composition may potentially reduce the stability of the community which would become more vulnerable to the next disturbance (Sheng et al., 2015). Although not accounting for natural agricultural systems, our results hint that  $\text{TiO}_2\text{NPs}$  exposure can disturb the compositional and functional profiles of soil microbial communities, warranting consideration of the  $\text{TiO}_2\text{NPs}$  applications. The plant-soil systems therefore needs to be included in future studies for better understanding of the impacts of  $\text{TiO}_2\text{NPs}$  on the rhizosphere community as well as plants performances. Given that more often  $\text{TiO}_2\text{NPs}$  are released into soil through biosolids or directly used as additives in fertilizers repetitively (Simonin et al., 2016a), the accumulated effect of multiple exposure on soil bacterial community due to repeated discharge or applications should be evaluated in the future.

## 5. Conclusions

Overall, This study investigated the effect of different  $\text{TiO}_2\text{NP}$  concentrations at different time-points considering the taxonomic composition and catabolic potential of a soil bacterial community. Our results indicated that the effect of  $\text{TiO}_2\text{NP}$  on soil bacterial taxonomic composition depended on exposure concentration and incubation time. Low-concentration inhibition and high-concentration stimulation of the taxonomic composition were observed directly following exposure. The relative abundances of core bacterial taxa were further observed to vary over time, with no obvious shifts after 15 days of exposure, and an apparent decrease with increasing exposure concentration after 60 days





KEGG pathway

Level 1	Level 2	Level 3
Metabolism	Metabolism of Terpenoids and Polyketides	Limonene and pinene degradation Geraniol degradation Polyketide sugar unit biosynthesis
	Energy Metabolism	Photosynthesis
	Amino Acid Metabolism	Lysine degradation Tryptophan metabolism beta-Alanine metabolism
	Lipid Metabolism	Fatty acid metabolism
	Carbohydrate Metabolism	Butanoate metabolism
	Glycan Biosynthesis and Metabolism	Lipopolysaccharide biosynthesis Lipopolysaccharide biosynthesis proteins
	Xenobiotics Biodegradation and Metabolism	Benzoate degradation Nitrotoluene degradation Naphthalene degradation Aminobenzoate degradation Caprolactam degradation

Fig. 6. Distribution of the significantly changed KEGG functions related to metabolism in response to TiO<sub>2</sub>NP treatments. Nodes represent the KEGG functions and treatments. The size of each arc connection represents the relative abundance of each function. Red connections are up-regulated functions, green connections are down-regulated functions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of exposure. However, although significant shifts in bacterial community composition in response to TiO<sub>2</sub>NP were observed over time, there was no significant change in the community functional profile as well as in enzyme activity after long-term exposure. The observed genus specific response induced by TiO<sub>2</sub>NP exposure not yet necessarily produced functional significance. This indicates functional redundancy likely contributed to the TiO<sub>2</sub>NP tolerance of the bacterial community. These results thus pointed out the need to take community compositional and functional responses into consideration when assessing the

impact of nanoparticle on soil microbial activities and soil ecosystem functioning.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2019.108713>.

## Data deposition

All DNA sequences were submitted to the NCBI database as a bio-project. The accession number is PRJNA491925.

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