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Compositional and predicted functional dynamics of soil bacterial community in response to single pulse and repeated dosing of titanium dioxide nanoparticles

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2NP) are often released into the soil through repeated discharge of wastewater and repetitive applications as fertilizers. Adverse effects of a single pulse on soil bacterial communities have been widely studied, while the impact of repeated exposure is poorly understood. This study compared the impacts of single and repeated exposure scenarios on the soil bacterial community. The repeated exposure promoted the total bacterial biomass but reduced the community diversity and induced larger alterations in community composition compared to the single exposure. Regarding the dosing frequencies of repeated exposure, community divergence increased in initial dosing cycles, and community stability was re-established and remained in subsequent dosing cycles. According to the different tolerance to dosing frequencies, the dynamic response patterns of the featured OTUs and functional genes could be classified into four types: 1) promotion, 2) suppression-recovery-promotion, 3) promotion-suppression-stable, and 4) suppression. These results suggest that chronic exposure with repetitive low-dosing of nanoparticles induced a tendency towards larger alteration of both community composition and functioning than in case of application of a single pulse of the same dosage. This study brings new insight into understanding the compositional and predicted functional dynamics of the soil bacterial community in response to nanoparticles and identifies a data gap in realistic time-variable exposure testing.

1. Introduction

Titanium-dioxide nanoparticles (TiO_2NPs) are used within a broad range of commercial and industrial applications and are consequently entering the environment in high quantities (Brunet et al., 2009). One area of specific concern is the accumulation of TiO_2NPs in agricultural soil through agricultural amendments and additives to improve soil fertility and quality (Keller et al., 2013; Sun et al., 2014).

Adverse impacts of TiO_2NPs on soil microbial activities and associated ecosystem functioning (McKee and Filser, 2016; Simonin and Richaume, 2015) have been detected by inhibiting soil respiration (Ge et al., 2011; Simonin et al., 2015), nitrification (Simonin et al., 2017), denitrification (Simonin et al., 2016b). Up until now, most studies on the impacts of TiO_2NPs on soil microbial communities were conducted using a one-time dosing (single exposure) of TiO_2NPs. Considering that TiO_2NPs were often released into the environment through the repetitive applications of biosolids, irrigation, or through the use of additives on agricultural soils (Liu and Lal, 2015; Simonin et al., 2016a), a more environmentally relevant scenario is the repeated exposure of TiO_2NPs on soil microbial community. It is likely that fluctuations of

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NPs exposure dynamics could change their impact on soil microorganisms (Zhai et al., 2017). Therefore, it is critical to investigate the soil bacterial community in response to repeated TiO_2NPs exposure as compared to a single exposure pulse.

When the bacterial community received repetitive dosing of stressors, the selection pressure from the previous exposure might produce promoted, supressed, or independent impact depending on the community tolerance (Reinert et al., 2002). Previous research using single bacterial culture has shown that bacteria could be more adaptive to stressors after repeated exposure as compared to single dosing (Thomas et al., 2000). For example, a tolerant bacterium (*Rhodanobacter* sp.) emerged in soils exposed to silver nanoparticles (Samarajeewa et al., 2019), and a persistent phenotype of *Pseudomonas putida* F1 with a more rigid membrane and higher tolerance was detected after several re-dosing cycle of nanoscale zerovalent iron (Kotchaplai et al., 2017). In contrast, for ammonia-oxidizing bacteria, the repetitive dosing of TiO_2NPs was found to be more harmful than one-time exposure (Simonin et al., 2016a). It might also be possible that bacteria stressed by the previous exposures had less energy to cope with additional disturbances (Griffiths and Philippot, 2013). More severe responses induced by repeated dosing were also found in the medical application, where repeated administration with a high dose rate of TiO_2NP induced significantly greater inflammation compared to one-time injection (Baisch et al., 2014). However, the resistance and resilience of natural microbial communities in response to the repeated exposure of TiO_2NPs remains unknown.

Within our study we aimed to: 1) compare the bacterial community responses – both compositional and functional – to TiO_2NPs applied as single pulse versus repeated exposure; and 2) investigate the dynamic changes of bacterial communities along with dosing frequencies. To this end, TiO_2NPs were chosen as representative, non-soluble nanoparticles, which were applied into soil microcosms in two different scenarios, namely, a one-time pulse dosing compared to a four-times repetitive dosing that have the same final concentration. The response of the soil bacterial community to single and repeated TiO_2NPs exposure were compared, and the dynamic changes in the compositional, structural and functional profiles of the soil microbial community after each new dosing cycle were also monitored.

2. Methods and materials

2.1. Soil, nanoparticles, and experimental design

Forest soil (top 15 cm) was collected from a non-polluted site dominated by deciduous trees (52°07′06.7"N 5°11′23.1″E, The Netherlands) as described previously (Zhai et al., 2016). The soil has been free of anthropogenic pollution such as wastewater discharge, agrochemical application, waste pollution, or nanoparticles addition. This makes the soil suited for use in this study. The soil was sieved (2 mm) and homogenized, and then stored at 4 °C with soil moisture at 18.4% of the dry soil weight (Zhai et al., 2017). Spherical TiO_2NPs (mixture of anatase (80%) and rutile (20%) crystal structure, 99.5% purity) with diameter of 25 nm and surface area of 35–65 m^2/g were purchased from Sigma-Aldrich (St. Louis, MO, USA). The soil physicochemical properties were reported previously (Zhai et al., 2016, 2017). The soil used had a loamy texture, soil pH was 6.2 and the dissolved organic matter concentration in the pore water was 400 mg/L. The Ti concentration in the control soils was 109.8 ± 12.3 mg/kg soil. TiO_2NP stock dispersions (5 and 20 mg/mL) were produced according to the Risk Assessment of Engineered Nanoparticles protocol by sonicating the suspensions at 4 °C at 38 ± 10 KHz for 16 min (Kermanizadeh et al., 2012).

The soil was pre-incubated at 20 °C for one week before the experiment. The soil was exposed to TiO_2NPs using either a single dose or four repetitive doses and placed in microcosms in 200 mL sterile plastic bottles (61 g soil with 50 g dry weight equivalent for each microcosm). The concentration of TiO_2NPs in soil that may come from agrochemicals was predicted to be 1 mg/kg TiO_2NPs (Sun et al., 2014). Our previous work investigated the impact of TiO_2NP concentration (ranging from environmental concentration at 1 mg/kg, medium concentration at 500 mg/kg, to seriously contaminated concentration at 2000 mg/kg) on the microbial community over a 60-day exposure, and we found that the high treatment could alter the soil bacterial community composition after 60 days of exposure, while no significant effect was found in the medium treatment for either 15- nor 60-day of exposure (Zhai et al., 2019). This study is a further step based on the previous results, and is designed to investigate the dynamic changes of microbial community along with dosing frequencies. Therefore, in this study, we exposed the soil microcosms for 60 days, and based on the long-term effect observed in the medium and high treatments, we chose the 2000 mg/kg TiO_2NP as single exposure, and 500 mg/kg TiO_2NP for each application for the repeated exposure. Based on this knowledge, we build up the following experimental design: 1) For the single exposure experiment, a TiO_2NP suspension was mixed into the soil microcosms once to reach an exposure concentration of 2000 mg TiO_2NP/kg dry soil and incubated for 60 days. 2) For the repeated exposure, TiO_2NP was mixed into the soil microcosms with four repetitive dosing cycle of 500 mg TiO_2NP/kg dry soil for each dosing separated by a 15-day interval. 3) Microcosms without application of TiO_2NP were used as controls. Each treatment was conducted in triplicate. During the dosing frequencies in the process of the repeated exposure, the same amount of sterilized water was added in the control and the single exposure. The TiO_2NP suspensions were applied to each microcosm drop by drop using a pipet, to achieve the target exposure doses (Ge et al., 2011), and thoroughly mixed manually for 5 min to homogeneously distribute the TiO_2NP within the microcosms (Sillen et al., 2015). Soil microcosms were incubated for 60 days in the dark at 20 °C, with soil moisture maintained at 24% by replenishing the water lost with sterile water every two weeks. At the end of the incubation (day 60), soil samples were collected for each treatment for further analysis. In addition, to study the dynamics of soil bacterial community along with dosing frequencies, sub-samples were also collected before each new dosing cycle of the repeated exposure (day 15, 30 and 45). Control samples were randomly collected on day 0, 15 and 60. This experimental design resulted in a total of 24 microcosms with three replicates per exposure dose and sampling time.

2.2. DNA extraction

Soil DNA was extracted from 0.25 g of each soil sample using the Qiagen DNeasy PowerSoil Kit (Hilden, Germany) following the manufacturer's instructions. DNA concentrations of each soil sample were estimated using ND-1000 Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The detailed information on the DNA samples is listed in Table S1. This is a pre-quality control step, the downstream sequencing further confirmed that the DNA samples passed the quality control checks.

2.3. Quantification of total bacterial biomass

After DNA extraction, the total bacterial abundance was measured using the QX200™ Droplet Digital™ PCR System (ddPCR, Bio-Rad Supermix, Bio-Rad, Hercules, CA, USA). The ddPCR reaction mixture contained: 11 µl Evagreen (Bio-Rad, Hercules, CA, USA), 1 µl forward primer (10uM stock) (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′), 1 µl reverse primer (10 µM stock), (909R: 5′-CCCGTCAATTCMTTTRAGT-3′), 2 µl template DNA (×10,000 diluted), 7 µl Milli-Q per sample. Droplets were generated using the QX-100 droplet generator (Bio-Rad), and loaded onto a 96-well PCR plate, heat-sealed with a foil seal and placed in the GeneAmp 9700 thermocycler (Life Technologies, Inc. Gaithersburg, MD, USA). Cycling consisted of 95 °C for 5 min, followed by 40 cycle of 95 °C for 30 s and 61 °C for 1 min, then 1 cycle of 5 min at

4 °C, 95 °C for 5 min with a 4 °C hold. After reaction, the droplets were read using QX-200 Droplet Reader (Bio-Rad) and QuantaSoft™ Software (Life Technologies).

2.4. High-throughput sequencing

The DNA samples were amplified with a primer set (515F: 5′-GTG CCAGCMGCCGCGGTAA-3′ and 909R: 5′-CCCGTCAATTCMTTTR-AGT-3′), targeting the V4-V5 hypervariable regions of both the Bacteria and Archaea domains. The paired-end sequencing of the amplicons (2 × 300 bp) were processed using 2 × 300 bp Illumina Miseq platform (Illumina, Inc., San Diego, CA, USA) by BaseClear (Leiden, the Netherlands). The sequences have been deposited in the NCBI database with project number: PRJNA517300, 491,925, and the sample origin of each sequencing library is provided in Table S2. Quantitative Insights Into Microbial Ecology (QIIME version 1.8.0) pipeline (Caporaso et al., 2010) was used to analyze the 16S rRNA gene amplicon sequences. After trimming, screening and filtering, the qualified sequences were clustered into Operational Taxonomic Units (OTUs) at a 97% sequence similarity level. De novo OTU clustering method was performed in QIIME by using the pick_de_novo_otus.py. Chimeric sequences and singletons were removed, chloroplasts, mitochondria, archaea and eukaryotes were filtered. Rarefaction was performed to remove sampling depth heterogeneity (Fig. S1 and Table S3). Taxonomy of each representative sequence was assigned using the uclust consensus taxonomy classifier. The OTU representative sequences and phylogeny inference were aligned with Python Nearest Alignment Space Termination (PyNAST) (Caporaso et al., 2009). To predict functional responses of the soil bacterial communities to different treatments, the 16S rRNA gene amplicon data sets were further normalized by dividing each OTU by the known/predicted 16S copy number abundance, and translated into predicted metagenomes to predict the functional capabilities of bacterial communities using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) (Langille et al., 2013). The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to annotate the predicted metagenomes (Ballou et al., 2016). Although there are limitations of PICRUSt due to the insufficient coverage of unknown microbial genomes in the Greengenes reference (Tong et al., 2014), the application of PICRUSt to diverse metagenomic data sets from humans, soils, mammalian and microbial mat communities shows that the phylogenetic information contained in the 16S marker gene sequences is sufficiently well correlated with the genomic content to yield accurate predictions when related reference genomes are available (Langille et al., 2013). The nearest sequenced taxon index (NSTI) was calculated to quantify the availability of nearby genome representatives for each microbiome sample, and the NSTI index for the tested samples were presented in the following Table S4, which should allow for accurate metagenome prediction (Langille et al., 2013).

2.5. Enzyme activity

Dehydrogenase activity was measured to further evaluate the effect of TiO_2NPs treatments on the metabolic state of soil microorganisms. Soil samples were analyzed by means of the 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) assay (Von Mersi and Schinner, 1991). In brief, 1 g soil for each soil sample was mixed with Tris Buffer (Tris(hydroxymethyl)aminomethane, 1 M, pH = 7.0, Sigma-Aldrich) and substrate solution (2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (iodonitrotetrazolium chloride (INT), 10 mM, Sigma-Aldrich). After incubation for 2 h at 40 °C in the dark, the developed iodonitrotetrazolium formazan (INTF) was extracted using the extraction solution *N*,*N*-dimethylformamide/ethanol in a 1:1 ratio. After 30 min of extraction, INTF was measured by using spectrophotometry at 464 nm (UV-1800, Shimadzu, Kyoto, Japan).

2.6. Statistical analysis

To test whether bacterial communities in the single or repeated exposure differed from the control, as well as the dynamic change of the communities along with dosing frequencies, the community dissimilarities were illustrated using principal coordinate analysis (PCoA) based on the weighted UniFrac distance matrices (Liu et al., 2014). The significance of dissimilarity was further tested by QIIME (beta_significance.py). OTUs with presence in 100% of the triplicate samples as well as relative abundance above 0.1% were selected as dominant OTUs. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to determine the abundant featured OTUs that most likely explain the differences between single or repeated TiO_2NP treatment and control samples using R v3.4.4. The cut-off logarithmic LDA score was set to 2.0 (Xu et al., 2017). LDA value of each significantly changed OTU in different treatment is given in Fig. S2 and S3. The relative abundance of these significantly changed OTUs in different treatment compare with the control is given in Fig. S4. The significantly changed OTUs that were specifically induced by the repeated exposure were defined as the featured OTUs. The evolutionary relationship of the featured OTUs were illustrated by a phylogenetic tree using MEGA 7.0 based on the Neighbor-Joining method (Fig. S5). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Kumar et al., 2016). After metagenomes prediction, the category of metabolism in KEGG pathways was selected for further analysis. LEfSe was applied to determine the featured functions among treatments, and the significantly changed functions that were specifically induced by the repeated exposure were defined as the featured KEGG functions. PERMANOVA was conducted to test the significance of both compositional and functional dissimilarities between samples using QIIME (compare_categories.py) and Rv3.4.4 (vegan package). A canonical correspondence analysis (CCA) was conducted to study the correlation between the featured OTUs and featured functions. The fitness curves of the dynamic changes of the featured OTUs along with dosing cycles are modelled using either exponential or polynomial equation based on the trend in OTU relative abundance. The parameters of fitness curves are presented in Table S9, and the grouping of the featured OTUs into Type I-IV is based on the rate constants in the fitness modelling (supplementary data). One-way analysis of variance (ANOVA) was performed to test whether there is a difference in either enzyme activity or total bacterial abundance or alpha diversity among different exposure scenarios (control, single and repeated exposure), respectively. We also did one-way ANOVA again to analyze the differences in either enzyme activity or total bacterial abundance or alpha diversity among exposure cycles within the repeated exposure scenarios, respectively. A Tukey post-hoc test was performed to test the significance between each treatment where the global effect was significant ($p < .05$). ANOVA and Tukey post-hoc tests were performed using IBM SPSS Statistics 23.

3. Results

3.1. Single versus repeated exposure responses on soil bacterial community

3.1.1. Enzyme activity and total bacterial biomass

The total soil bacterial biomass (quantified by 16S rRNA gene copy number × 10^7/g soil) decreased significantly from 93.1 ± 11.3 to 59.6 ± 3.3 in the single exposure. On the contrary, the repeated exposure significantly increased total soil bacteria from 93.1 ± 11.3 to 118.8 ± 9.5 (Table 1). However, as revealed by dehydrogenase activity (Table 1), no significant change in the enzyme activity was observed in the soil bacterial community treated by single or repeated exposure.

3.1.2. Bacterial community diversity and composition

In this study, the OTUs ID were assigned before the filtration, and in

Table 1
The changes in enzyme activity and bacterial abundance in different exposure scenarios.

Comparison	Treatment	Enzyme activity (mg INTF/g dry soil/2 h)*	Total bacterial abundance (16S gene copy number × 10^7/g soil)*
Single versus repeated exposure	Control	47.7 ± 4.5[a]	93.1 ± 11.3[a]
	Single exposure	51.2 ± 2.3[a]	59.6 ± 3.3[b]
	Repeated exposure	55.6 ± 4.0[a]	118.8 ± 9.5[c]
Effect of dosing cycle	Initial state	48.5 ± 8.2[a]	88.3 ± 13.0[a]
	1st control	50.1 ± 4.9[a]	98.9 ± 4.1[a]
	1st redosing cycle	65.8 ± 12.3[a]	105.8 ± 5.3[ab]
	2nd redosing cycle	58.9 ± 4.4[a]	112.0 ± 3.9[b]
	3rd redosing cycle	62.6 ± 9.0[a]	110.6 ± 4.2[b]
	4th control	47.7 ± 4.5[a]	93.1 ± 11.3[a]
	4th redosing cycle	55.6 ± 4.0[a]	118.8 ± 9.5[b]

* Different letters represent a statistically significant difference ($p < .05$). The data presented are the mean values of triplicates ± the standard deviation, $n = 3$.

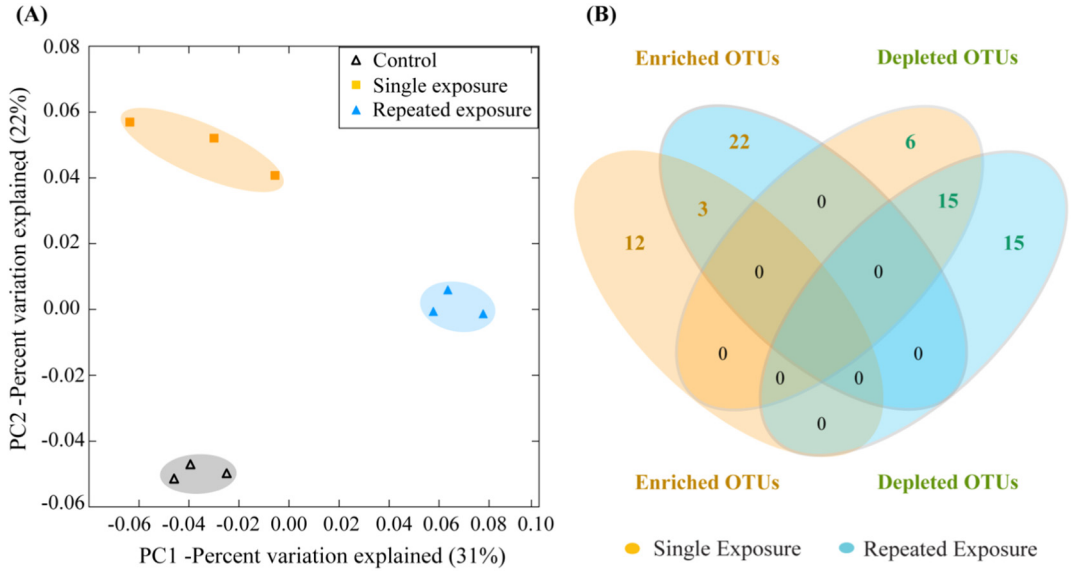

Fig. 1. Comparison of the impact of either single or repeated exposure to TiO_2NP on the taxonomic composition of the soil bacterial community. The soil exposed to a single addition of TiO_2NP is shown in yellow, whereas the repeatedly exposed soil is shown in blue. All measurements were taken after 60 days of incubation. (A) Principle coordinate analysis (PCoA) of the bacterial community structure based on the taxonomic composition. The percentages in parentheses indicate the proportion of variation explained by each ordination axis. n = 3. (B) The shared and unique dominant OTUs that were significantly increased or reduced compared with the control, due to single and repeated exposure to TiO_2NP.

total 186,130 OTUs were observed (summed up for all microcosms samples). After quality check, the summary of the observed OTUs and sequences of the bacterial communities in each sample are listed in Table S3. Statistics of the significant test on community alpha diversity index in response to TiO_2NP treatments are given in Table S5. The single exposure had minor impacts on diversity, while repeated exposure significantly reduced all diversity-parameters, including the number of shannon index, chao1 index and phylogenetic diversity. The community composition at the phylum level (with relative abundance above 0.1%) in different treatments is given in Fig. S6. The bacterial communities of the control, single and repeated exposure treatments were dominated by Proteobacteria (30.1–34.8%), Actinobacteria (12.9–16.3%), and Acidobacteria (11.7–12.9%). The relative abundance of OTUs was further compared to identify the changes in the bacterial community induced by the single and repeated exposure (Fig. 1 and Table S6). As shown in Fig. 1(A) and Table S6, the samples in the control, single and repeated exposure were significantly separated into three clusters, suggesting that both single and repeated exposure induced significant shifts in soil bacterial community. The shared and unique dominant OTUs that were significantly different in the single or repeated exposure compared with control are given in Fig. 1(B). Both single and repeated exposure altered the taxonomic composition of the bacterial community compared with control. Moreover, the single exposure induced 36 significantly changed OTUs (15 OTUs were significantly increased in relative abundance compared with the control, and 21 OTUs were significantly decreased). However, the repeated exposure induced more significantly changed OTUs than the single exposure (in total 55 OTUs, 25 were significantly increased in relative abundance compared with the control, and 30 OTUs were significantly decreased), which indicated a higher impact of repeated exposure on the taxonomic composition of the bacterial community.

3.1.3. *Community functional profile*

The effects of single and repeated TiO_2NP exposure on potential functioning of the soil bacterial community were further predicted based on the 16S rRNA gene sequences (Fig. 2). The community comparison among the different treatments (control, single and repeated exposure) based on the functional composition is given in Fig. 2A and Table S8. Although not statistically significant, all the samples separated into three groups based on the treatments. The effects of single and repeated exposure on specific functions of the soil bacterial community were further investigated based on the category of metabolism in the KEGG pathway at the level 3. Nine significantly changed KEGG functions (featured functions) were identified in the repeated exposure compared with the control (5 enriched and 4 depleted featured functions). However, no significantly changed function was observed in the

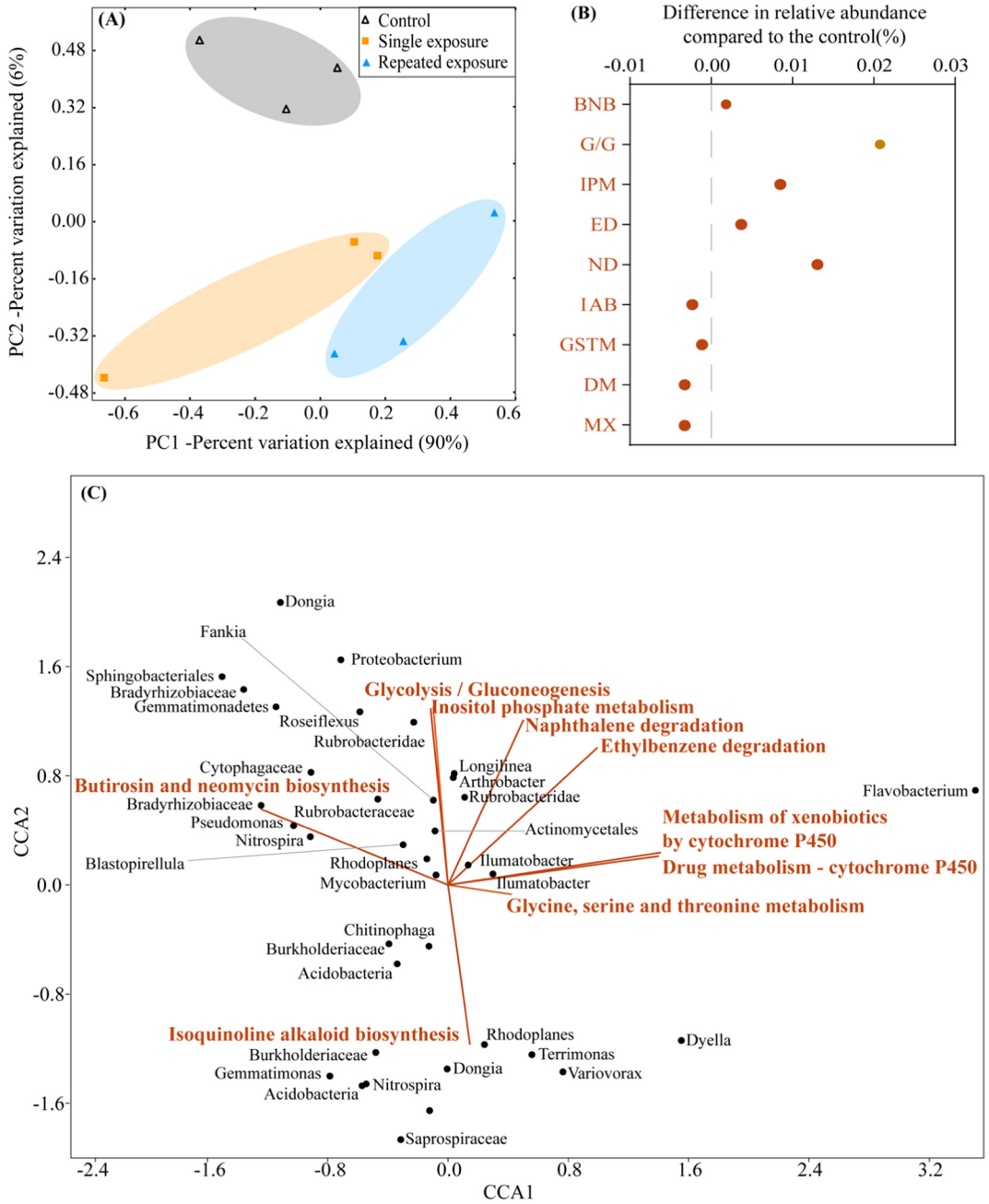

Fig. 2. Comparison of the impact of single or repeated exposure to TiO_2NP on the functional profile of the soil bacterial community. (A) Principle coordinate analysis (PCoA) of the bacterial community structure based on functional composition. (B) Difference in average relative abundance (ARA, %) of significantly altered KEGG functions compared with the control. BNB-Butirosin and neomycin biosynthesis, G/G-Glycolysis/Gluconeogenesis, IPM- Inositol phosphate metabolism, ED-Ethylbenzene degradation, ND-Naphthalene degradation, IAB-Isoquinoline alkaloid biosynthesis, GSTM-Glycine, serine and threonine metabolism, DM-Drug metabolism-cytochrome P450, MS-Metabolism of xenobiotics by cytochrome P450. (C) Canonical Correlation Analysis (CCA) showing the correlation of core OTUs and core functions based on Pearson correlation coefficient.

single exposure (Fig. 2B). The relationships between the featured OTUs and featured functions were further investigated, and the results are given in Fig. 2C. There were strong associations between microbial clade and predicted functions: 1) *Bradyrhizobiaceae*, *Pseudomonas*, *Nitrospira* and butirosin, neomycin biosynthesis; 2) *Rubrobacteridae*, *Frankia*, *Actinomycetales* and glycolysis/gluconeogenesis, inositol phosphate metabolism; 3) *Flavobacterium* and drug metabolism-cytochrome P450, metabolism of xenobiotics by cytochrome P450; 4) *Rhodoplanes* and isoquinoline alkaloid biosynthesis.

3.2. Effect of dosing cycles in process of repeated exposure on soil bacterial community

3.2.1. Enzyme activity and total bacterial biomass

All the four dosing cycles stimulated the enzyme activity of the soil bacterial community although the stimulation was not statistically significant (Table 1). The total bacterial biomass increased from 88.3 ± 3.0 to 105.8 ± 5.3 (expressed as 16S rRNA gene copy number × 10^7/g soil) after the 1st dosing cycle, and significantly increased to 112.0 ± 3.9 by the next cycle and remained stable after the 3rd and 4th dosing cycles, suggesting that the significant effect of repeated exposure predominantly occurred in the initial dosing cycles

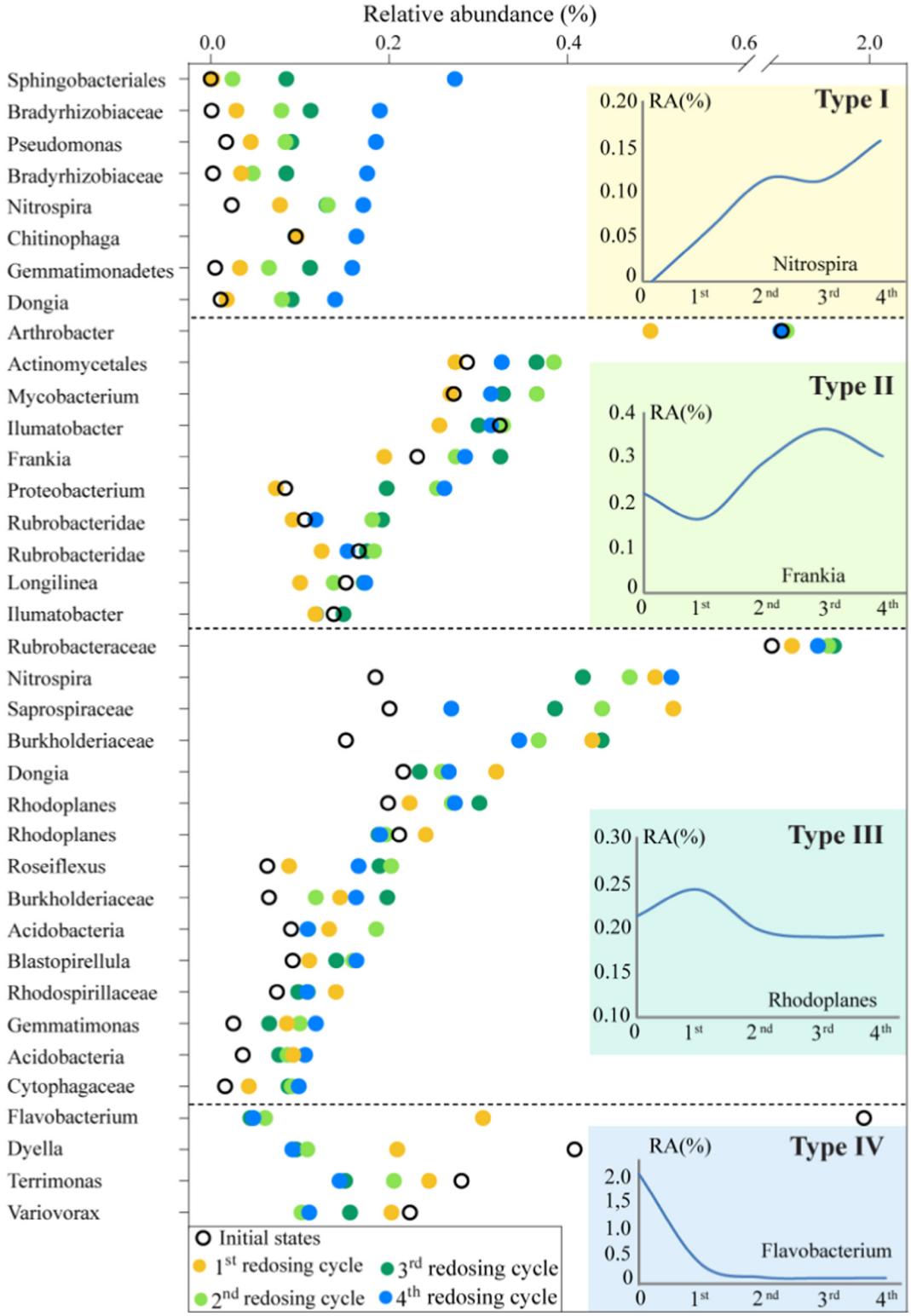

Fig. 3. Changes in the relative abundance of the selected core OTU along with dosing cycles in repeated exposure of TiO_2NP. For each OTU, the relative abundance (x-axis) is changing along with the dosing frequencies, the yellow colors represent the relative abundance of each OTU after the 1st dosing cycle, light green colors represent the relative abundance of each OTU after the 2nd dosing cycle, dark green represents the relative abundance of each OTU after the 3rd dosing cycle, and blue represents the relative abundance of each OTU after the 4th dosing cycle. All relative abundances in the 4 dosing cycles for each OTU are presented in the same x-axis.

(Table 1).

3.2.2. Community diversity and composition

The compositional dynamics of the soil bacterial community along with dosing frequencies were investigated based on the taxonomic composition (Fig. S7A and Table S7). The control groups from the 1st sampling to the 4th sampling cycles were not changed significantly. The samples in the 1st TiO_2NP dosing cycle were clustered with the

controls, while the bacterial communities in the 2–4 dosing cycles clustered together and significantly separated from the controls. This indicated that the impact of TiO_2NP on the soil bacterial community increased with dosing cycles, and the bacterial communities tended to be more stable in the later dosing cycles.

To further investigate the effect of dosing cycles on the bacterial community, the dynamic change of the featured OTUs were further analyzed (Fig. 3). The dynamics of the featured OTUs along with the dosing frequencies could be classified into four types. The fitness curves as well as the parameters of the fitness for the dynamic change of the featured OTUs along with dosing frequencies are given in Fig. S8 and Table S10. Type I represents a successive improvement of the bacterial tolerance, where the relative abundance of 8 featured OTUs increased successively with increasing dosing cycle. In addition, there were 25 featured OTUs changed in fluctuation, with 10 OTUs suppressed in the 1st or 2nd dosing cycle and then promoted in the later dosing cycle (Type II), as well as 15 OTUs enriched by the 1st or 2nd dosing cycle, but suppressed in the next dosing cycle and maintained stable in the later dosing cycle (Type III). Moreover, the relative abundance of 4 OTUs were observed to decrease along with increasing dosing cycle and remained stable after the 3rd dosing cycle (Type IV).

3.2.3. Community functional profile

To understand the functional dynamics of the soil bacterial community in process of repeated exposure, soil bacterial communities along with dosing frequencies were further analyzed based on the predicted functional profile (Fig. S7B and Table S9). Although none of the dissimilarities among the soil bacterial communities were statistically significant, samples in the 2–4 dosing cycles clustered together and separated from samples in the control and the 1st dosing cycle.

The effect of dosing cycles on each featured KEGG function were further analyzed (Fig. 4). The dynamics of the featured functions along with dosing frequencies could also be classified into four types. The relative abundance of butirosin and neomycin biosynthesis increased with increasing dosing frequencies and remained stable after the 2nd dosing cycle (Type I). In addition, the relative abundance of glycolysis/gluconeogenesis, inositol phosphate metabolism, ethylbenzene degradation, and naphthalene degradation were reduced after the 1st dosing cycle but significantly promoted after the 2nd dosing cycle and remained stable afterwards (Type II). The relative abundance of isoquinoline alkaloid biosynthesis was enriched by the 1st dosing cycle, then significantly declined after the 2nd dosing cycle and remained stable (Type III). The relative abundance of glycine, serine and threonine metabolism, drug metabolism-cytochrome P450 and metabolism of xenobiotics by cytochrome P450 were found to be suppressed after the 1st dosing cycle and remained stable in the later dosing cycle (Type IV).

4. Discussion

4.1. Consequences of single and repeated exposure on soil bacterial community

After two-month incubation with TiO_2NP, significant changes on total bacterial biomass were observed (quantified by 16S rRNA gene copy number), while the changes of bacterial activity were not statistically significant (measured by dehydrogenase activity). The changes of total bacterial biomass were significantly different when applying a single pulse versus a repeated exposure. This was seen in the total number of 16S rRNA gene copies that were significantly reduced by the single exposure but promoted by the repeated exposure. TiO_2NP is known to induce membrane damage and cytotoxicity by producing reactive oxygen species, which could have negative effect on bacterial growth even in the absence of light (Brunet et al., 2009; Heinlaan et al., 2008; Sohm et al., 2015). Regarding the alpha diversity of bacterial community (Table S5), our results suggested a change in evenness with a lot of taxa being lost due to repeated exposures that are replaced by a few taxa with a high relative abundance and biomass. Combing the results of bacterial biomass and community diversity, it is conceivable that the repeated exposure acted as selection pressure by inhibiting the susceptible bacteria and promoting the survived tolerant ones. The enrichment of tolerant bacteria surpassed the loss of susceptible ones, which in the end was observed as the increase of total bacteria (Ren et al., 2015).

Both single and repeated exposure significantly impacted the bacterial community composition (Fig. 1A), whereas the repeated exposure induced more changes on the dominant OTUs than the single exposure (55 OTUs vs. 36 OTUs, Fig. 1B). The larger alteration in the community composition caused by repeated exposure compared to single exposure suggests that the low stability of the bacterial community after an additional exposure could be ascribed to the decreasing diversity caused by the previous exposures (Griffiths and Philippot, 2013). The observed community diversity reduction and community composition alteration in the soil bacterial community caused by repeated exposure are expected to induce larger community divergences, as directly confirmed by the beta diversity analysis on the bacterial community similarity.

Generally speaking, no significant changes were observed regarding the bacterial community functional composition in neither single nor repeated exposure (Fig. 2A). The non-significant changes of enzyme activity among different treatments further confirmed the insignificant impact of TiO_2NPs on soil bacterial community functioning (Table 1). When focusing on the specific functional response at KEGG pathway level 3, significant changes were observed on 9 KEGG functions in the soil samples subjected to repeated exposure, whereas no significant changes were found after single exposure (Fig. 2B). This finding indicated that the repeated exposure also induced larger alterations on the specific functional response than the single exposure. It is reasonable to suppose that the suppression in susceptible bacterial populations would result in a decline of the associated functional properties, while the relative success of the resistant bacteria would promote the functions they perform (Ren et al., 2015. This hypothesis is confirmed by the obtained CCA results which clearly illustrated that the changes in the featured functions were closely related with the corresponding changes of the featured OTUs. For instance, change regarding the function of xenobiotics degradation was found to be related to the suppression of *Flavobacterium*, which is characterized as oxidative detoxification of hydrophobic xenobiotics including pollutants, drugs and pesticides (Ning and Wang, 2012; Xun and Orser, 1991). Another example is the observed correlation between carbohydrate metabolism (the functions of glycolysis/gluconeogenesis and inositol phosphate metabolism) and *Actinomycetales* (Fig. 2C), which plays an important role in organic matter cycling by degradation of high molecular weight compounds like hydrocarbons (Bhatti et al., 2017).

4.2. Dynamics in soil bacterial community during the repeated exposure

Compared to the single exposure, the TiO_2NP repeated exposure induced more significant impacts on the biomass, diversity, composition and function of the soil bacterial community. Beyond the comparison, it is important to further investigate the dynamic changes of the bacterial community in process of the repeated exposure. Both the bacterial quantity (16S rRNA gene copies) and community (diversity and composition) were significantly separated from the control and stabilized in 2–4 dosing cycles (Table 1 and Fig. S7). This indicated that the bacterial community shift along with dosing cycles could be classified into two stages: bacterial selection (predominated occurred in the 2nd dosing cycle), and bacterial community stabilization (during 2–4 dosing cycles). In other words, the tolerance of the bacterial community to TiO_2NP was likely developed after the 2nd dosing cycle, and subsequent dosages posed no more pressure to the stability of the newly established bacterial community. It is suggested that within a complex bacterial community, the selection of resistance within a given selective

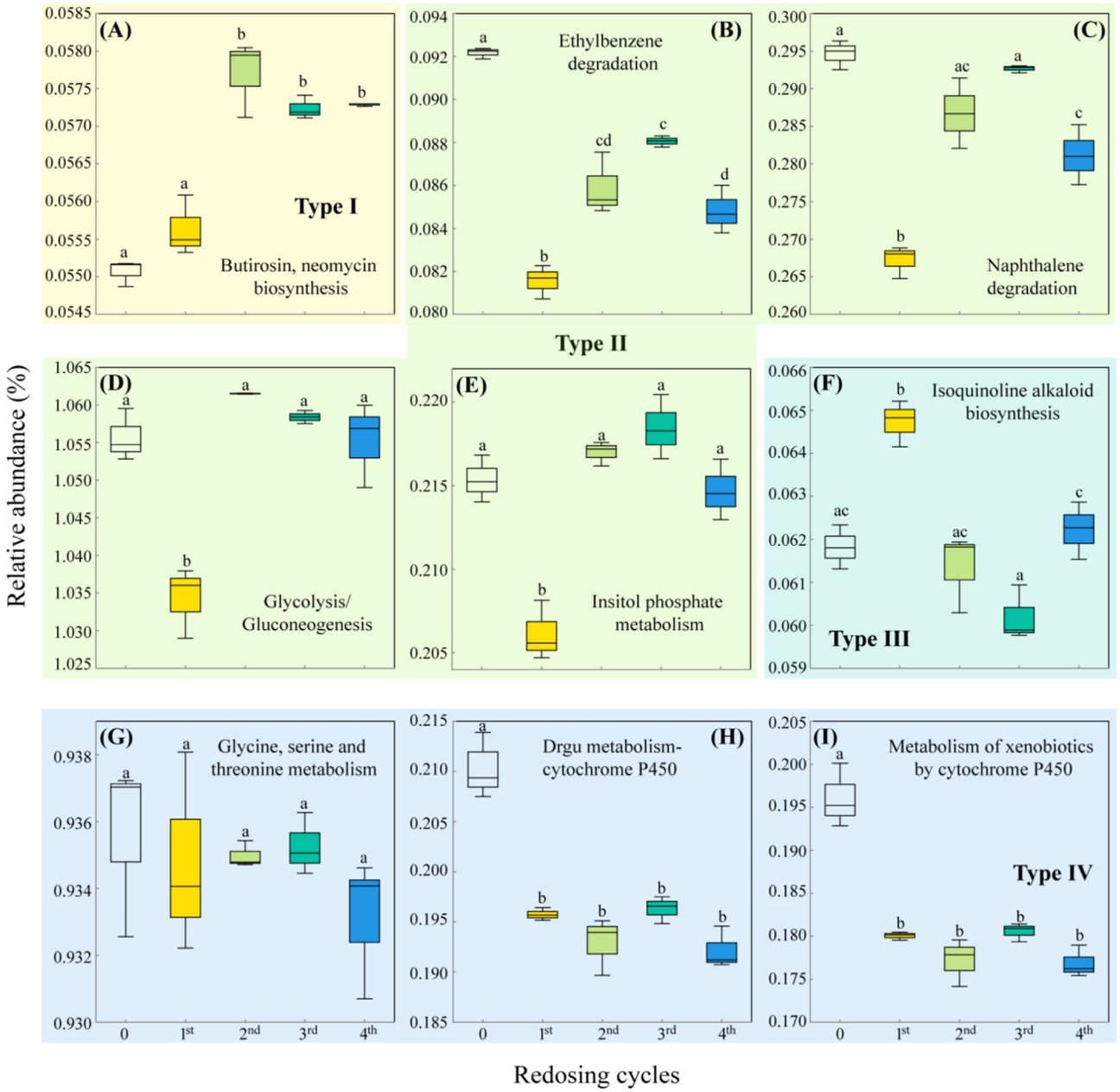

Fig. 4. Changes in the relative abundance of each core KEGG function along with dosing cycles in repeated exposure of TiO_2NP. Different letters indicated a statistically significant difference ($p < .05$, One-way ANOVA with Tukey test).

space could remain constant due to the cross-protection by the resistant bacteria of the susceptible ones (Dugatkin et al., 2003; Murray et al., 2018). This indicated that the TiO_2NP selection of resistant bacteria is degradative to favor the maintenance of a relatively stable community in the later dosing cycle. Since community instability is important for microbial ecology and the associated functions (Knight et al., 2018), the significant community shift along with increasing dosing cycles leads to potentially affected functioning. The disruption of the bacterial community in the early application stage as well as the tolerance of the newly-established community in later stage thus are suggested to be taken into account when assessing ecotoxicological impact of TiO_2NP contained agrochemicals under realistic scenarios.

When focusing on the bacterial community composition at the genus level, the dynamic responses of featured OTUs and functional genes to the repeated exposure along with dosing frequencies can be classified into four types: i.e. promotion (Type I), suppression-recovery-promotion (Type II), promotion-suppression-stabilization (Type III) and suppression (Type IV) (Figs. 3 and 4). Type I represents OTUs with enhanced adaptation over TiO_2NP dosing frequencies that showed a successive increase in relative abundance, which is similar with the reported response of *Pseudomonas putida* F1 to repeated exposure of nanoscale zerovalent iron (nZVI) (Kotchaplai et al., 2017). In return, the established persistent phenotypes (through acclimation, detoxification enzymes, and horizontal transfer of resistant genes) will lead to the promotion of the resistant taxa and the associated functions (Ernst et al., 2016). Type II is similar to the trend observed for *Mycobacterium* response to repeated treatment of atrazine (Fang et al., 2015), the relative abundance of which decreased in the initial dosing cycle, then recovered and was even stimulated in later dosing cycle. For this type of response, the first dosing caused acute toxicity, while the bacteria could recover during the dosing-free period, and the toxic effects of the earlier exposure could be reduced and the tolerance to the next dosing cycle could be improved (Rodriguez-Gil et al., 2017). For Type III, the tolerance of the bacteria was promoted in the initial exposure

stage at a lower amount of TiO_2NP, while the accumulated effect in the later dosing cycle limited the promotion of these taxa and the associated functions (Simonin and Richaume, 2015). Type IV experienced continuous stress from the previous dosing and consumed more energy to detoxify and to repair the caused damage, leaving less energy to cope with further disturbances (Griffiths and Philippot, 2013). As a result, the next dosing cycle would inhibit the bacterial survival as well as the associated functions.

Although the changes found in the enzyme activity and community functional profile along with dosing frequencies were not statistically significant (Table 1 and S6), community diversity and composition changed significantly along with increasing dosing frequencies. Previous studies have found that community functional redundancy played an important role when receiving single NPs exposure (Sheng et al., 2015; Moore et al., 2016). Compared to the single pulse of high dosage, repetitive exposure with low-dosing of TiO_2NP induced a tendency towards larger alteration in both community composition and functioning. The community instability following chronic low-dose exposure thus needs to be taken into consideration when assessing the impact of NPs on soil bacterial communities and the associated ecosystem functioning.

5. Conclusions

This study was designed to examine a scenario that is more likely to occur in real-life settings, due to the nature of for instance repeated exposures through fertilization with biosolids that might contain TiO_2NP. Our findings on the different responses of a soil bacterial community to the single and repeated TiO_2NP exposure reveal that one-time pulse addition with a high dose does not capture the environmentally relevant impact of repetitive dosing on soil microbial activity and ecosystem functioning. In most studies found in literature on nanomaterials, the nanoparticles were added as a single dose and responses were recorded after one-time injection. The assessment of the soil bacterial community dynamics in response to repeated NPs exposure led to new insights regarding time-varying exposure testing of nanoparticles. Moreover, the alterations of the soil bacterial community were found to be mainly driven by the initial two redosing cycles, indicating that the dosing allocation of NPs exposure at each time was more critical in influencing the soil bacterial community than the total NPs load over a period. When dosage is at the level that no direct mortality of the microbial community occurs, the soil bacterial community might alter towards tolerance after the first few dosing cycles of NPs. The dynamic patterns of the featured taxa could help understanding bacterial evaluation and adaptation in the process of repeated NPs exposure. The follow up scientific question is then: what is the biologically significant impact of the altered microbial community? To answer this question, further studies are needed for elucidating the critical NPs load for each dosing cycle, and the length of the dosing-free period for “recovering”, as well as for “training” of the in-situ soil bacteria towards community resistance and resilience in receiving NPs disturbances.

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Data deposition

All DNA sequences were submitted to the NCBI database with accession numbers of PRJNA517300, 491925.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2019.100187.

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