Time-Dependent Effect of Graphene on the Microbial Activity of the Soil Under Single and Repeated Exposures

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ABSTRACT

Graphene (GR) has huge industrial and biomedical potential, and its adverse effect on soil microorganisms has been evaluated in ecotoxicological studies. These studies focused on single exposure to GR, but repeated exposures with low concentration are more likely to occur under actual exposure scenario. In this study, we compared the impact of single and repeated exposures of GR on structure, abundance and function of soil bacterial community based on soil enzyme activity and high-throughput sequencing. The results displayed that after 4 days of incubation the activities of urease and fluorescein diacetate esterase increased by approximately 12% and 4% upon repeated exposure to GR (biweekly 15 mg doses GR for the two-exposure experiment, 10 mg doses GR for the three-exposure experiment, resulting in the same final concentration of 300 mg/kg), respectively. Instead, the activities of urease and fluorescein diacetate esterase decreased by 13% and 6% upon single exposure (30 mg for the single-exposure experiment), respectively. As the incubation time increased to 60 days, these activities showed little difference. The alpha diversity of soil bacterial community under repeated exposures increased more than that under single exposure, demonstrating that a low concentration chronic exposure to GR increase the diversity within a specific bacterial community. The PCoA and sample level clustering tree showed single exposure to GR after 4 days alter the soil bacterial community to some extent. During the entire incubation process, no matter what kind of exposure scenarios to GR, the majority of bacterial phylotypes at the phylum level had no great change except for Proteobacteria and Actinobacteria according to the relative abundance of phylotypes. These results elucidated the repeated exposures to GR increased the metabolic activity and diversity of the soil microbial community as compared with single exposure. This study can provide a new perspective on the impact of carbon nanomaterials on soil microbial community.

1. Introduction

Graphene (GR) is an atomically thin, tough two-dimensional, carbon-based material consisting of sp² hybridized carbon atoms (Rao et al. 2010). It is widely studied because of unique physicochemical properties that make them rapidly expanding in agricultural, engineering and environmental applications such as filtration membranes and adsorbent materials for contaminant, novel catalyst systems for degrading pollutants and optical devices and biosensors with different transduction modes for detecting hazardous analytes.
(Luigi et al. 2023). By 2023, it is estimated that the worldwide market volume of graphene composites will reach 1521 tons (Mendona et al. 2019). With the increment of production and utilization, GR will inevitably be discharged into the environment during the course of production and application, exerting potential environmental effect on ecosystems, such as nutrients losses and the transformation of other pollutes. And due to the weak migration ability, aerial deposition and irrigation, the soil system has been considered as the main and ultimate recipient for GR. The released GR could be accumulated in soil and impact the microorganism in soil ecosystem. Probabilistic material-flow modeling estimates that carbon nanomaterials (for example, carbon nanotubes, graphene and fullerenes) enter into soil with release rate of 0.004–1.6 µg/kg annually (Holden et al. 2014). Thus, graphene is nowadays considered as emerging contaminants in soil along with the concomitant need to understand its potential ecological effects.

Soil microbial communities are an indispensable part in nutrient cycling of soil system, the remediation of contaminated soil and especially sensitive indicator of soil’s response to the environmental changes (Liu et al. 2015). Therefore, some studies were conducted to study the effect of GR on soil microbial communities. Ren et al. (2015) observed that graphene (10, 100 or 1000 mg/kg) had a significant influence on the number of micro-organisms and community structure in soil, and these effects are clearly related to the contact time with graphene and microorganisms. Especially, when the concentration of GR in the soil was up to 1000 mg/kg, the bacteria population which was involved in nitrogen biogeochemical cycles and degradation of organic compounds were significantly reduced. And some studies stated that low concentration of graphene oxide (100 µg/g) could promote the growth of microorganism, which might due to the increase of the cell attachment and proliferation (Zhao et al. 2020). Furthermore, Wu reported that compared to short-term exposure (30d), long-term exposure (360d) to 300 µg/g GR, SW and fullerene(C60) more strongly altered the beta diversity of soil bacterial communities (Wu et al. 2021). Further, GR relative to SW and C60 exposure more significantly altered soil bacterial community composition. In brief, we can clearly notice that the effect’s degree of GR on soil microbial communities have certain relationship with the concentration of GR and the length of exposure time.

In these studies, GR was exposed to soil in a single homogeneous and acute exposure method. This exposure model may lead to have more resistant microbial communities to GR perturbation if sensitive communities are replaced by resistant communities (Simonin et al. 2016). However, given the practical environmental contamination, GR is more likely to be released into the soil chronically through repeated application. Zhai et al. (2019) have already suggested that repeated exposures of titanium dioxide nanoparticles altered the microbial community more than a single exposure (Zhai et al. 2019). Nevertheless, there are too few studies of investigating the effect of repeated exposure of GR on soil microbial communities. Therefore, a comparative study between single and repeated exposures to GR on soil bacterial communities not only can provide a reference for future studies that which exposure scenario should be paid more intensive attention but also offer broadly insights into microbial responses to GR disturbances.

As a first attempt to address this gap, the differences in the structure, abundance and function of soil microbial community between single and repeated exposures to GR were investigated through microcosm experiments. The changes in the bacterial abundance and the structure of the soil bacterial community were determined by high-throughput
sequencing of 16S rRNA genes. And the effect of graphene on microbial function was evaluated by soil enzyme activities, which are considered as sensitive indicators of changes in the microbial community under nanomaterials stress. This study is expected to provide new insights into the effect of GR on soil microbial community under different exposure scenarios.

2. Materials and methods

2.1. Graphene

Graphene (500–1000 m²/g specific surface area, 0.5–3.74 nm thickness), with purity>95% wt., was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. The other reagents were obtained from Tian-jin Reagent Factory. All reagents of analytical grade were used without prior purification. The solutions used in this work were prepared with deionized distilled water.

2.2. Soil sample collection and physicochemical characterization

Soil sampling was performed on October, 2021. Surface soil sample was collected from cropland at Dongyang Agroecological Experimental station of Shanxi Academy of Agricultural Sciences (112°40’E, 37°32’N). In the sample area, soil type is cinnamon soil. The crop on the experiment plot is sorghum and there is no crop rotation. Five soil samples gathered from the top 5–10 cm were mixed to form one composite sample. The sample was gathered with wooden spades, sealed in polyethylene bags in the field, and then brought back to the laboratory. Large root fragments of sorghum and particles were removed, and the soil sample was sieved to 2 mm. A small fraction of soil was air-dried for measuring physical and chemical properties, and the other fraction was stored in a fridge at 4°C for microbial experiments.

Soil pH value was determined with pH-meter (FE20-pH, Mettler Toledo, Switzerland). The pH value detection was determined by dipping the electrode in the supernatant solution which is prepared from 10.0 g soil and 25.0 mL distilled water. Organic matter (OM) was detected by titrating the sample in an acidic medium, with the end point followed by a redox reaction. Soil moisture was detected by drying for 8 h at 105°C. The total nitrogen was analyzed by element analyzer (VARIO EL3, Germany). The available phosphorus was detected by Infinite 200 Pro microplate reader (Tecan, Männedorf, Switzerland). The available potassium was detected by flame photometry (novAA800, Germany). The characteristics of the soil were as shown: pH, 8.42; organic matter, 6.92 g/kg; soil moisture 13.6%; total nitrogen, 0.81 g/kg; available phosphorus, 4.43 mg/kg; available potassium, 131.52 mg/kg.

2.3. Exposure experiment

The soil used for microbial experiments was pre-cultured at 25°C for three days to restore soil bacterial activity. A total of 4 sets of experiments including the control experiment, single-GR-exposure experiment, two-GR-exposure experiment and three-GR-exposure experiment, which were abbreviated C, CR1, GR2 and GR3 were set up under the same
conditions to explore the microbial responses of soil microbial community under different exposure scenarios. Specifically, 100 g soil samples were added to each 500 ml sterilized triangular flasks and specific amount of GR was added to the soil: 30 mg for the single-exposure experiment, 15 mg for the two-exposures experiment, 10 mg for the three-exposures experiment, resulting in the same final concentration of 300 mg/kg (Table 1) which was selected based on the model estimation and previous studies on exposure experiments of GR in soil (Gottschalk, Sun, and Nowack 2013). Soil without GR added served as the control. Sterilized water was added weekly to adjust the soil moisture to 13.6%. The successive addition of GR was separated by a 15-day delay, during which the soil samples were incubated at 25°C in the dark. A total of 12 sets of subsamples were collected from C, GR1, GR2 and GR3 on 4th, 21st and 60th days after the last addition of GR to soil. These subsamples were defined as C1, C2, C3, GR11, GR12, GR13, GR21, GR22, GR23, GR31, GR32 and GR33, respectively. All treatments were performed with three replicates.

### 2.4. Soil enzyme activities

The enzyme activities of urease and FDA esterase for all soil subsamples were determined according to the previous methods (Liu et al. 2015). The enzyme assays were carried out in triplicate for each treatment.

### 2.5. DNA extraction and high-throughput sequencing

For all the cultured soil samples, 0.2 g soil was used to extract the total genomic DNA with a E.Z.N.A. Soil DNA Kit (Omega, USA). The concentration and quality of the extracted DNA were measured using a Qubit 3.0 (life, USA) to ensure that adequate amounts of high-quality genomic DNA had been extracted.

The V3-V4 region of the bacterial 16S rRNA genes was amplified using KAPA HiFi Hot Start Ready Mix (2×) (TaKaRa Bio Inc., Japan). The universal bacterial 16S rRNA gene amplicon PCR primers 341F/805 R (CCTACGGGNGGCWGCAG, GACTACHVGGGTATCTAATCC) was applied to amplify the V3-V4 region of bacterial 16S rRNA genes. The reaction was set up as follows: microbial DNA (10 ng/μL) 2 μL; amplicon PCR forward primer (10 μM) 1 μL; amplicon PCR reverse primer (10 μM) 1 μL; 2× KAPA HiFi Hot Start Ready Mix 15 μL (total 30 μL). The plate was sealed and PCR performed in a thermal instrument (Applied Biosystems 9700, USA) using the following program: 1 cycle of denaturing at 95°C for 3 min, first 5 cycles of denaturing at 95°C for 30 s, annealing at 45°C for 30 s, elongation at 72°C for 30 s, then 20 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and a final extension at 72°C for 5 min. The PCR products were checked using electrophoresis in 1% (w/v) agarose gels in TBE buffer (Tris, boric acid, EDTA) stained with ethidium bromide (EB) and visualized

![Table 1. The experiment layout.](image-url)
under UV light. The AMPure XP beads were used to purify the free primers and primer dimer species in the amplicon product. Samples were delivered to Sangon BioTech (Shanghai) for library construction using universal Illumina adaptor and index. Before sequencing, the DNA concentration of each PCR product was determined using a Qubit® 2.0 Green double-stranded DNA assay and it was quality controlled using a bioanalyzer (Agilent 2100, USA). Depending on coverage needs, all libraries can be pooled for one run. The amplicons from each reaction mixture were pooled in equimolar ratios based on their concentration. Sequencing was performed using the Illumina MiSeq system (Illumina MiSeq, USA), according to the manufacturer’s instructions.

After sequencing, data were collected as follows: (1) The two short Illumina readings were assembled by PEAR (v0.9.6) software according to the overlap and fastq files were processed to generate individual fasta and qual files, which could then be analyzed by standard methods. (2) Sequences containing ambiguous bases and any longer than 480 base pairs (bp) were dislodged and those with a maximum homopolymer length of 6 bp were allowed. And sequence shorter than 200bp were removed. (3) All identical sequences were merged into one. (4) Sequences were aligned according to a customized reference database. (5) The completeness of the index and the adaptor was checked and removed all of the index and the adaptor sequence. (6) Noise was removed using the Pre.cluster tool. Chimeras were detected by using Chimera UCHIME. All the software was in the mothur package. We submitted the effective sequences of each sample to the RDP Classifier again to identify bacterial sequences. The modified pipeline is described on the mothur website. Finally, all effective bacterial sequences without primers were submitted for downstream analysis.

2.6. Statistical analysis

The Shannon index was used to describe the soil bacterial alpha diversity, because it evaluates both evenness and richness. The Bray-Curtis distance was calculated as a measurement of the dissimilarity of control-to-treatment in bacterial community composition. And the Principal coordinate analysis (PCoA) and sample clustering tree based on Unweighted-Unifrac distance were conducted to visualize the difference between the control and the treatment in microbial community structure. A Venn diagram was constructed to count intuitively the unique and shared number of operational taxonomic units (OTUs) between the control and treated samples. The relative abundance was summarized at phylum level to compare the community structure among the control and the treatments.

3. Results and discussion

3.1. Effect of GR on soil enzyme activities

The soil enzyme activities are considered as excellent indicators of changes of soil microbial activity under GR stress. Figure 1 summarizes the enzyme activity of soil exposure to GR through different exposure scenario. Compared with the control, after 4 days of incubation following the last application of GR, the urease and FDA esterase activities for single exposure were inhibited by 13% and 6% respectively, which is consistent with the studies of some studies (Fu et al. 2023; Ren et al. 2015). Due to the high surface-to-volume and low homeostatic capacity of microorganisms, their activity decreased a little under high concentration of GR
exposure for a short time (Shrestha et al. 2013). However, the activity of urease and FDA esterase for repeat exposures all increased, indicating that a chronic contamination of GR with low concentration have positive effect on soil microbial activity. Some studies found that GR promoted the growth of the bacterial communities at low concentrations by increasing cell attachment and proliferation (Ruiz et al. 2011). And when the incubation time was up to 20 days, the urease and FDA esterase activities for single exposure and repeated exposures both increased by more than 10%. And the enzyme activities under the three exposures increased more than under two exposures. This is probably because the acute and high concentration of GR exposure repressed the growth of some soil microorganisms which produced these enzymes. And the bioavailability of GR was decreased due to the increasing interaction between GR and organic matter in soil, thus no significant effect on the bacterial growth as the incubation time increased. But the chronic and low concentration of GR proliferated to a much greater extent than the bacteria that were inhibited. Previous study indicated that soil functions were associated with the soil microbial community (Griffiths et al. 2004). As the increase of exposure time, the gradual growth of the relevant microorganisms narrowed the gap between the single and repeated exposures after exposure for a longer time. Therefore, for single and repeated exposures, there were little difference for the urease and FDA esterase activities when the incubation time is up to 60 days. A possible explanation is that the soil microbes start to tolerate and adapt the inhibitor after some time which is consistent with the environ-mental change by transformation of metabolism (Simonin and Richaume 2015). In the course of the whole experiment, the change of urease activities under GR stress for different exposure scenario was greater than that of the FDAE activities. Urease is a representative extracellular enzyme and the key source of bacterial nitrogen appears to be more sensitive to pollution than FDAE activities which represent overall microbiological activity of soil.

3.2. Effect of GR on soil bacterial community alpha diversity

Based on high-throughput sequencing, the alpha diversity (Shannon index) of different treated soil is displayed in Figure 2. The alpha diversity at the beginning of exposure to GR through different methods was almost identical because the bacterial community was from the same soil
Figure 2. Temporal trends of alpha diversity of soil bacterial communities exposed to GR under three different exposure scenarios. The bars indicate the standard deviations. (GR1: 1 exposure; GR2: 2 exposures; GR3: 3 exposures).

Although the final concentration of GR was the same, alpha diversity followed an order of GR31>GR21>GR11 after 4 days incubation. This result demonstrated that high concentration of GR exposure for the short term decreased the diversity within a specific bacterial community. Due to the evenness enhancement of the soil bacterial community, soil microbial diversity increased when the applied GR concentration was low. Hence the alpha diversity of repeated exposures was bigger than that of single exposure after 4 days incubation (Wu et al. 2021). When the incubation time reached 21 days, the alpha diversity of repeated exposures reached a maximum, which indicated that the repeated exposures exhibited pronounced increased after a relatively longer time. When the incubation time was prolonged to 60 days, the alpha diversity for single and repeated exposures all decreased. Song et al. (2018) reported that the alpha diversity of soil bacterial under exposure to GR decreased when the incubation time was up to 90 days, regardless of its concentration (0, 100, and 1000 mg/kg). This is probably due to that the relatively tolerant and resistant species became more abundant and occupied the niche of the sensitive species that were eliminated, leading to a lesser diverse (Song et al. 2018). The death of these sensitive species probably because that the sharp edge of GR could puncture the cell membrane, causing intracellular content leakage even bacterial cell death. The oxygen-containing oxygen on GR surface interact with the hydrophilic lipopolysaccharides of the bacterial cells’ outer membrane, thereby posing toxicity effect to bacterial cells. And the alpha diversity of the control soil increased even no extra substances were added, implying that the soil bacterial community tended to be more even over time (Wu et al. 2021). Overall, the results suggested that with the extension of exposure time the repeated exposures have more positive effect on the soil alpha diversity than the single exposure.

3.3. Effect of GR on soil bacterial community beta diversity

In order to compare similarities of soil bacterial community compositions under different exposure method, the Bray-Curtis distance of soil samples was compared relative to the
control. From Figure 3, the Bray-Curtis distance of the single exposure of GR was 0.40 which was nearly twice times compared with that of the repeated exposures after 4 days incubation, indicating single exposure of GR played a stronger effect on soil bacterial communities, while their dissimilarity tended to be smaller gradually with the extension of incubation time. The principal co-ordinates analysis (PCoA) based on Unweighted-UniFrac distance which incorporates phylogenetic distance into relative abundance measurement was also conducted to study the difference of soil bacterial community. The PCoA results showed that, the soil bacterial community after exposure to GR in a short-term time

![Figure 3](image-url)

**Figure 3.** Bray-Curtis distances between each treatment and the control (a) PCoA of Unweighted-UniFrac distance (b) of soil bacterial community exposed to GR under three different exposure scenarios (C: control; GR1: 1 exposure; GR2: 2 exposures; GR3: 3 exposures). The percentage of the total variance explained by each axis is shown.
were totally separated from the control, especially for the soil under single exposure. And the soil bacterial communities changed constantly with the growth of culture time, and the distinction between the single exposure and repeated exposures got smaller. In addition, after a longer period, the control, the repeated GR exposure were clustered with single GR exposure, suggesting similar microbial community structures. And adonis analysis on the bacterial community further confirmed that the difference in soil bacterial communities under different incubation time to GR was significant ($R^2 = 0.573$, $p = .001$).

Different exposure model induced certain shifts in the soil bacterial community composition, some core taxa still remained under all treatments. Via high-throughput sequencing, in Figure 4, the Venn diagrams showed that a total of 2334 OTUs, 2965 OTUs and 2910 OTUs were detected in agricultural soil to all treatments after 4-, 21- and 60-days incubation, respectively. The 11.57%, 5.18%, 12.96% and 13.73% of all OTU observed were specific to control, 1 exposure, 2 exposures and 3 exposures after 4 days incubation. However, as the incubation time lengthened to 60 days, these data were 9.49%, 7.83%, 10.09% and 10.58%, respectively. These results indicated that in the short term, adding a certain amount of graphene to a soil at a time may inhibit the growth of some unique species. Together, both beta diversity and core taxa analysis suggest that, within 4 days exposure, single exposure to GR exerted more significant effects on the bacterial community composition.

### 3.4. Effect of GR on soil bacterial populations at phylum level

To further identify the changes in the bacterial community of soil exposure to GR through different adding method, the sample level clustering tree and the relative abundance (RA) of phylotypes were summarized at the phylum level based on the pyrosequencing data. For the sample level clustering tree, the smaller the difference between the samples, the closer the samples will be to the same branch. According to the Figure 5a, when the incubation time was lower than 21 days, GR1 was on a branch, GR2 and GR3 were on the same branch, indicating that the soil bacterial communities of single exposure were obviously different from that of repeated exposures. And the difference got smaller and smaller with the extending of incubation time. And according to the sample level clustering tree the soil bacterial community of all soil samples including control had already changed when the incubation time reached 60 days. Figure 5b revealed that there was high bacterial diversity in all treated soil and control samples, and 85% of the total bacterial counts could be represented by eight predominant bacterial phyla among

![Figure 4. Venn diagrams of common and unique OTUs in soil bacterial communities under three different exposure scenarios (C: control; GR1: 1exposure; GR2: 2 exposures; GR3: 3 exposures).](image)
which Proteobacteria, Acidobacteria, Planctomycetes, Actinobacteria, and Bacteroidetes are the dominant phyla in agricultural soils (Buckley and Schmidt 2003). The Proteobacteria is the most predominant phylum in the soil bacterial community (33.62%-52.01%), which consists of a majority of Gram-negative bacteria (Mai-Prochnow et al. 2016). And the RA of Proteobacteria in soil under repeated exposures was increased by approximately 3% which is higher that of single exposure after 4 days incubation, the gap had been narrowed with the extension of time. The Gram-negative bacteria have a higher tolerance to external stress than Gram-positive bacteria because of their outer membrane and cell wall (Premanathan et al. 2011). And the phylum Proteobacteria could participate in degradation of carbonaceous compounds (Cebron et al. 2008; Spain, Krumholz, and Elshahed 2009). Hence an increase in RA of Proteobacteria under multiple exposure to GR reflected its high resistance and high carbon mineralization rate in soil. It is obvious that Proteobacteria and Acidobacteria were the primary phylum under a short period (4 and 21 days). The acidobacteria and actinobacteria were slightly reduced under GR exposure compared to control, and the rest of phylum did not change significantly under any of the GR exposure way, except for these three phyla. Normally, soil microbial abundance is priority than microbial structure in indicating the surroundings change. Intriguingly, with the extension of time, up to 60 days, for all the soil sample, not only the Proteobacteria increased, but also the actinobacteria increased for all the soil samples. The phylum Actinobacteria has the potential to degrade lignocellulose; some tentative cellulose degrading enzymes were found in Actinobacteria genomes (Tetrovsky et al. 2014). So, the lignocellulose degradation rate for all soil increased because of the potential to degrade lignocellulose of phylum Actinobacteria. Based on sample level clustering tree and the relative abundance ( RA ) of phylotypes, it could be inferred that GR changed the soil bacterial community to a certain extent and the changes became weaker or disappeared with extending of exposure time no matter what exposure scenarios were.
4. Conclusion

In summary, our work provides useful information for comprehensive understanding the ecotoxicological effect of GR on soil bacterial communities under different exposure scenarios. The activities of urease and fluorescein diacetate esterase demonstrated that the single exposure played a negative effect on the structure, function and diversity of the soil bacterial community, particularly during early stages of incubation. The richness and diversity indices of bacteria community in soil under repeated exposures to GR increased more than under single exposure to GR, implying that different exposure scenarios to GR in soil exhibit diverse effect on soil bacteria community. And the relative abundance of Proteobacteria and actinobacteria could enhance the associated functional processes they mediated. These systematic works implied that the exposure scenarios should be considered when assessing the ecological risks of GR except for the concentration and the incubation time of GR.

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Disclosure statement

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