Environment International 132 (2019) 105116

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Soil biofilm formation enhances microbial community diversity and metabolic activity

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ARTICLE INFO

Keywords:
Soil biofilms
Nutrient availability
Microbial biomass
Microbial community
Heterotrophic respiration
Microbial activity

ABSTRACT

Biofilms have been extensively studied in aquatic and clinical environments. However, the complexity of edaphic microenvironment hinders the advances toward understanding the environmental functionalities and ecological roles of soil biofilms. In this work, artificial soil was employed to investigate the soil biofilm formation and corresponding impacts on community structure and microbial activities. Our results showed that extracellular polymeric substances (EPS) production was significantly enhanced and micro-meter sized cell aggregates formed with high glucose amendment. Biofilm development exhibited significant effects on the soil microbial processes. 16S rRNA gene sequencing demonstrated the soils with biofilms and free-living cells shared similar microbial communities. But the Shannon diversity and evenness indices of communities with soil biofilms were significantly enhanced by 18.2% and 17.1%. The soil with biofilms also revealed a rapid response to nutrient provision and robust microbial activity, which consumed 65.4% more oxygen in the topsoil (0–1.5 mm). Kinetic respiration analysis showed that the enhanced metabolic activity was attributed to 23-times more active microbes in soil biofilms. In summary, this study revealed that soil biofilms can sustain a diverse and robust community to drive soil biogeochemical processes.

1. Introduction

In natural environments, > 99% of bacteria are estimated to reside in the biofilm matrix (Costerton et al., 1987). Soil ecosystem harbors a myriad of microbes. Increasing evidence shows that the sessile lifestyle was also the predominant mode of life for soil microorganisms (Kuzyakov and Blagodatskaya, 2015; Gutiérrez Castorena et al., 2016; Flemming and Wuertz, 2019). Soil biofilms composed of single or multiple species were found to colonize various substrata and interfaces including mineral surfaces, pore spaces and plant roots (Holden, 2001; Gallé et al., 2004; Ramey et al., 2004; Zhang et al., 2014). During biofilm development, extracellular polymeric substances (EPS) are excreted which form the scaffold of biofilm matrix, mediating surface associations and maintaining biofilm cohesiveness (Flemming and Wingender, 2010; Costa et al., 2018).

Similar to biofilms in other environments, the sessile lifestyle also provides a number of competitive advantages over free-living cells, such as enhanced fitness during desiccation and elevated opportunities for horizontal gene transfer (Molin and Tolker-Nielsen, 2003; Moyano et al., 2013; Lennon and Lehmkuhl, 2016). On the other hand, the development of soil biofilms impacts the local soil physicochemical properties. For example, biofilm formation causes bioclogging in the soil pores which influences water permeability (Hand et al., 2008; Pintelon et al., 2012). The biofilm-secreted EPS develops connection among minerals and strengthens the internal cohesion of soil microaggregates (Cosentino et al., 2006; Henao and Mazeau, 2009; Lin et al., 2016). Therefore, soil biofilms increase survival of microorganisms under environmental stress and play a determinant role in shaping soil microenvironments.

Although the protective features of soil biofilms against environmental challenges, like desiccation, grazing and heavy metals are generally recognized, there are still fundamental questions to be answered. For example, the biofilm development processes in soil matrix, the environmental and biological determinants of soil biofilm formation and the influences of soil biofilms on microbial metabolic activity and community structure are largely unknown. Standardized approaches to characterize biofilms and free-living cells in soil matrix were also lacking. One recent study has attempted to separate planktonic and

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https://doi.org/10.1016/j.envint.2019.105116
Received 16 June 2019; Received in revised form 12 August 2019; Accepted 21 August 2019

biofilm cells in natural soils via washing (Bystrianský et al., 2019). The specialization of certain taxa to the planktonic life mode was identified. However, due to the coexistence of biofilm and free-living modes of life in natural soil, it's difficult to distinguish their contributions to microbial metabolism.

In this study, artificial soil was used to investigate the microbial growth and soil biofilm formation. 16S rRNA amplicon sequencing was performed to reveal the changes in the microbial community and major biofilm-forming bacteria. To further evaluate the effects of biofilms on soil biogeochemical processes, microbial metabolic activities of soil with biofilms/free-living cells were compared and the fraction of active microbes was estimated by kinetic modeling. This study provides mechanistic insights into the soil biofilm formation process and the potential ecological roles of soil biofilms, which are critical to understanding soil biogeochemical processes.

2. Materials and methods

2.1. Extraction of soil microorganisms

Surface soil (0–20 cm depth) was collected from paddy fields in Wuxue (Hubei Province, China, 29°51′ N, 115°33′ E) (Table S1). Biofilm-forming microorganisms in the soil were extracted by following the previous approach (Burmølle et al., 2007). One hundred gram of 2-mm sieved soil was mixed with 300 mL deionized water and sterile glass slides were immersed into the soil slurry. After 7-day incubation, biofilms attached on glass slides were washed with phosphate-buffered saline (PBS) thoroughly. The resultant bacterial suspension was pre-cultivated in lysogeny broth (LB) medium to generate sufficient biomass for subsequent experiments.

2.2. Microplate biofilm assay

The 96-well microplate-based biofilm assay is widely applied to investigate the influence of nutrient input on biofilms formation (O'Toole, 2011; Wu et al., 2015). After overnight cultivation in LB medium, the soil bacterial culture was centrifuged and resuspended in a minimal salt medium (OD_{600} = 0.1). The minimal salt medium was prepared as described previously (Macur et al., 2004), which consisted of KH_2PO_4 (1×10^{-5} M), KOH (1.25×10^{-3} M), $MgSO_4$ (1×10^{-3} M), $CaCl_2$ (1×10^{-4} M), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (3×10^{-9} M), H_3BO_3 (4×10^{-7} M), $CoCl_2{\cdot}6H_2O$ (3×10^{-8} M), $CuSO_4{\cdot}5H_2O$ (1×10^{-8} M), $MnCl_2{\cdot}4H_2O$ (8×10^{-8} M), $ZnSO_4{\cdot}7H_2O$ (1×10^{-8} M) and $FeSO_4{\cdot}7H_2O$ (1×10^{-6} M). The pH of the minimal salt medium was adjusted to 7.0. Different amount of glucose and ammonium nitrate were amended as carbon and nitrogen source. One hundred μL of bacterial suspension was transferred to each well of microplate. After 24-h incubation under 28 °C, the planktonic growth was monitored by measuring OD_{600}. The planktonic culture was then removed and the surface-associated biofilm was washed three times using sterile PBS buffer. The biofilm was stained with 1% crystal violet for 15 min. After removal of excess dye, 200 μL of 95% ethanol was added and the amount of biofilm was determined by OD_{590}. Six replicates were carried out for each condition.

2.3. Biofilm formation in artificial soil

As all the soil microorganisms were estimated to exist in biofilms (Flemming and Wuertz, 2019), the artificial soil was used in this study to eliminate the influence of pre-existing soil biofilms and EPS. The artificial soil was composed of 30 wt% quartz sand (70–100 μm), 40 wt % quartz flour (30–40 μm), 20 wt% montmorillonite, 5 wt% goethite and 5 wt% hematite (Pronk et al., 2017). The artificial soil was autoclaved prior to use. To simulate the nutrient composition in the field, the ratio of total organic carbon (TOC) to total nitrogen (TN) in the minimal salt medium remained the same with that under practical condition (TOC/TN = 27.7 mg/L/2.4 mg/L ≈ 12/1). Based on the microplate biofilm assay, 4.62 mmol glucose/kg soil and 1.0 mmol ammonium nitrate/kg soil were chosen as high-nutrient condition to stimulate soil biofilm formation (Fig. S1). Only one-tenth of TOC and TN were supplied under the low-nutrient condition. Soil bacteria were re-suspended in the minimal salt medium to a final OD_{600} of 0.1. One point two mL of the bacterial suspension was mixed with 1.5 g sterilized artificial soil and then cultivated in 24-well microplates under 28 °C.

2.4. Quantification of biomass growth, nutrient consumption and oxygen concentration

The microbial biomass in artificial soil was determined by the fumigation-extraction method (Vance et al., 1987). In brief, microbial cells were lysed by chloroform fumigation. The organic carbon from fumigated and non-fumigated samples was extracted with 0.5 M K_2SO_4, which was then quantified by a TOC analyzer (Multi N/C 2100, Analytik Jena). The difference between organic carbon extracted from fumigated and non-fumigated samples was the K_2SO_4-extractable microbial biomass. The K_2SO_4-extractable biomass carbon was then multiplied by a factor of 2.22 to obtain the total microbial biomass carbon (Wu et al., 1990).

Glucose concentration in soil solution was measured using a glucose assay kit (Biosino Bio-Technology and Science). The artificial soil was extracted with 1 M KCl at liquid/soil ratio of 10:1. After shaking for 1 h, the supernatant was collected and ammonium concentration was determined by continuous flow analysis (Seal Analytica). The oxygen concentration in soil was measured by an oxygen microoptode with a microprofiling setup (Pyroscience).

2.5. Biofilm EPS extraction and quantification

Biofilm EPS was extracted from artificial soil by cation exchange resin (CER) (Frølund et al., 1996; Redmile-Gordon et al., 2014). During growth, the soluble microbial products (SMP) were excreted into the soil water, which were not associated with cells or minerals (Ni et al., 2011). As SMP would induce overestimation of EPS concentration, they were removed prior to EPS extraction. Three gram of soil samples were suspended in 35 mL pre-cooled 0.01 M $CaCl_2$ solution and shaking for 30 min. The supernatant with soluble microbial products was removed after centrifugation at 4 °C and 3200 *g* for 30 min. The remaining soil samples were mixed with 1.2 g CER and 35 mL EPS extraction buffer. The extraction buffer was composed of 2 mM Na_3PO_4, 4 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl. After shaking at 4° for 2 h, the supernatant was collected and filtered through 0.22 μm membrane filters. Protein concentration was quantified by the bicinchoninic acid (BCA) assay. Polysaccharide content was determined via the phenol-sulphuric acid method (Dubois et al., 1956).

2.6. Biofilm imaging by SEM and CLSM

After incubation, the soil samples with biofilms/free-living cells were collected. The freeze-dried soil samples were sputter-coated with gold and examined by scanning electron microscopy (SEM, JSM-6700F, JEOL). To visualize soil biofilm cells under confocal laser scanning microscope (CLSM), the collected soil samples were stained with SYTO 9 (2.5 μM). After 15-min staining, the soil biofilms were imaged via CLSM (FV1000, Olympus).

2.7. 16S rRNA amplicon sequencing

To investigate the changes in microbial communities, artificial soil samples were collected during biofilm development process. Total DNA from the soil samples was extracted using EZNA Soil DNA Kit (Omega). The universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3′) and 806R (5'-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the

V3-V4 region of 16S rRNA gene. A 7-bp barcode sequence was incorporated in the forward primer for multiplexing. The amplification was done using Q5 High-fidelity DNA polymerase (NEB). The PCR conditions were as follows: 2 min initial denaturation at 98 °C; 25 cycles at 98 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s; and a 30-s final extension at 72 °C. The amplicons were purified using AxyPrep DNA Gel Extraction Kit (AxyPrep) and then sequenced on the Illumina Miseq sequencing platform with 250 bp paired-end reads in Personalbio Company (Shanghai). Three biological replicates were performed for each treatment. The raw sequencing data was deposited in the NCBI SRA database with the accession number of PRJNA510169.

2.8. Bioinformatic analysis

The raw sequencing data was filtered using QIIME 1.8 to remove low quality reads (quality < Q20, length < 150 bp) (Caporaso et al., 2010). The paired reads were merged with Flash 1.2.7 with a minimum overlap of 10 bp (Magoč and Salzberg, 2011). After chimera checking using USEARCH 7.0, the assembled sequences were then clustered into operational taxonomic unit (OTU) using UCLUST 5.2.236 with 97% sequence identity (Edgar, 2010). The representative sequence was blasted against Silva database to classify the taxonomic information. The low-abundance OTUs (frequency below 0.001%) were removed (Bokulich et al., 2013). The resultant OTU table was rarefied to the same sequencing depth (23,563 sequences per sample) (Fig. S2). Detrended correspondence analysis (DCA) and principal component analysis (PCA) were used to assess the changes of microbial communities by CANOCO 5.0 (ter Braak and Šmilauer, 2012). As the gradient length of the first axis determined by DCA was lower than 3, the linear method (PCA) was adopted (Ramette, 2007).

2.9. Soil respiration measurements

To compare the microbial metabolic activity of soil with biofilms/free-living cells, substrate-induced respiration was evaluated. Artificial soil with biofilms/free-living cells was developed as mentioned above. After the depletion of glucose in soil solution, 0.1 mL nutrient solution with 15 mg glucose, 2.85 mg $(NH_4)_2SO_4$, 3.375 mg K_2HPO_4, and 5.43 mg $MgSO_4$ was homogeneously spread to each well (Salazar et al., 2018). Upon addition of extra nutrient solution, the soil respiration was monitored using an Automated Soil CO_2 Flux System (LI-COR) every 15 mins for 6 h. Six grams of soil were monitored at one time and three replicates were performed.

The soil respiration rates were calculated as:

$$R_s(t) = \frac{V(C_i - C_{i-1})}{W}$$

where $R_s(t)$ is soil respiration rate at time t (mg C/(g soil * h)), V is the volume of the survey chamber, $C_i - C_{i-1}$ is CO_2 concentration change in 15 min (mg/L), W is the dry mass of the soil ($W = 6$ g) (Salazar et al., 2018).

2.10. Microbial parameter estimation

The substrate-induced respiration was modeled using the following kinetic equation:

$$R_s(t) = R_u + R_c \times \exp(\mu t)$$

where R_u is the respiration rate irrelevant with growth, R_c represents the initial respiration rate coupled with microbial growth, t is the incubation time and μ is the microbial specific growth rate. R_u, R_c and μ were estimated by fitting the predicted data to the experimental respiration rate via nonlinear least-squares regression (Salazar et al., 2018). R^2 was calculated as a measure of goodness of fit (Table S2).

The fraction of active microbial biomass (FAMB) and total microbial biomass (TMB) in the initial soil system can be calculated as:

$$FAMB = \frac{R_c(1-\lambda)}{R_u + R_c(1-\lambda)}$$

$$TMB = \frac{R_c \lambda Y_{CO_2}}{FAMB \times \mu}$$

where λ is the ratio of productive respiration to total respiration which equals to 0.9 (Akimenko et al., 1983), Y_{CO_2} is the biomass yield per unit of CO_2 which taken as 1.5 (Payne, 1970).

Active microbial biomass (AMB) in soil can be estimated as:

$$AMB = TMB \times FAMB$$

2.11. Statistical analysis

All experiments were performed in three biological replicates. The statistical significance of differences was evaluated using Student *t*-test. P value < 0.05 was considered statistically significant.

3. Results

3.1. High nutrient input induced biofilm formation

To assess the influence of nutrient input on biofilm development of soil bacteria, microplate biofilm assays were performed. At the lowest nutrient level (glucose: 0.58 mM, NH_4NO_3: 0.13 mM), the lowest OD_{590}/OD_{600} value of biofilm formation (4.85 ± 1.12, $P < 0.01$) and highest optical density of the bacterial suspension (OD_{600}: 0.11 ± 0.01, $P < 0.01$) indicated that minimal biofilm was developed and most soil bacteria were remained as planktonic form (Fig. S1). Either ten-fold higher glucose (5.77 mM) or ammonium nitrate (1.25 mM) concentration contributed to elevated biofilm formation (OD_{590}/OD_{600}: 10.25 ± 1.62 and 8.36 ± 1.58) and less planktonic cells (OD_{600}: 0.08 ± 0.01 and 0.06 ± 0.01). When both carbon and nitrogen nutrients concentrated 10 times, biofilm production was enhanced by 1.3-fold ($P < 0.01$). This result was consistent with previous findings that sufficient nutrient supply is a prerequisite for the development of soil biofilms (Nunan et al., 2003; Redmile-Gordon et al., 2014; Reischke et al., 2015). In the subsequent experiments, the glucose and ammonium nitrate concentrations for the low-nutrient condition were 0.58 and 0.13 mM respectively, which corresponded to 0.46 mmol glucose/kg soil and 0.1 mmol ammonium nitrate/kg soil. 4.62 mmol glucose/kg soil and 1.0 mmol ammonium nitrate/kg soil were provided for the high-nutrient condition.

3.2. Soil biofilms growth and EPS production in artificial soil

When cultivated in artificial soil, the microbial biomass was increased by 13% in 7 h under the low-nutrient condition ($P < 0.05$), which coupled with the consumption of glucose (Fig. 1A). After 10 h, the biomass carbon decreased slightly from 1700 to 1600 mg/kg soil. Similar microbial growth was observed under the high-nutrient condition. The microbial biomass carbon increased to 1700 mg/kg soil within 10 h ($P < 0.01$) (Fig. 1B). Although > 3.5 mmol glucose/kg soil and 0.7 mmol ammonium nitrate/kg soil remained in the soil solution at 10 h (Fig. 1B), the growth of microbial biomass wasn't sustained and started to decrease gradually. When glucose was depleted, the biomass carbon left in the soil was much less than that under the low-nutrient condition (1260 ± 18 vs. 1586 ± 24 mg/kg, $P < 0.01$). Nevertheless, biofilm EPS content was found to be significantly elevated. After the whole incubation period, the protein and polysaccharide concentrations for the low-nutrient condition were 0.18 ± 0.13 mg/g soil and below detection limit (detection limit 0.005 mg/g soil). Meanwhile, the protein and polysaccharide contents were increased to 1.19 ± 0.30 mg/g soil and 1.55 ± 0.21 mg/g soil under the high-nutrient condition.

After the whole incubation period, soil samples were observed

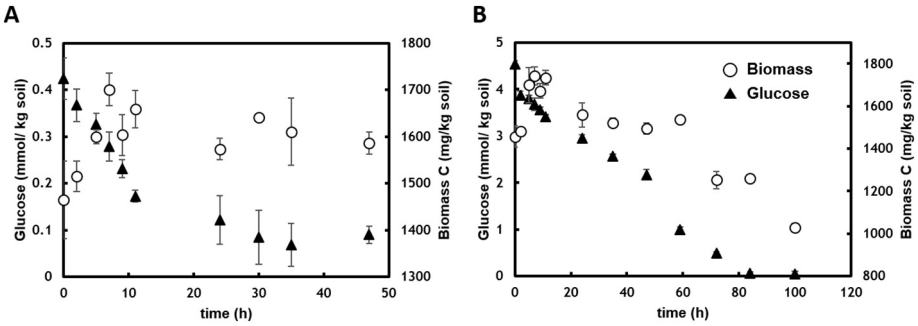

Fig. 1. The microbial growth and glucose consumption in soil under low-nutrient (A) and high-nutrient conditions (B).

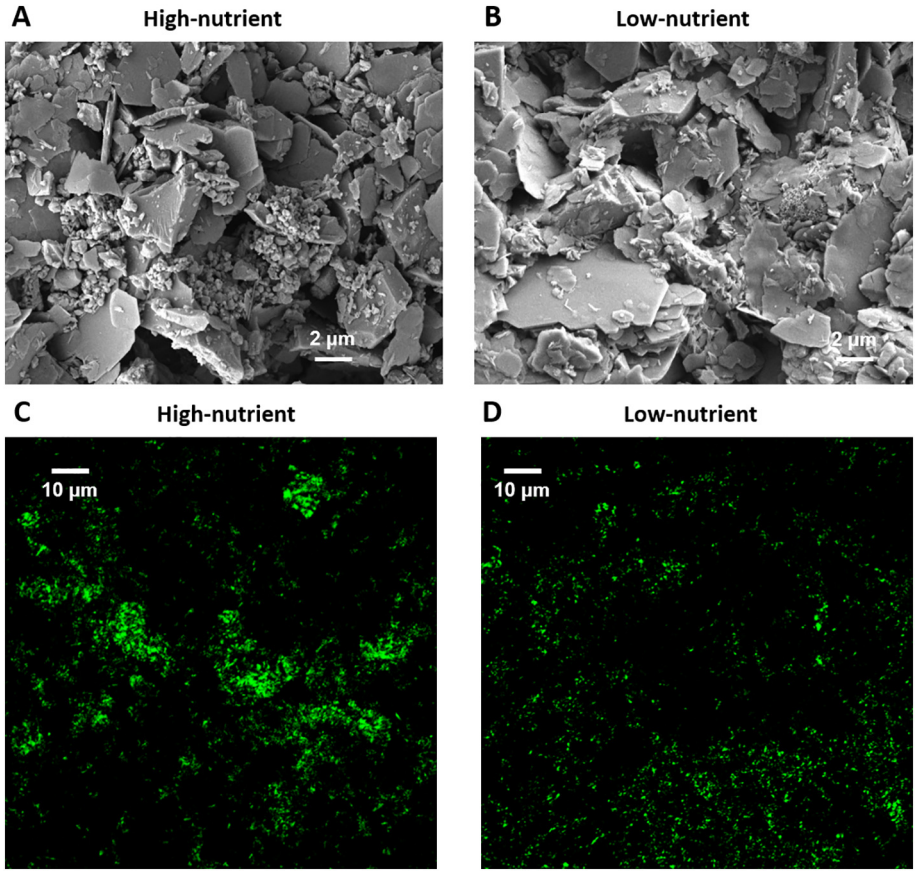

Fig. 2. SEM and Confocal images showing multicellular biofilm developed under high-nutrient condition (A, C), but not under low-nutrient condition (B, D).

under SEM to verify biofilm formation. A number of micro-meter sized aggregates were formed under the high-nutrient condition, which consisted of bacterial cells and mineral particles (Fig. 2A). Meanwhile, no aggregates were observed under the low-nutrient condition (Fig. 2B). The similar phenomenon was observed under CLSM (Fig. 2C, D). It confirmed that high nutrient input induced biofilm formation in artificial soil.

3.3. Soil biofilm formation increased microbial diversity and evenness

In order to characterize the changes in microbial communities during soil biofilm development, 16S rRNA gene sequencing was performed. The diversity decreased under both nutrient conditions after inoculation (Table 1). Although the soil microbial biomass amount reached a peak value at 8 h, the Simpson diversity indices for both nutrient conditions decreased significantly ($P < 0.05$). Under the low-nutrient condition, the evenness and diversity further decreased afterwards. At 45 h, the evenness index decreased by 22.2% compared with initial community ($P < 0.05$), while the Shannon and Simpson indices declined by 34.6% and 26.7%, respectively ($P < 0.05$). Although α-diversity of soil communities under the high-nutrient condition declined during the initial 8 h, the evenness and diversity indices showed negligible difference from 8 to 96 h. When nutrient depleted, the α-diversity and evenness indices of the microbial community with the soil biofilms were much higher than those with free-living cells ($P < 0.05$) (Table 1).

Based on the abundance on class level, *Gammaproteobacteria* and *Bacilli* were dominant which made up about 90% of soil microbial communities (Fig. S3). On the basis of the Bray-Curtis similarity index, the resultant communities of the soil with biofilms/free-living cells shared > 90% similarity with the initial community (Fig. S4). During the incubation period, the relative abundance of *Gammaproteobacteria* under the low-nutrient condition increased from 74.1 ± 2.7 to 82.1 ± 1.0% in 8 h ($P < 0.05$), while *Bacilli* decreased slightly from

Table 1
Richness and diversity of soil microbial communities cultivated under low- and high-nutrient conditions.

Samples	Richness estimators		α-diversity		Evenness
	Ace	Chao	Shannon	Simpson	
0H	343 ± 14 ^{a}	345 ± 24 ^{a}	1.85 ± 0.27 ^{a}	0.45 ± 0.04 ^{a}	0.45 ± 0.05 ^{a}
LOW-8H	334 ± 97 ^{ac}	321 ± 76 ^{ac}	1.58 ± 0.14 ^{ab}	0.35 ± 0.04 ^{b}	0.39 ± 0.03 ^{ab}
LOW-45H	180 ± 25 ^{b}	176 ± 17 ^{b}	1.21 ± 0.02 ^{c}	0.33 ± 0.01 ^{b}	0.35 ± 0.03 ^{b}
HIGH-8H	319 ± 85 ^{ac}	316 ± 86 ^{ac}	1.51 ± 0.10 ^{ab}	0.34 ± 0.01 ^{b}	0.38 ± 0.03 ^{ab}
HIGH-45H	275 ± 114 ^{abc}	281 ± 129 ^{abc}	1.43 ± 0.19 ^{abc}	0.38 ± 0.04 ^{ab}	0.37 ± 0.05 ^{ab}
HIGH-96H	224 ± 47 ^{bc}	213 ± 34 ^{bc}	1.43 ± 0.07 ^{b}	0.42 ± 0.00 ^{a}	0.41 ± 0.03 ^{a}

^{abc}Different superscript letters in the same column indicate significant differences ($P < 0.05$).

16.7 ± 1.4 to 14.6 ± 1.2% ($P < 0.05$). It suggested the growth of biomass in the soil was mainly attributed to the increased relative abundance of *Gammaproteobacteria*. Under the high-nutrient condition, the relative abundance of *Gammaproteobacteria* was also increased to 81.7 ± 1.0% during the initial 8 h ($P < 0.05$), while *Bacilli* abundance decreased to 11.5 ± 2.6% ($P < 0.05$). Then the trend was reversed during the subsequent period. The relative abundance of *Gammaproteobacteria* commenced to reduce, while the proportion of *Bacilli* doubled after 96 h.

On genus level, *Acinetobacter* and *Bacillus* were the most abundant in the community (Fig. 3A). As a major group of microbes in *Gammaproteobacteria*, the proportion of *Acinetobacter* increased from 73.7 ± 2.8 to 81.5 ± 0.9% under the low-nutrient condition ($P < 0.05$), while the abundance of *Bacillus* was maintained at about 15%. With the high-nutrient amendment, the relative amount of *Acinetobacter* increased to 81.4 ± 0.9% in the initial 8 h ($P < 0.05$) and decreased to 74.6 ± 0.8% after 96-h incubation ($P < 0.01$). The proportion of *Bacillus* under high-nutrient condition showed an opposite variation trend compared with that of *Acinetobacter*. In the first 8 h, its abundance reduced from 15.4 ± 1.7 to 10.6 ± 2.5% ($P < 0.05$). Then the abundance reverted to increase to 21.3 ± 2.0% at 96 h ($P < 0.01$). Therefore, the enriched *Bacillus* and reduced *Acinetobacter* in the soil with biofilms were the major differences compared with the soil with free-living cells. Based on principal component analysis (PCA), the arrow of another genus *Paenibacillus* in *Bacilli* pointed in the similar direction with that of *Bacillus*, which indicated a high correlation between these two genera (Fig. 3B).

3.4. Soil biofilm formation enhanced microbial metabolic activity

To further investigate the influence of soil biofilms on microbial activity, the soil oxygen distribution was measured after the whole incubation period. In the soil with biofilms, the oxygen concentration was sharply decreased to 50 μM at the depth of 0.5 mm (Fig. 4A). The oxygen was depleted at approximately 1.5-mm depth. Beyond that depth, the soil was under anoxic condition. Meanwhile, in the soil with free-living cells, oxygen was slowly decreased to 130 μM at the depth of 1 mm, which was much higher than that with biofilms ($P < 0.01$). With the increment of depth, soil oxygen concentration tended to be constant. Even at the bottom of vessel, 50 μM oxygen still remained in the soil. It suggested microbial respiration was mainly occurred in the surface soil. The surface soil (0–1.5 mm) with biofilms consumed 65.4% more oxygen than that with free-living cells ($P < 0.01$). Although the total microbial biomass of the soil with biofilms was less (1260 ± 18 vs. 1586 ± 24 mg/kg, $P < 0.01$), it had increased microbial respiration rate which indicated higher microbial activity and portion of active microbes.

To evaluate the microbial metabolic activity of soil biofilms, the same amount of nutrient (0.46 mmol glucose/kg soil and 0.1 mmol ammonium nitrate/kg soil) was resupplied after the depletion of initial nutrient. Upon the addition of extra nutrient, the glucose in the soil with biofilms was consumed dramatically (Fig. 4B). The glucose

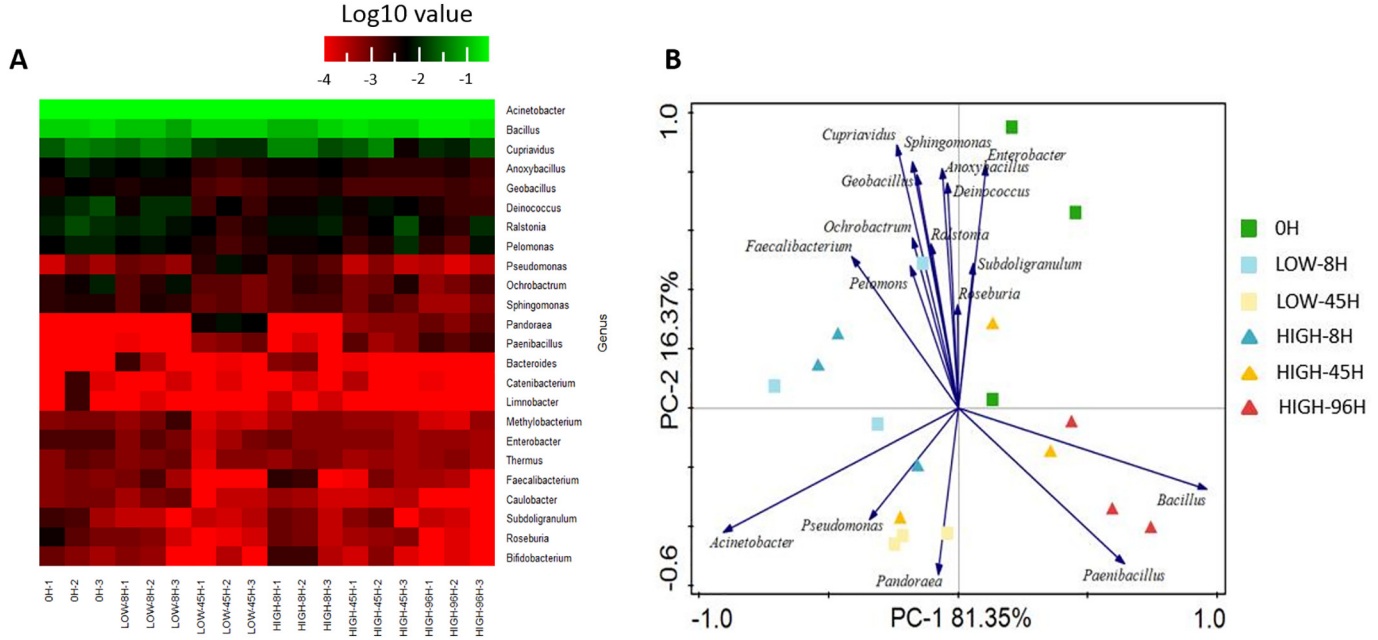

Fig. 3. The heatmap of major bacteria genera (> 0.1% abundance) (A). Principal component analysis (PCA) of microbial community structure on genus level (scaling 2) (B). Arrows represent the seventeen most abundant genera which accounting for > 95% of the total community.

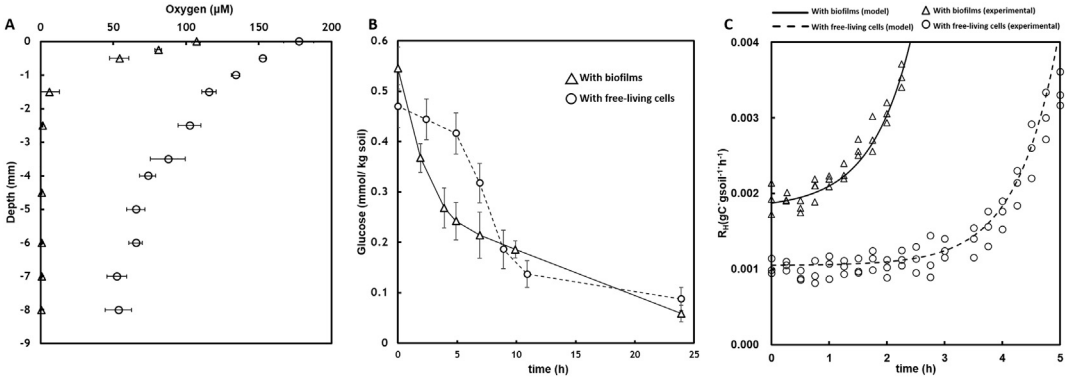

Fig. 4. The oxygen distribution in soil with biofilms/free-living cells (A). Additional glucose was amended into the soil after the depletion of initial nutrient. The glucose consumption revealed soil with biofilms having a rapid response to the substrate provision (B). Substrate-induced microbial respiration of soil with biofilms/free-living cells (C). The stimulated data is presented by the solid line for soil with biofilms and the dashed line for the one with free-living cells.

consumption rate in the soil with biofilms was much higher than that with free-living cells (−56.6 ± 5.0 vs. -11.0 ± 5.1 μmol/(h·kg soil); $P < 0.05$). The higher substrate consumption rate was in a good agreement with the higher respiration rate for the soil with biofilms. Therefore, it indicated that soil biofilms sustained more active microbes in soil which responded rapidly to the changes in nutrient availability.

To further decipher the kinetics of microbial activity of soil with biofilms/free-living cells, we monitored the substrate-induced respiratory response. As shown in Fig. 4C, the soil with biofilms had one-fold higher basal respiration rate than that with free-living cells at the initial incubation stage ($P < 0.01$). After the supplement of substrate, the respiration rate for the one with biofilms started to rise. Meanwhile, the soil with free-living cells showed a 3-h lag period before the respiration rate began to increase. The microbial kinetic parameters were determined by fitting experimental data. Soil with biofilms demonstrated significantly higher growth-coupled (R_c) and uncoupled (R_u) respiration rates (Table 2). Due to the short lag period and rapid increase in respiration rate, the R_c for soil with biofilms was approximately 23 times higher than that with free-living cells ($P < 0.01$) (Table 2). Meanwhile, the biofilm formation didn't impact the specific growth rate (μ) of the soil microbial community. Based on estimation, the fraction of active microbe in the soil with biofilms was 0.397 ± 0.096%, which was 14.6-times higher than that with free-living cells ($P < 0.05$). The total amount of active microbes in the soil with biofilms was 23-fold higher than that with free-living cells (0.069 ± 0.022 vs. 0.003 ± 0.003 mg C/g soil, $P < 0.05$). Therefore, the formation of biofilms in soil matrix significantly enhanced the fraction of active microbe, which contributed to the higher nutrient transformation rate.

4. Discussion

Soil biofilms were known to occur under copiotrophic conditions. In agreement with previous studies on natural soil microorganisms (Nunan et al., 2003; Redmile-Gordon et al., 2014; Reischke et al., 2015), the present work demonstrated that high-nutrient input induced biofilm formation in artificial soil matrix. Although 10-fold higher nutrient supply under high-nutrient condition induced less biomass growth than that under low-nutrient condition, the EPS concentration was significantly enhanced. It suggested that a large proportion of carbon source was incorporated into EPS which was energy-intensive to synthesize, but not chloroform-labile (Redmile-Gordon et al., 2014; Redmile-Gordon et al., 2015). With stimulated biofilms formation in artificial soil, we further showed their impacts on soil microbial community and metabolic activities.

4.1. The changes of soil microbial community

The soil biofilms communities like rhizosphere biofilms are known to be distinct from those in the bulk soil (Marschner et al., 2012; Blagodatskaya et al., 2014; Kuzyakov and Blagodatskaya, 2015). Meanwhile, our results revealed that the soil with biofilms or free-living cells shared similar community members which achieved a Bray-Curtis similarity above 90% (Fig. S4). The high similarity was related with the dominance of *Gammaproteobacteria* and *Bacilli* in the communities (Fig. S3), which may be caused by the same inoculum and cultivation conditions except for the nutrient level. One recent study also showed that random microbial ecosystems converged to similar “family”-level structure when supplied with the same carbon source (Goldford et al., 2018). In soil habitats, the glucose uptake by different groups of bacteria was found to be proportional with the amount of amended glucose (Dungait et al., 2011). Therefore, the soil biofilm development wouldn't lead to significant variation in microbial community structure in short term.

Although the community compositions were similar, soil with biofilms exhibited an increased microbial diversity and evenness. When nutrient deprived, the total biomass concentration of the soil with biofilms was lower than that with free-living cells (1260 ± 18 vs. 1586 ± 24 mg/kg, $P < 0.01$). But the soil biofilm formation significantly enhanced the microbial diversity and evenness by 17% and 18% compared to that with free-living cells (Table 1). In other natural and engineered environments especially aquatic systems, biofilm

Table 2
Fitted kinetics of microbial activity in soil.

	R_u (mg C/g soil/h)	R_c (mg C/g soil/h)	μ (h^{-1})	FAMB[a] (%)	TMB[b] (mg C/g soil)	AMB[c] (mg C/g soil)
High-nutrient	1.799 ± 0.043^{A}	0.0716 ± 0.0163^{A}	1.43 ± 0.12^{A}	0.397 ± 0.096^{A}	17.18 ± 1.44^{A}	0.069 ± 0.022^{A}
Low-nutrient	1.045 ± 0.149^{B}	0.0030 ± 0.0028^{B}	1.42 ± 0.25^{A}	0.027 ± 0.022^{B}	10.36 ± 3.59^{A}	0.003 ± 0.003^{B}

^{ABC}different capital letters in the same column indicate significant differences ($P < 0.05$).
[a] FAMB: fraction of active microbial biomass.
[b] TMB: total microbial biomass.
[c] AMB: active microbial biomass.

formation was generally found to increase microbial community diversity (Rickard et al., 2004; Douterelo et al., 2013). Meanwhile, the edaphic environments are distinct from the others, in which the resource distribution renders high spatial and temporal heterogeneity. Therefore, it is of great importance to elucidate the influence of soil biofilms formation on the microbial community. Our results have demonstrated that biofilms development in soil can sustain a more active and diverse community. The close proximity between microbes in biofilms promotes intensive competition and cooperation in the confined space which contributes to the constant adaptation of population fitness (Stewart and Franklin, 2008; Flemming and Wingender, 2010; Vlamakis et al., 2013). A previous study has demonstrated that a diverse soil microbial community was more efficient in resource utilization which is beneficial for the soil fertility and ecosystem health (Mäder et al., 2002).

During the development of soil biofilms, *Bacillus* and *Paenibacillus* were the main biofilm-forming bacteria in the soil. The abundance of dominant *Acinetobacter* increased initially under both nutrient conditions, which contributed to the increased biomass in the soil. Meanwhile, its abundance decreased concurrently with the enrichment of *Bacillus* and *Paenibacillus* under high-nutrient condition. *Bacillus* and *Paenibacillus* were well recognized as major contributors to soil biofilms formation (Timmusk et al., 2005; Vlamakis et al., 2013; Dervaux et al., 2014). In the soil community, *Bacillus* was found to produce toxins to kill non-biofilm-forming bacteria (Shank et al., 2011). Moreover, soil biofilms can block the pore space which smothers their competitors and gains themselves more opportunities to access oxygen and nutrient (Xavier and Foster, 2007; Coyte et al., 2017). The rapid oxygen depletion could be caused by the development of *Bacillus* biofilm, which may explain the decline of *Acinetobacter* and total biomass under high-nutrient condition.

4.2. Enhancement of microbial metabolic activity

Our findings showed that microbial metabolic activities have been greatly impacted after the formation of soil biofilms. In spite of relative lower biomass concentration, the soil with biofilms exhibited higher respiration activity and increased nutrient turnover rate (Fig. 4), which were attributed to 23-times more active microbes in the soil biofilms. In the soil ecosystem, > 80% of microorganisms were found in the dormant state and growing slowly (Lennon and Jones, 2011). The microbial dormancy is known as *K*-strategy which conferred microbes fitness under nutrient-limited conditions. On the contrary, the active microbes were *r*-strategists which responded rapidly to the addition of labile substrate and had a low nutrient use efficiency (Blagodatsky et al., 2000). The rhizosphere soil dominated by *r*-strategists had shown higher basal respiration rate than root-free soil, while the microbial carbon use efficiency of root-free soil was 1.5 times higher (Blagodatskaya et al., 2014). Under the low-nutrient condition, the amended glucose was not enough to trigger inactive bacteria to exit dormancy (Blagodatsky et al., 2000; Hill et al., 2008; Blagodatskaya et al., 2009). The low-nutrient input (0.46 mmol glucose/kg soil) was approximate to the threshold (0.28 mmol glucose/kg soil) to stimulate soil microbial growth (Reischke et al., 2015). High nutrient supplement triggered the transition of dormant microbes to active state and induced EPS secretion and soil biofilms formation. The reduced resource use efficiency of *r*-strategists also partly explained the less microbial progression with high nutrient amendment (Wu et al., 1993; Nguyen and Guckert, 2001; Schneckenberger et al., 2008; Dungait et al., 2011).

After the formation of soil biofilms, a diverse and metabolically active microbial community is developed. The higher proportion of active microbes rather than higher microbial biomass can accelerate the biogeochemical cycles. The increased organic carbon transformation rate and microbial respiration were shown in this study. And the soil biofilm formation was also revealed to elevate the mineralization rate of N and P (Seneviratne and Jayasinghearachchi, 2005). Besides the active microbial metabolism, the unique physiochemical properties of biofilm can further impact edaphic environment. The excreted EPS triggers the formation of soil microaggregates and improves aggregate stability. Biofilms developed in soil cause clogging of pores, which induce a substantial reduction of hydraulic conductivity and retard the convection and diffusion of various substrates like organics and oxygen (Hand et al., 2008; Pintelon et al., 2012). Consequently, soil biofilm formation can greatly contribute to the spatial and biochemical heterogeneity of soil.

5. Conclusion

The results of this study showed that soil biofilms can be stimulated in artificial soils with increased provision of labile nutrients. Although the biomass growth under high-nutrient condition was lower, EPS production was significantly enhanced and microaggregates were developed. 16S rRNA gene sequencing revealed similar microbial community structures for the soil cultivated under different nutrient conditions. Meanwhile, the diversity and evenness indices of soil with biofilms were significantly higher than that with free-living cells. *Bacillus* and *Paenibacillus* were the main biofilm-forming bacteria in the soil, which enriched at the late incubation stage. After the formation of soil biofilms, the microbial respiration rate was increased which responded rapidly to the change of nutrient availability. Based on substrate-induced respiration, soil biofilms sustained 23-times more active microbes than that with free-living cells, which contributed to increased organic turnover rate. Taking these factors together, this study provides novel environmental implications on the soil biofilms development processes and their corresponding ecological impacts.

Acknowledgements

We thank Prof. Shaobing Peng, Prof. Kehui Cui and Prof. Yonghong Bi for their helps in experiments. This work was supported by the National Natural Science Foundation of China (41807024, 41877029), the National Basic Research Program of China (2016YFD0800206) and the Fundamental Research Funds for the Central Universities (Program no. 52902-0900201674).

Declaration of competing interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105116.

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