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Metagenomic analysis of soil microbial community under PFOA and PFOS stress

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ABSTRACT

Perfluorinated compounds (PFCs) contamination of soil has attracted global attention in recent years but influences of PFCs on microorganisms in the soil environment have not been fully described. In this study, the effects of perfluorooctane sulphonate (PFOS) and perfluoroctanoic acid (PFOA) on bacterial communities were determined by Illumina Miseq sequencing and Illumina Hiseq Xten. The stimulation of PFCs pollutants on soil bacterial richness and community diversity were observed. Sequencing information indicated that *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, and *Gemmatimonadetes* were the dominant bacterial phyla. Two genera, *Bacillus* and *Sphingomonas*, exhibited adverse responses toward PFCs pollution. Carbohydrate-active enzymes (CAZy), Kyoto Encyclopedia of Genes and Genomes (KEGG) and NCBI databases were used to elucidate the proteins and function action of soil microbial to PFCs pollution. Pathways such as Carbohydrate metabolism, Global and overview maps and Membrane transport in the soil microbes were affected by PFCs stress. CAZy analysis revealed that glycosyl transferases (GTs) in PFCs-polluted soils showed more active, while glycoside hydrolases (GHs) were inhibited severely.

1. Introduction

Perfluorinated compounds (PFCs) are a class of anthropogenic synthesis chemicals with unique physical, chemical and biological characteristics (Lindstrom et al., 2011). In the past six decades, PFCs have been used widely in various consumer and industrial applications, causing a worldwide distribution and creature exposure. Of particular concern was the fact that the compounds are of environmentally persistent, bioaccumulation, and long half-lives in humans (Olsen Geary et al., 2007), which seemed to satisfy the definition of persistent organic pollutants (POPs). Moreover, perfluorooctane sulphonate (PFOS), perfluoroctanoic acid (PFOA), both salts and related compounds had been included in the Stockholm Convention on POPs in 2009 and 2019, respectively. Previous studies have detected that the environmental persistence of PFCs has made it ubiquitously found in water, soil, and creature (Ahrens and Bundschuh, 2014; Zhu and Kannan, 2019). Besides, in the light that environmental behaviors of PFCs could be influenced by their chain length and functional group, long chain PFCs, such as PFOA and PFOS, have higher sorption to soils compared to short chain ones (Bräunig et al., 2019; Zhang et al., 2020). In rural areas from eastern China, the total concentrations of PFCs in soils were 0.34–65.8 ng/g dry weight, while in comparison, merely the concentrations of PFOA detected in PFCs-contaminated soils were up to 130 ng/g dry weight in average (Chen et al., 2016; Zhu and Kannan, 2019). Moreover, due to the bioaccumulation, PFCs would be potential to accumulated from contaminated soil by both biota and plants, then move away into the terrestrial food web (Rich et al., 2015).

For the moment, research on the ecotoxicity of PFCs in soil is mainly focused on some conventional soil-related creatures, for example, soil enzymes, earthworms, crop plants and soil microbial. Zhang et al. (2013) analyzed the changes of soil enzymes (urease, catalase, and phosphatase) induced by PFOA in laboratory conditions. After an entire incubation period, the activities of the three soil enzymes decreased with increasing PFOA concentration. Previous researches investigated the damages of PFOA and PFOS to the antioxidant system in *Eisenia fetida*, and revealed that PFCs can induce oxidative stress in the earthworm, causing biochemical effects (Xu et al., 2013; Zhao et al., 2017). PFOA at high concentrations can inhibit germination rate and root growth of wheat significantly (Zhou et al., 2016). Soil microorganisms are an important part of the soil ecosystem, which play a

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vital role in the biogeochemical cycle (Peralta et al., 2013). Some studies have explored soil microbial community responses to PFCs pollution by adopting 16S rRNA gene sequences and high-throughput sequencing technology. Through Illumina high-throughput sequencing analysis, the contamination of PFOA and PFOS could change the diversity and richness of the bacterial community in sediments and soils, with the influences factors as concentrations and carbon chain length (Bao et al., 2018; Li et al., 2017; Qiao et al., 2018; Sun et al., 2016). In these studies, the major bacterial groups were detected to differ in relative abundance but same in species, and even some specific genus were found to be resistant to PFOA pollution. However, it is barely enough for the integrated soil health under contamination to assess microbial community diversity solely. Microbial functions and metabolism pathways are subjected to contribute to it.

In general, Metagenomics approach can reveal the microbial community's response to disturbance, which means that it can provide diversification on the structure of microbial communities, their function and the interaction when occurring to external interference without cultivation of microbes, and currently microbial communities in contaminated environment would have less metabolic and phylogenetic diversity (Bouhajja et al., 2016). These methods have been applied for a number of metals and POPs pollution on the microbial community. For example, by using Illumina sequencing, sediment contamination by heavy metals, polychlorinated biphenyls and polycyclic aromatic hydrocarbon (PAHs) has been detected to alter microbial diversity in coastal areas significantly. More than that, the richness of microorganisms gradually decreased with the increase in pollution concentrations, and the communities were developing towards the choice of species that can use organic pollutants as carbon and energy sources, which suggested the potential contribution of resident microbial to contaminated bioremediation actions (Quero et al., 2015). Another study utilized functional metagenomics to analyze some interactions produced between native microbiota and PAHs-degrading microbial communities during the bioremediation of PAHs-contaminated soils. Functional metagenomics showed a superiority of PAHs-degrading microbial consortium on PAH mineralization, since changes in gene abundance suggest a bias towards aromatics and intermediate degradation pathways and the increase of co-metabolic degradation caused by the degradation of large amounts of PAHs in soils (Zafra et al., 2016). Qiao et al. (2018) detected the effects of perfluoroalkyl substances (PFASs) on bacterial communities by Illumina Miseq sequencing and found that the normal function of soil microorganisms was significantly damaged by PFASs pollution. In addition, PICRUSt was used to analyze the bacterial COG gene prediction and reveal a reduction of soil functional genes related to the cellular structure, gene expression, immune system of soil microorganisms, providing a discussion on the effects of PFASs on gene levels of microorganisms theoretically. Even so, researches on the environmental impacts of PFCs on soil microbial community and metabolism function pathway are still deficient.

Therefore, in the present study, 16S rRNA and metagenomic methods were used to measure the responses of soil bacterial communities to PFOA and PFOS exposure. The specific aims of this study were: (1) to analyze the influences of PFOA and PFOS on bacterial community structure; (2) to explore the feedback of community function when suffered PFCs pollution; (3) to evaluate the potential threats to soil ecosystem caused by PFOA and PFOS.

2. Materials and methods

2.1. Chemicals and materials

PFOA (98%, Sigma-Aldrich) and PFOS (98%, J&K Chemical) were used in the experiments, both of which were of analytical grade. The solutions of PFOA and PFOS were prepared by dissolving the solid chemicals in Dimethyl sulfoxide (DMSO, AR). The surface soil samples (0–20 cm) were collected from Heilongjiang Province in China, whose soil microbial activity showed a clear growth trend and full of vigorous in our previous study (Cai et al., 2019). Soils were air-dried and sieved (100-mesh), and stored at 4 °C before further analysis.

2.2. Experimental design

20 g soil were weighed into 15 polypropylene centrifuge tubes respectively. Then divided these 15 tubes into 5 groups so that each group had 3 parallel samples. Refer to our previous study, the microbial in selected soils exhibited approximate 20% inhibitory in 800 μg/g PFOA and PFOS in 3 days acute toxic experiment, and the half inhibitory ratio (IC_{50}) of PFOA calculated was nearly 3000 μg/g (Cai et al., 2019). Therefore, each group was mixed with PFCs pollutants to keep the mass concentration of PFCs in soils at 0 mg/kg, PFOA-1000 mg/kg, PFOA-3000 mg/kg, PFOS-1000 mg/kg, PFOS-3000 mg/kg. All treatments were performed in triplicate. The 0 mg/kg concentration group was the control group with an equal amount of distilled water. All tubes were added equally the nutrient solution made of glucose together with ammonium sulfate. All soil samples were continuously cultured in the dark. After 10 incubation days, the soil samples were prepared for the analysis of microbial communities.

2.3. DNA extraction

DNA was extracted from soil samples using E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, US) following the manufacturer's protocols. The quality of the extracted DNA samples was measured using 1% agarose gel electrophoresis, and the DNA concentration and purity were quantified using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA).

2.4. 16S rRNA gene sequencing and analysis

The primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the hypervariable region V3-V4 of the bacterial 16S rRNA gene by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The 16S rRNA gene PCR amplification process was as follows: initial denaturation at 95 °C for 3 min, then followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, and single extension at 72 °C for 10 min, and end at 4 °C. PCR products were extracted from a 2% agarose gel and purified according to the manufacturer's instructions using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using a Quantus™ Fluorometer (Promega, USA). Purified amplicons were equimolarly combined according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China), and paired-end sequencing (2 × 300) was performed on the Illumina MiSeq platform (Illumina, San Diego, USA). The original readings have been deposited into the NCBI Sequence Read Archive (SRA) database. The original 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH, after which these sequences were clustered in operational taxonomic units (OTUs) with a cutoff value of 97% similarity using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and deleted. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the 16S rRNA database (eg. Silva SSU128) using a confidence threshold of 0.7. Mothur was used to calculate the α-diversity indices of microbial community (Schloss et al., 2009). Circos figures obtained from Circos-0.67-7 (http://circo.ca/) reflected the proportion of prominent species in each sample and also the distribution ratio of each dominant species in different samples. Heatmap analyses were produced in R using Vegan package.

2.5. Metagenome sequencing and annotation

Covaris M220 (Gene Company Limited, China) was used to fragment DNA extracts to an average size of about 300 bp for the construction of paired-end library. A paired-end library was constructed using NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA) and then sequenced on Illumina Hiseq Xten (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using HiSeq X Reagent Kits according to the manufacturer's instructions (www.illumina.com). Sequence data associated with the project has been stored in the NCBI database. The adapter sequences were cleaned and assembled using SeqPrep and Sickle, and contigs with the length being or over 300 bp were selected as the final assembling result, then used for further gene prediction and annotation. MetaGene was used to predict open reading frames (ORFs) from each assembled contig (Noguchi et al., 2006). The predicted ORFs with a length of 100 bp or more were retrieved and translated into amino acid sequences using the NCBI translation table. CD-HIT was used to cluster all predicted genes with 95% sequence identity (90% coverage) (Fu et al., 2012), and the longest sequence was selected from each cluster as the representative sequence to construct a non-redundant gene catalog. The SOAPaligner was used to map the quality-controlled reads to a representative sequence with 95% identity (Li et al., 2008), and gene abundance in each sample were assessed.

BLASTP (Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for classification and annotation, the representative sequences of the non-redundant gene catalog were compared with the NCBI NR database with an e-value cutoff of $1e^{-5}$ (Altschul et al., 1997). The KEGG annotations were performed using the BLASTP (Version 2.2.28+) on the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/keeg/) with an e-value cutoff of $1e^{-5}$ (Xie et al., 2011).

Annotation of carbohydrate-active enzymes was performed using hmmscan (http://hmmer.janelia.org/search/hmmscan) for the CAZy database Version 5.0 (http://www.cazy.org/) with a cutoff value of $1e^{-5}$.

3. Results and discussion

3.1. Effects of PFCs on soil bacterial community richness and diversity

By using Illumina high-throughput sequencing technology, 727074 effective sequences were obtained from the soil samples, with an average microbiota length of 418.55 bp. The sequences were clustered to 3896 OTUs of bacterial with a threshold of 0.97. As shown in Fig. 1, the group PFOA-1000 mg/kg showed the most abundant diversity with an OTUs value of 2703, simultaneously, groups PFOS-3000 mg/kg and PFOS-1000 mg/kg behaved lower diversity but had higher OTUs numbers than the control, indicating a stimulation of PFCs on soil microbial community diversity. The particular and shared bacterial OTUs of five tested groups were exhibited by Veen diagram, where soil samples treated un-amended and PFCs exposure (PFOA-1000 mg/kg, PFOA-3000 mg/kg, PFOS-1000 mg/kg and PFOS-3000 mg/kg) contained 282, 197, 137, 134, and 177 unique OTUs respectively and shared 1518 OTUs. The PFOA-3000 mg/kg shared a minimal OTUs with the control as 36, whereas the shared OTUs between the PFOS-1000 mg/kg and the control was the maximum as 65. Shannon and Simpson index (Table 1, coverage estimator ≥ 99%), which were used to measure the diversity of microbial communities, also illustrated the stimulation performance when the index values of the control displayed the lowest diversity. On the other way, Ace and Chao richness indexes of soil bacterial under toxin exposure were higher than un-amended soil, representing that PFCs could increase soil bacterial richness. Actually, according to the previous studies, the richness had correlations with the functional properties of the ecosystem, the C and N mineralization and the increase in total stores of C, N and P (McGill and Cole, 1981; Vitousek and Hooper, 1993). These correlations mean that the change in richness could cause disturbance on soil environment. In addition, microbial diversity and richness are considered to be intrinsic characteristics of microbial community to defend interference, but limited research has been performed to detect the influences of PFCs on soil microbial community from such aspects.

For example, Li et al. (2017) revealed that microbial abundance and richness got promotion when the content of perfluorohexane sulfonate (PFHxS) in soil increasing, and the result is consistent with the present study. However, Zhang et al. (2019) reported that the addition of perfluoroakyl acids (PFAA) would decrease the microbial richness and diversity. In a research from Montagnolli et al. (2017), 3.9 g/kg concentrations of fluorine-containing aqueous film-forming foams (AFFF) may compromise microbial activity then decreased richness. Bao et al. (2018) detected the response of bacterial communities in PFAS-contaminated soils and found a negative influence of 10 μg/g concentrations of PFOS on bacterial richness and diversity, furthermore, researchers investigated the determinants to bacterial richness and diversity by taking Spearman rank correlation analysis, and put forward the considerable significance of soil organic carbon. Apart from this, researchers (Qiao et al., 2018) determined that soil contaminated by PFBS and PFHxS had higher richness and diversity than those contaminated by PFOS, indicating the difference in microbial richness regulation by different kinds of PFCs, which means the species of PFCs were considered to be one of the key factors in governing bacterial community structure when caused differences in richness and diversity.

3.2. Effects of PFCs on soil bacterial community structure

The Circos figures (Fig. 2) and heatmaps (Fig. 3) showed the distribution and abundance of bacterial in test soils on phylum and genus levels. On the phylum level, *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, and *Gemmatimonadetes* were the dominant bacterial phyla in the present study, accounting for more than 95.8% and had distinguishing differences in proportion. In the soils polluted by heavy-PFAS over 10 years, bacterial communities were mainly composed of *Chloroflexi*, *Actinobacteria*, *Proteobacteria*, *Cyanobacteria*, and *Acidobacteria,* while *Firmicutes*, and *Gemmatimonadetes* were minor bacterial groups (Bao et al., 2018). To measure the impacts of PFASs on bacterial communities, Qiao et al. (2018) conducted a greenhouse experiment for 80 days and found out the distribution of dominant phyla as *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Acidobacteria*. When investigating the microbial community in soils contaminated by Cr and PFASs, researchers detected *Actinobacteria*, *Chloroflexi*, *Proteobacteria* and *Firmicutes* as the main bacterial phyla (Li et al., 2017). Moreover, in comparison to the control, the proportion of *Acidobacteria* in PFCs-exposed soils were markedly higher, as well as *Gemmatimonadetes*, *Chloroflexi* and *Actinobacteria*. As for the other two phyla, converse appearances were found as the proportion was lower in the soil microbial community under exposure of PFCs, specifically the *Firmicutes*, which seemed that the activity of microbial was depressed or inactivated. In the previous studies, PFASs were considered to have a clear link to the overall structure of soil bacteria: the proportions of *Acidobacteria*, *Chloroflexi* and *Actinobacteria* were regulated by the levels of PFBS, PFHxA and PFOS, while the proportion of *Proteobacteria* was regulated by the level of PFOS (Bao et al., 2018). Besides that, PFASs were regarded to stimulate the growth of *Firmicutes*, *Actinobacteria* and *Acidobacteria* but inhibit other bacterial such as *Chloroflexi* and *Latescibacteria* (Qiao et al., 2018). High concentrations of PFOA may lead to a distinct increase in the relative abundance of *Proteobacteria* in sediment, which was inconsistent with the present study (Sun et al., 2016). As exhibited above, one kind phylum of bacterial can show different growth trend under PFOA stress. Although there have no systematic researches conducted to explain these differences, we can draw inspiration from a study investigating the effects of pesticide residues on soil microbial communities, which mentioned that the

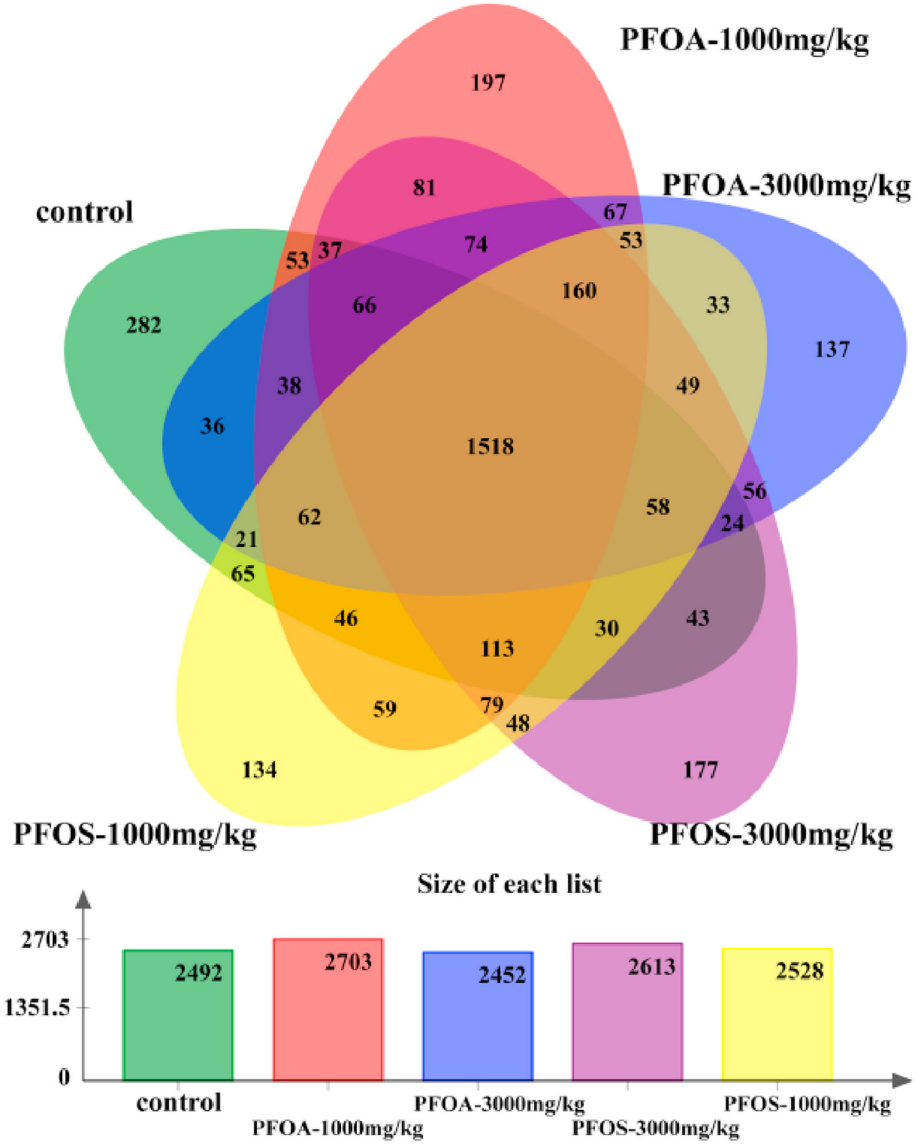

Fig. 1. Veen of OTU numbers in soil samples. Different colors represent different samples, the numbers of overlapping sections represent the number of species common in multiple samples, and the numbers of non-overlapping sections represent the number of species unique to the corresponding sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

response of soil bacteria to cultivation years was different under greenhouse conditions (Wang et al., 2020a).

In general, members of *Proteobacteria* are widespread in soil environments and comprise one of the largest bacterial phyla within prokaryotes (Gupta, 2000). In Betaproteobacteria phylum, researchers found some Gram-negative bacteria with thin cell walls and complex ingredients could respond quickly to pollution stress then tolerate contamination (Wang et al., 2020b). But in a research conducted to study interactions between PFCs and simplified bacterial membranes modeled with phospholipid Langmuir monolayers, Gram-negative bacterial presented much PFCs susceptible than Gram-positive bacterial and PFCs molecules interacted mainly with the phosphate group of the phospholipid polar head group which was a unique texture in Gram-negative bacterial (Wojcik et al., 2018). More than that, a genus called *Pseudomonas*, which belonging to *Proteobacteria*, could defluorinated fluorotelomer alcohols (FTOH) by removing multiple -CF_2- groups to form shorter-chain PFCAs (Kim et al., 2012). Since the shorter-chain PFCAs have been proved to have less toxicity than longer-chain ones by some studies (Cai et al., 2019; Mahapatra et al., 2017), the usage of *Proteobacteria* in microbial degradation shortening carbon chain of PFCs

Table 1
Alpha-diversity of the soil microbial community.

Sample	shannon	simpson	ace	chao	coverage
control	5.717 ± 0.118	0.011 ± 0.003	1909 ± 94	1904 ± 85	0.994 ± 0.000
PFOA-1000 mg/kg	6.093 ± 0.109	0.006 ± 0.002	2097 ± 81	2090 ± 80	0.993 ± 0.001
PFOA-3000 mg/kg	6.166 ± 0.091	0.005 ± 0.000	2196 ± 205	2209 ± 192	0.993 ± 0.000
PFOS-1000 mg/kg	5.773 ± 0.333	0.012 ± 0.007	2005 ± 100	1990 ± 104	0.993 ± 0.001
PFOS-3000 mg/kg	6.178 ± 0.046	0.005 ± 0.000	2018 ± 26	2031 ± 26	0.994 ± 0.001

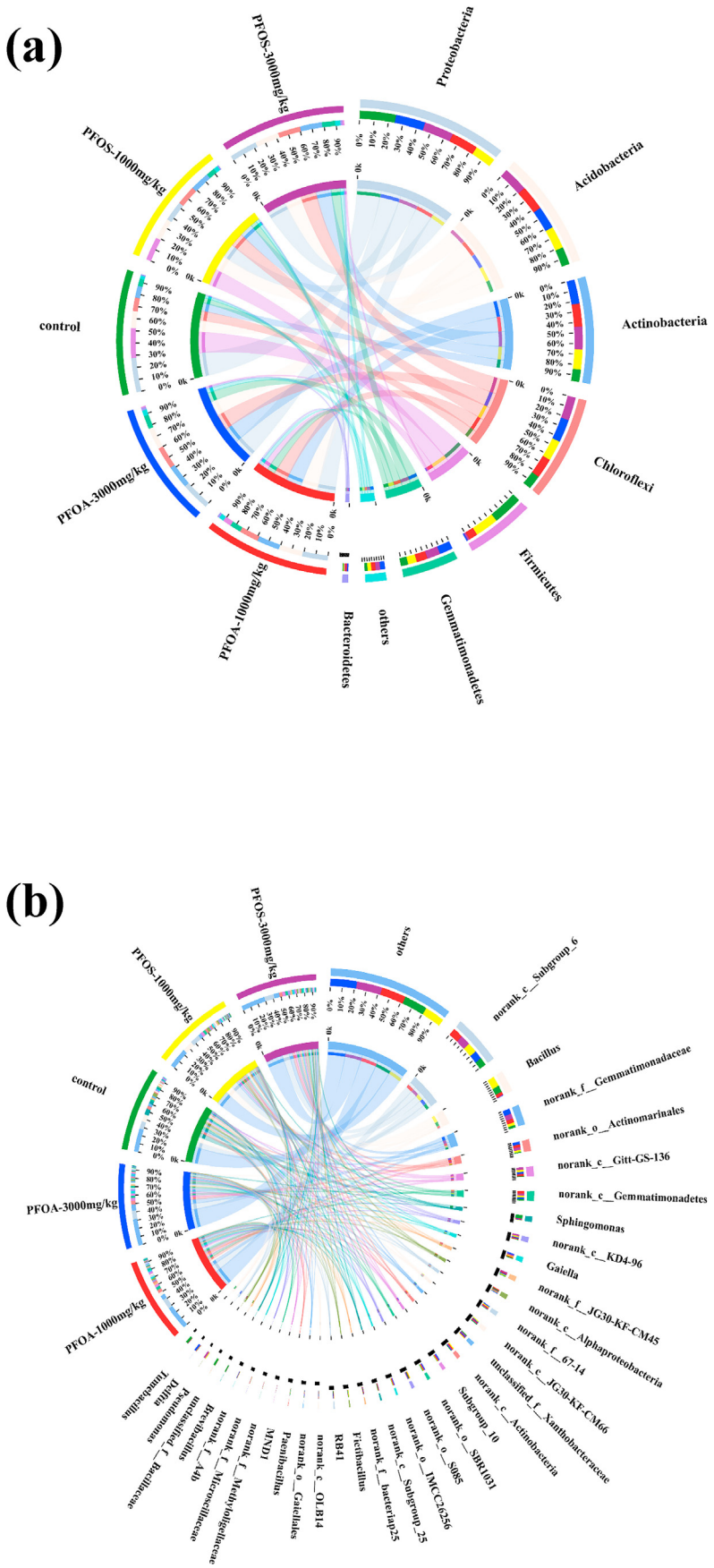

Fig. 2. Composition and abundance of soil samples at phylum (a) and genus (b) levels.

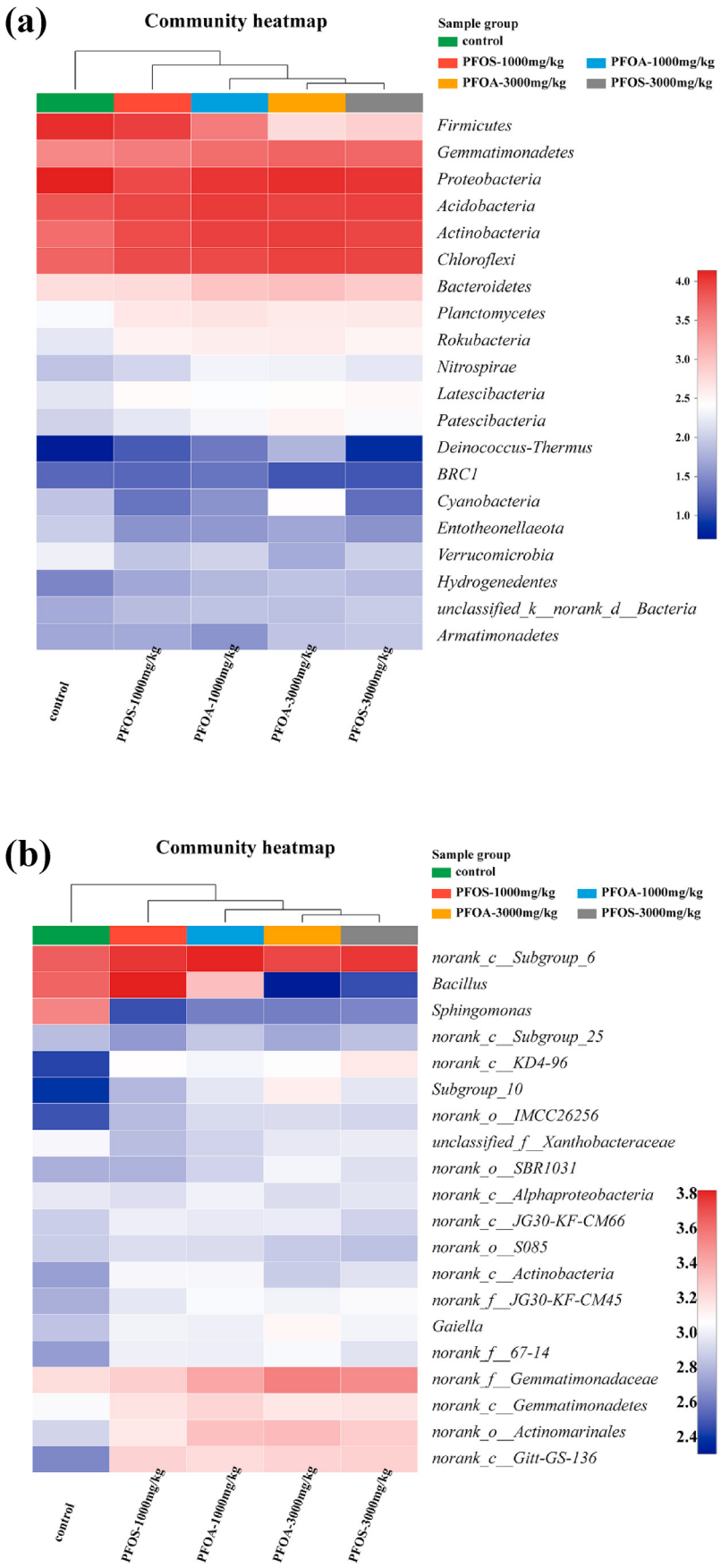

Fig. 3. Heatmap on phylum (a) and genus (b) level. The cluster tree showed the similarity of phylum/genus abundance in each sample. The intensity of the color in the heat map represents the phylum/genus abundance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

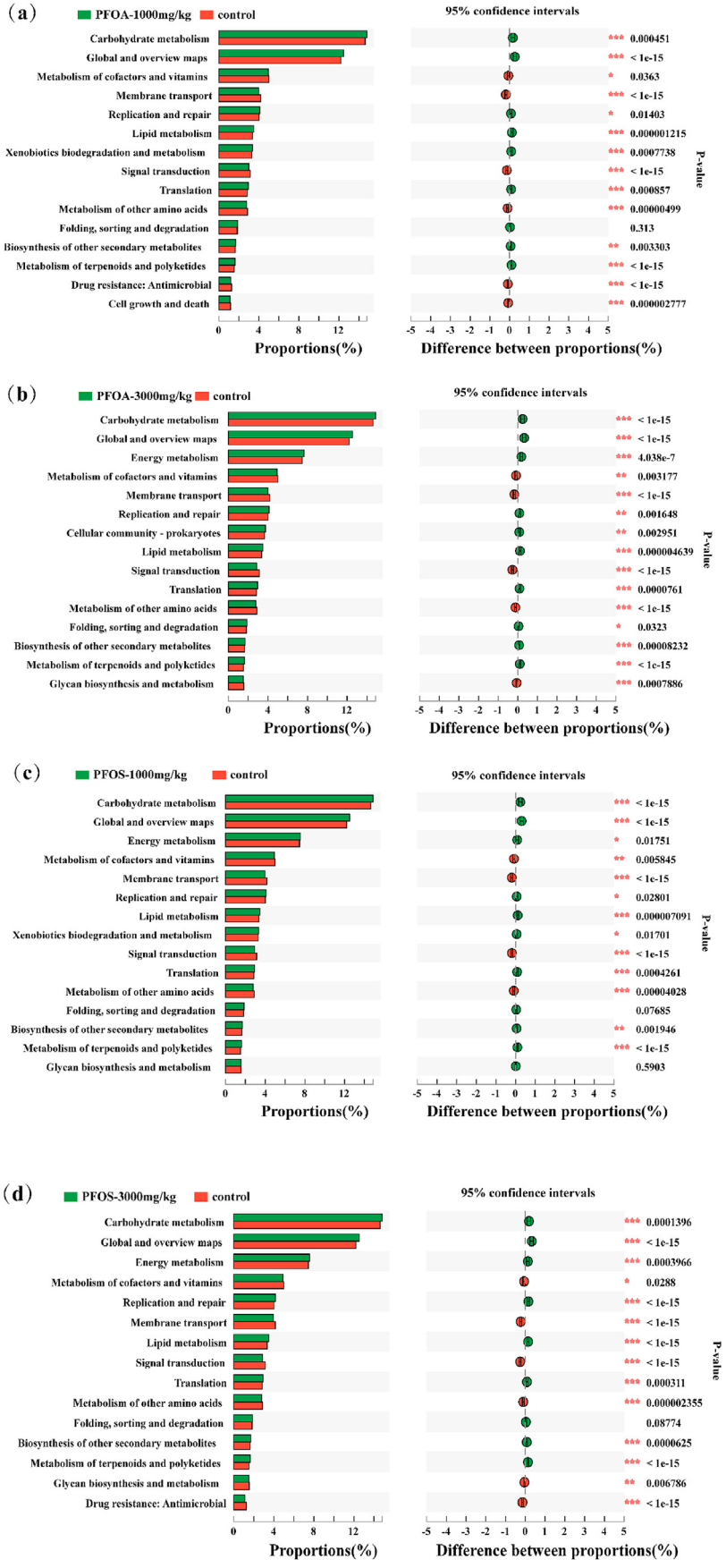

Fig. 4. Fisher's exact tests on pathway level 2.

Table 2
KEGG pathway with significant differences.

ko number	Function description	level 2	level 1
01200	Carbon metabolism	Global and overview maps	Metabolism
01230	Biosynthesis of amino acids	Global and overview maps	Metabolism
02010	ABC transporters	Membrane transport	Environmental Information Processing
02020	Two-component system	Signal transduction	Environmental Information Processing
00720	Carbon fixation pathways in prokaryotes	Energy metabolism	Metabolism
00630	Glyoxylate and dicarboxylate metabolism	Carbohydrate metabolism	Metabolism
00970	Aminoacyl-tRNA biosynthesis	Translation	Genetic Information Processing
00010	Glycolysis/Gluconeogenesis	Carbohydrate metabolism	Metabolism
00680	Methane metabolism	Energy metabolism	Metabolism
00520	Amino sugar and nucleotide sugar metabolism	Carbohydrate metabolism	Metabolism
01212	Fatty acid metabolism	Global and overview maps	Metabolism
01210	2-Oxocarboxylic acid metabolism	Global and overview maps	Metabolism
03010	Ribosome	Translation	Genetic Information Processing
00500	Starch and sucrose metabolism	Carbohydrate metabolism	Metabolism
00030	Pentose phosphate pathway	Carbohydrate metabolism	Metabolism

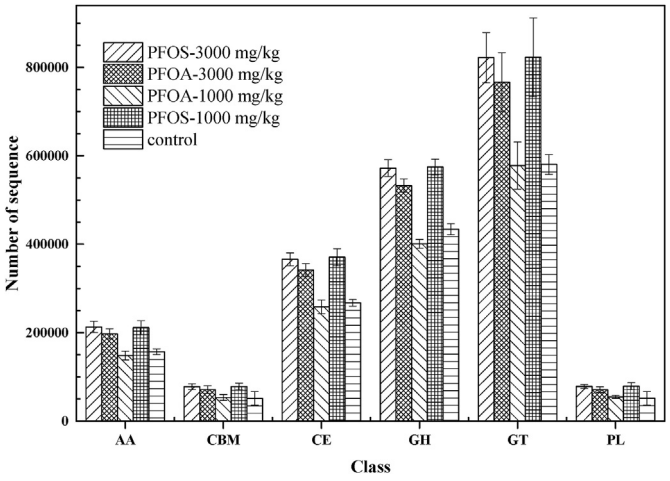

Fig. 5. Carbohydrate active enzyme gene abundances in each sample.

would be highly promising. Besides, as a copiotrophic taxa, the relative abundance of *Proteobacteria* usually increased in high nitrogen regions, while the oligotrophic taxa (mainly *Acidobacteria*) presented converse pattern, which was consistent with the relative abundance behaviors of these two bacteria in our study (Fierer et al., 2012). Apart from the great susceptibility to the input of organic nutrients, *Acidobacteria* exhibited tolerance to a variety of pollutants such as petroleum and pesticide, bring about a surmise that *Acidobacteria* could have degradation potential for some pollutants (Abed et al., 2002; Wang et al., 2020a).

On the genus level, two typical genera were worth noting except for some subgroup in other levels. *Bacillus* were present at the highest proportion in the PFOS-1000 mg/kg sample, followed by the control, and PFOA-1000 mg/kg sample, PFOS-3000 mg/kg sample, PFOA-3000 mg/kg sample, which seemed that the bacterial was of adverse sensitivity to PFCs exposure. The comparison of relative abundance between PFOA and PFOS dosages at 1000 mg/kg and 3000 mg/kg exhibited that higher PFCs concentration caused more severe inhibitory on growth and metabolism of *Bacillus*. One kind of special bacterial called *Bacillus licheniformis* strain SL10 isolated from water and sludge in an effluent treatment plant, whose growth showed a linear relationship with 2,4-Dichlorophenol (2,4-DCP) concentration with the inhibitory concentration of 55.74 mg/L, at the same time, the bacterial proved to be potential in biodegrading 2,4-DCP at a relatively faster rate (Chris Felshia et al., 2020). As for *Sphingomonas*, the proportions in amended PFCs soils were highly lower than the control, exhibiting significant inhibitory caused by PFCs. Similarly, *Sphingomonas* was surveyed to be great sensitive in Cd-contaminated soils (Wang et al., 2019b), and as a kind of Gram-negative bacteria, *Sphingomonas* was capable to endure stress disturbance and was potential in some antibiotic resistance when isolated from drinking water (Vaz-Moreira et al., 2011). Moreover, *Sphingomonas* can promote the growth of *Dendrobium officinale* by nitrogen fixation, confirmed through the sequencing of the *nifH* gene (Yang et al., 2014). Based on that, the function of *Sphingomonas* on nitrogen cycle might be impaired significantly under the exposure of PFCs.

3.3. Effects of PFCs on soil bacterial community function

Based on KEGG database, the comparison results of Fisher's exact tests on pathway level 2 between control group and four PFCs amended soil groups were presented in Fig. 4. As can be seen in the figure, there are significant differences exist on the proportions of pathways between PFCs-polluted samples and control group, which means that soil bacterial function was influenced by the addition of PFCs. In overview, the selected pathways, for instance, Carbohydrate metabolism ($p < 0.001$), Global and overview maps ($p < 0.001$), Membrane transport ($p < 0.001$), Lipid metabolism ($p < 0.001$) are mainly classified into two level-1 pathways, Metabolism and Environmental Information Processing. The impacts of PFCs on cell membrane have been reported in a few studies. Due to relatively non-specific detergent-like effects on the membrane, PFOS could increase membrane fluidity of fish leukocytes in a dose-dependent manner (Hu et al., 2003). As described by Liu et al. (2016), the exposure to high concentrations PFOS and PFOA could transform cell surface hydrophobicity, and hydrophobic parts of PFOA and PFOS bonding to lipid bilayer of cell membrane could cause the increase in membrane fluidity, thereby disrupting the membrane structure, leading to the cell inactivation and mortality.

To obtain more detailed information on pathways, we conducted Fisher's exact tests on pathway level 3, and the top 15 pathways with significant differences in abundance were shown in Fig. S1 and Table 2. As well known, carbon metabolism pathways (ko01200) is one of the core metabolisms in soil bacterial and antibiotics and heavy metals pollution could undermine the utilization of carbon source, such as amino acids, saccharides and metabolites by the microorganisms (Ning et al., 2019; Wang et al., 2019a). Heavy metal pollution inhibited microbial biomass carbon (MBC) formation in dose effects, which would not mean less organic carbon mineralization. The inhibitory effect of heavy metals on MBC efficiency while unitizing TOC suggested that soil microorganisms were utilizing increased energy to repair and maintain under metal stress (Xu et al., 2019). Actually, in the present study, the relative abundance of carbon metabolism in PFCs-polluted soils were higher than the control, more than that, the performance of Replication and repair on level 2 was similar to carbon metabolism, which was

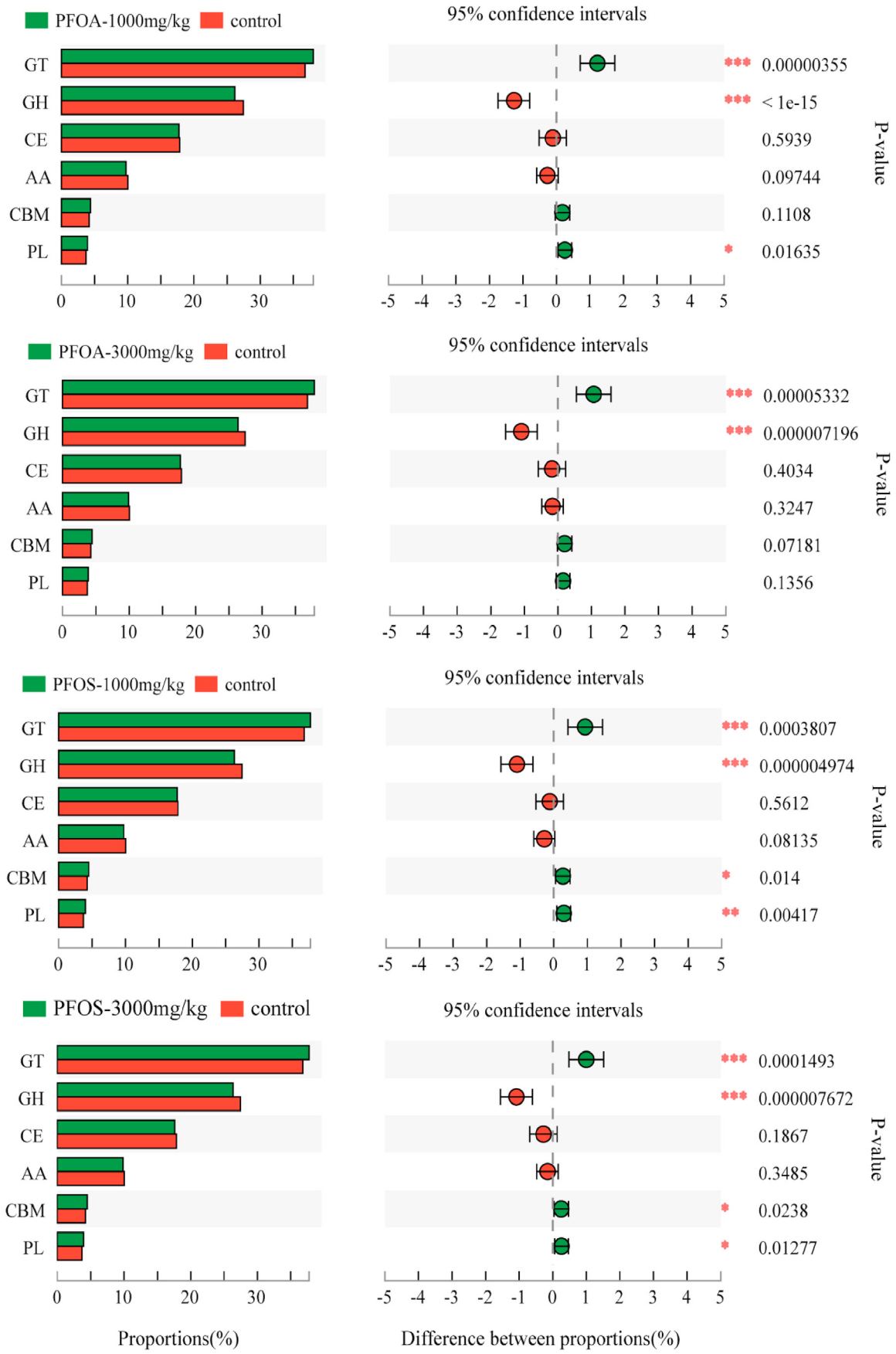

Fig. 6. Fisher's exact test on Class level of CAZy. On the left is the bar graph represents the certain CAZy function abundance in each sample, the right graph shows the proportion of differences in species abundance within a set confidence interval. 0.01 < p ≤ 0.05*, 0.001 < p < =0.01**, p ≤ 0.001***.

consistent with the previous study. The relative abundances of some microbial metabolic pathways, such as anaerobic carbon fixation, fermentation, lignolysis, glycolysis, and methanogenesis were reported to have an association with the dissolved organic matter composition, which represented the main obstacle of soil biogeochemistry (Li et al., 2018a). Glycolysis (ko00010) is an indispensable pathway for organisms to catabolize glucose, converting glucose to pyruvate and producing a small amount of ATP and NADH (van Maris et al., 2004). The active glycolysis could favor a higher phenol degradation rate in oxygen-diffused microbial electrochemical systems (MESs) (Zhou et al., 2020). Pb stress was proved to cause serious impacts on upstream process of energy production via enzymes and control genes in the process of glycolysis (Li et al., 2018b), in comparison to, another heavy metal, copper, whose addition on co-fermentation of food waste and waste activated sludge enhanced the activity of glycolysis, increasing lactic acid production (Ye et al., 2018).

KO (KEGG Orthology) is a classification system for proteins (enzymes) with highly similar sequences, and proteins with similar functions on the same pathway are grouped. There are some specific KO selected through differences analysis on two level 3 pathways, Carbon metabolism and glycolysis (Fig. S2, Fig. S3). KO with significant differences and higher relative abundance in ko01200 were K03520 (aerobic carbon-monoxide dehydrogenase large subunit, EC:1.2.5.3), K01895 (acetyl-CoA synthetase, EC:6.2.1.1), K00164 (2-oxoglutarate dehydrogenase E1 component, EC:1.2.4.2) and K00382 (dihydrolipoamide dehydrogenase, EC:1.8.1.4), while in ko00010 were K00382, K00627 (dihydrolipoamide acetyltransferase, EC:2.3.1.12), and K00873 (pyruvate kinase, EC:2.7.1.40). K00382 (DLD) in ko00010 participate in synthesis of Dihydrolipoamide-E and Lipoamide-E, while in ko01200, K00382 (DLD) shorten the process of glycine into ammonia. K01895 is involved in transforming Acetate into Acetyl-CoA. K00627 is the enzyme that catalytic formation of Dihydro-lipoamide-E and the mutual transformation of Acetyl-CoA and S-Acetyl-dihydrolipoamide-E. K00873 is the key enzymes to transform Phosphoenolpyruvate to Pyruvate.

So far, the analysis of metagenomics-based datasets mainly rely on alignment of sequences against those in databases, for example, the Carbohydrate-Active Enzyme (CAZy) database, which specialized in the enzymes that set up and breakdown complex carbohydrates and glycoconjugates (Cantarel et al., 2008). The variation of carbohydrate-active enzyme gene abundances based on CAZy analysis were shown in Fig. 5. The enzymes abundance ranked in decrement order as: GTs, GHs, carbohydrate esterases (CEs), auxiliary activities (AAs), carbohydrate-binding modules (CBMs) and polysaccharide lyases (PLs). Among each kind enzyme, the number of sequences in five soil groups ranked in order as: PFOS-1000 mg/kg and PFOS-3000 mg/kg > PFOA-3000 mg/kg > control and PFOA-1000 mg/kg. The results indicated that such enzymes were significantly elevated in PFOS-amended soils and inhibited in the PFOA-1000 mg/kg sample, scilicet the carbohydrate-active enzymes could resist more stress in PFOS-contaminated soils than PFOA's. The adoption of Fisher's exact test on Class level revealed two enzymes, GTs and GHs, had highest gene counts and distinct differences between the control sample and contaminated-soil samples (Fig. 6). As reported, GHs and GTs played key roles in the enzymatic breakdown to polymetric substrates (Roth et al., 2017). As one of the most abundant enzymes in previous metagenomic soil studies (Manoharan et al., 2015), GHs can be found in almost all organisms, and it can hydrolyze the glycosidic bonds of various sugar-containing compounds to form monosaccharides, oligosaccharides, or sugar complexes. Within the GHs family, lytic transglycosylases were essential in the metabolism of cell wall heteropolymer (Blackburn and Clarke, 2001). Fisher's exact test on Family level picked some enzymes differed in relative abundance between PFCs polluted soils and blank group (Fig. S4). For example, GH74 and GT83 were the shared enzymes in all test results. GH74 endoglucanase is an important family with highly specific, processive endo-xyloglucanase that can hydrolyze the polysaccharide backbone, also, it enhances saccharification of barley straw (Arnal et al., 2018; Badhan et al., 2018). GT83 was identified as one of the representative families after genome encodes in *Paludisphaera borealis* PX4(X) by analyzing its genome (Ivanova et al., 2017), and the bacterium is aerobe of Gram-negative, chemo-organotrophic and mildly acidophilic.

4. Conclusions

PFCs pollutants could increase soil bacterial richness and community diversity. Taxonomic profiling revealed that *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, and *Gemmatimonadetes* were the dominant bacterial phyla in the present study, accounting for more than 95.8%. Two genera detected, *Bacillus* and *Sphingomonas*, were subject to varying degrees of suppression by PFCs pollution. Based on KEGG analysis, some pathways such as Carbohydrate metabolism, Global and overview maps and Membrane transport in the soil microbes were affected by PFCs stress. CAZy analysis revealed the most abundant enzymes as glycosyl transferases (GTs) and glycoside hydrolases (GHs), and GTs were more active in PFCs-polluted soils, GHs were inhibited severely. The functional pathways, enzymes and community diversity were regarded to be regulated by microbial proteins and genes. Therefore, the practical operation on gene regulations influenced by PFCs can be a promising research on figure out the toxic mechanism.

Author contribution statements

Yanping Cai and Huilun Chen make equal contributions to conception and design, and/or acquisition of data, and/or analysis, interpretation of data and writing the manuscript; Fei Wang and Rongfang Yuan participate in revising the manuscript critically for important intellectual content; Zhongbing Chen and Beihai Zhou give final approval of the version to be submitted and any revised version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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