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RESEARCH ARTICLE

Effects of perfluorooctanoic acid (PFOA) on activated sludge microbial community under aerobic and anaerobic conditions

Duanyi Huang[1,2] · Rui Xu[3] · Xiaoxu Sun[3] · Yongbin Li[3] · Enzong Xiao[4] · Zhimin Xu[5] · Qi Wang[3] · Pin Gao[3] · Zhaohui Yang[1,2] · Hanzhi Lin[3] · Weimin Sun[3,6,7]

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Abstract

Per- and polyfluoroalkyl substances (PFASs) have received increasing attention due to their widespread presence in diverse environments including wastewater treatment plants (WWTPs) and their potential adverse health effects. Perfluorooctanoic acid (PFOA) is one of the most detected forms of PFASs in WWTPs. However, there is still a paucity of knowledge about the effect of PFASs on microorganisms of the key component of WWTP, activated sludge. In this study, lab-scale microcosm experiments were established to evaluate the influences of PFOA on activated sludge microbes under aerobic and anaerobic conditions. The diversity, structure, and microbe-microbe interaction of microbial community were determined by 16S rRNA gene amplicon sequencing and co-occurrence network analysis. After 90 days of exposure to PFOA, activated sludge microbial richness decreased under both aerobic and anaerobic conditions. Specifically, under aerobic condition, *Rhodopseudomonas* (mean relative abundance 3.6%), *Flavobacterium* (2.4%), and *Ignavibacterium* (6.6%) were enriched in PFOA-spiked activated sludge compared with that in the unspiked sludge (2.6%, 0.1%, and 1.9%, respectively). By contrast, after 90 days of exposure to PFOA, *Eubacterium* (2.1%), *Hyphomicrobium* (1.8%), and *Methyloversatilis* (1.2%) were enriched under anaerobic condition, and more abundant than that in the control sludge (0.4%, 1.5%, and 0.6%, respectively). These genera were the potential PFOA-resistant members. In addition, *Azospirillum* and *Sporomusa* were the most connected taxa in PFOA-aerobic and PFOA-anaerobic networks, respectively. Prediction of the functional gene showed that PFOA inhibited some gene expression of sludge microbes, such as transcription, amino acid transport and metabolism, and energy production and conversion. In summary, continued exposure to PFOA induced substantial shifts of the sludge bacterial diversity and composition under both aerobic and anaerobic conditions.

Keywords Perfluorooctanoic acid (PFOA) · Activated sludge · Microbial community · High-throughput sequencing · Co-occurrence network

Introduction

Per- and polyfluoroalkyl substances (PFASs, C_nF2_{n+1}–R) are a group of ubiquitous synthetic compounds, which is used extensively in a wide range of industrial and consumer applications. Owing to the unique strong carbon–fluorine (C-F) bond of the molecule, PFASs display oil and water repellency, high chemical and thermal stability, and surfactant properties (Lehmler 2005; Lindstrom et al. 2011). Among them, perfluorooctanoic acid (PFOA) is one of the most widely detected groups of PFASs. However, considerable studies show that PFOA exerts toxic effects on animals and human, such as endocrine disruption, reproductive toxicity, developmental toxicity, immunotoxicity, and neurotoxicity (Mariussen 2012; Sun et al. 2016). To be worse, high

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Duanyi Huang and Rui Xu contributed equally to this work.

✉ Zhaohui Yang
yzh@hnu.edu.cn

✉ Hanzhi Lin
hanzhi_lin@126.com

✉ Weimin Sun
wmsun@soil.gd.cn

Extended author information available on the last page of the article

concentrations of PFOA have been widely detected in various natural environments, including river, sediment (Wang et al. 2019), soil (Zareitalabad et al. 2013), and wastewater treatment plants (Higgins et al. 2005). Therefore, before designing any applicable removal protocols, it becomes more urgent for environmentalists to investigate and evaluate the impact of PFOA on these environments.

Among these impacted environments by PFOA, wastewater treatment plants (WWTPs) gained increasing attentions because of their pivotal function in modern cities to receive and treat municipal and industrial wastewater. Activated sludge is a major contributor of WWTP to pollutant removal (Jiang et al. 2008). However, activated sludge is likely to be affected by high concentration of PFOA. PFOA is ubiquitous in wastewater with various concentrations ranging from 58 to 1050 ng/L (Sinclair and Kannan 2006). Some wastewater, such as aqueous film forming foam (AFFF)–contaminated wastewater or specific industrial wastewater, contains high concentrations of PFASs up to milligram per liter (mg/L) level (Arias et al. 2015; Houtz et al. 2013). Due to the low lipophilicity and solubility of long-chain PFASs, PFOA is preferential partitioning in the sludge during wastewater treatment (Zhang et al. 2013). By getting into WWTPs via domestic and industrial wastewater, PFOA could remain and accumulate in these plants through the process of biological treatment attributed to its recalcitrance to biodegradation (Liou et al. 2010). Conventional water treatment processes are ineffective in removing PFOA (Abunada et al. 2020). Moreover, precursor biodegradation may lead to an extra addition of PFOA (Sinclair and Kannan 2006). Consequently, PFOA is usually dominant in wastewater and activated sludge of WWTPs, and cannot be efficiently removed by conventional wastewater treatment. Given the bioaccumulation and toxicity of PFOA in WWTPs, it is necessary to investigate the impact of PFOA on sludge microbiota.

The structure and function of activated sludge microbial community are important factors affecting the efficiency and stability of most WWTPs (Wang et al. 2014). Studies indicated that PFOA might influence the geochemical behaviors and activities in activated sludge. And PFOA might induce stress to inhibit the growth of specific microbiome (Nobels et al. 2010). For example, PFOA inhibited nitrification rate of activated sludge (Chen et al. 2020), and suppressed the acidification and methanation of activated sludge anaerobic digestion (Wang et al. 2021). However, to date, the research on effects of PFAS on microorganisms are mainly focused in soil and sediment with limited studies on activated sludge microbiota. Qiao et al. (2018) applied a greenhouse experiment to investigate the effects of PFASs on soil microbiota and found that PFAS pollution significantly disturbs the normal function of soil microorganisms. Zhang et al. (2017b) measured the effect of 6:2 FTOH on a sediment microbial community under aerobic condition and potential 6:2 FTOH degraders and tolerant strains were identified in the sediment samples. Moreover, previous studies primarily focused on the effects of PFAS on activated sludge under fixed redox condition (mostly under aerobic conditions), ignoring the fluctuating oxygen content during biological treatment processes (Kassab et al. 2010). Yu et al. (2018) set up a sequencing batch reactor system to monitor dynamics of microbial communities under aerobic condition. Yang et al. (2020) reported that PFOA inhibited the cyclic transformations of polyhydroxyalkanoates and glycogen by the batch experiments with PFOA and aerobic granular sludge. Therefore, a comparative analysis under diverse redox conditions is necessary; giving different oxygen contents may give rise to different effects on the fate of PFOA.

In the current study, microcosm experiment was designed to investigate the PFOA stress on microbiome in activated sludge. PFOA was selected as representative PFAS owing to its ubiquity. Aerobic and anaerobic culture conditions were set up to observe similarity and difference of microbiome affected by PFOA in activated sludge. The diversity, composition, and interactions of microbial communities of activated sludge were determined by the high-throughput sequencing and statistical analyses. The objective of the present study was to elucidate the impacts of PFOA on microbial communities of activated sludge under aerobic and anaerobic conditions.

Materials and methods

Sludge collection

Inoculum sludge was collected from a secondary sedimentation tank of a municipal waste water treatment plant (WWTP) located in Guangzhou, Guangdong province, China, whose mixed liquor suspended solid (MLSS) concentration was 14.7 g/L and mixed liquor volatile suspended solid (MLVSS) concentration was 6.3 g/L. This WWTP adopted traditional anaerobic-anoxic-aerobic processes. Collected sludge samples were stored in a cryogenic tank during transportation and then stored at 4℃ in the laboratory. The evenly mixed sludge samples were divided into two identical parts, one for molecular analysis and the other for microcosm establishment.

Chemicals and reagents

All the chemicals used in the experiments were of analytical grade. PFOA (CAS# 335–67-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The PFOA stock solution consisted of PFOA dissolved in methanol (> 99.8%) with the final concentration of 100 mg/L. Mineral salt medium (MSM) was used to construct the microcosms. It is

composed of $Na_2HPO_4{\cdot}12H_2O$ (10.55 g/L), NH_4Cl (0.3 g/L), $MgCl_2{\cdot}6H_2O$ (0.1 g/L), KH_2PO_4 (1.5 g/L), vitamin solution (1 mL/L), and trace element solution (1 mL/L) (Xu et al. 2021a).

Microcosm experimental design

Aimed to evaluate the effects of PFOA on activated sludge under two distinct redox conditions, aerobic and anaerobic microcosms with activated sludge as inoculum were established respectively. Microcosms were constructed in triplicate for each treatment at different time points (30, 60, 90 days). To set up the aerobic microcosms, 50 mL activated sludge sample and 150 mL autoclaved MSM were first transferred into a clean sterile 250-mL serum bottle followed by 5 days of equilibration (Xu et al. 2021d). Two treatments were then established after equilibration by adding (1) 500 µL PFOA stock solution whose final concentration in the experiment was 0.25 mg/L (hereinafter, denoted as "PA_O" group); or (2) equal volume of methanol without PFOA (denoted as "Ct_O") (Yu et al. 2018). To ensure adequate oxygen content, the microcosms were sealed with air permeable parafilm. All microcosms were then incubated at 25℃ in the dark (Li et al. 2021b).

The establishment and incubation of anaerobic microcosms were similar to those of aerobic microcosms, except for the addition of sodium nitrate (with a final concentration of 1000 mg/L) and nitrogen aeration for 30 min before sealing, in order to achieve anaerobic conditions. The serum bottles were sealed with butyl rubber septa and aluminum caps. Similar nomenclature was adopted in the two anaerobic treatments; i.e., group with PFOA was referred to "PA_N" and control group added with the same amount of methanol was referred to "Ct_N," respectively.

After being set up, all microcosms were cultured continuously for 90 days. Another 500 µL PFOA or methanol stock solutions were kept being added to the corresponding microcosms every 15 days (totally 6 times) to mimic the accumulation process of PFOA in sludge. After 30, 60, and 90 days of incubation, sacrificial sampling was performed on triplicate cultures from each treatment before the next addition of PFOA/methanol. A total of 39 sludge samples (including three original sludge samples, representing cultures at the 0th day) were obtained for subsequent DNA extraction and 16S rRNA gene amplicon sequencing.

Illumina high-throughput sequencing

The extraction of genomic DNA from each activated sludge sample was carried out using the Powersoil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA) in accordance with the manufacturer's protocol (Sun et al. 2020). The concentration of the extracted DNA was determined by Qubit (Invitrogen 3.0 Fluorometer) and the purity was determined by gel electrophoresis (Xu et al. 2021e). V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Kuczynski et al. 2011). PCR amplification was performed using the following thermal conditions: 60 s at 98 °C, 30 cycles of 10 s at 98 °C, 30 s at 50 °C, and 30 s at 72 °C, followed by a final extension step for 5 min at 72 °C. PCR products purified by gel extraction were sent for high-throughput sequencing on an Illumina MiSeq platform (Gao et al. 2021).

Bioinformatic and statistical analysis

After trimming of the barcodes and primers, generated raw sequences were merged and then filtered with USEARCH (v 10.0) (Xu et al. 2021d). UNOISE3 was applied to denoise the sequences retained after quality control and obtain the unique amplicon sequence variants (ASV). The ASVs were classified into taxonomy with the GreenGene database (v 13.5) (Xu et al. 2020). Functional gene prediction was performed with PICRUSt and annotated with the COG database (Langille et al. 2013).

Determination of alpha-diversity and beta-diversity was performed in R by "–alpha_div" and "–beta_div" function (Gao et al. 2021). MicrobiomeAnalyst platform was used for comprehensive statistical analysis, including analysis for the relative abundance (Xu et al. 2021b). Additionally, STAMP software was employed to analyze the difference in genus level with Welch's *t* test among groups and filtered the genera with a *p* value exceeding 0.05 and effect sizes smaller than 0.1 (Parks et al. 2014). Co-occurrence networks was calculated with the R package "ggcorrplot" and was visualized by Gephi v 0.29 software (Sun et al. 2021; Xu et al. 2021c). The significance level of all tests was set at 0.05.

Quantitative PCR

Total bacteria biomass was estimated in this experiment by quantitative PCR targeting 16S rRNA using the primers 338F and 518R (Xu et al. 2020). The quantitative PCR reaction was performed in a volume of 20 µL containing 10 µL TB Green Premix Ex Taq (Tli RNaseH Plus) (Takara, Japan), 0.2 µL of each primer (10 pmol/µL), 1 µL of tenfold diluted template DNA (1–10 ng), and 8.6 µL of double-distilled H_2O (ddH_2O). The qPCR was performed on a CFX96 Real-Time System (Bio-Rad, USA) using the following thermal conditions: 15 min at 95 °C, 45 cycles of 15 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, with a final extension step of 72 °C for 7 min. A standard curve was constructed using a serial of tenfold diluted known-copies plasmid solution

harboring 16S rRNA genes (Li et al. 2021a). Each sample was quantified in triplicates.

Results

Richness of sludge microbial community affected by PFOA addition

Biomass and richness of microbial community under aerobic condition

As mentioned, total bacteria biomass of activated sludge was measured by qPCR of 16S rRNA gene on 30, 60, and 90 days (Fig. 1). Mean copy number of 16S rRNA gene in the PA_O treatment group was significantly lower than that with Ct_O treatment during matched incubation times. Specifically, Chao1 index was used to further determine the richness of sludge microbial communities (Fig. 2). At the first 30 days, PA_O sludge had a higher bacterial Chao1 index (1078.72) than Ct_O sludge (1038.43). However, over the course of incubation, the Chao1 value of PA_O sludge decreased gradually. Finally, the Chao1 index of bacteria in sludge treated with PFOA (1033.26) was relatively lower compared with that in control sludge (1125.07) after 90 days of incubation.

Biomass and richness of microbial community under anaerobic condition

Similar to aerobic incubation, final microbiome biomass of activated sludge was suppressed under exposure to PFOA under anaerobic condition according to the copy number of 16S rRNA gene. In detail, compared with Ct_N samples, abundance of 16S rRNA gene of PA_N samples was lower after 90 days of incubation. However, richness of activated sludge first raised promoted by coexistence with PFOA at the first 60 days, and then fell at the end of the 90-day anaerobic incubation. The mean value of Chao1 index in Ct samples was 864.71, while the value in PFOA samples was 659.23 on the 90th day.

Microbial community structure affected by PFOA

Structure of microbial community under aerobic condition

Initially in original activated sludge samples, Proteobacteria (45.8%, relative abundance), Nitrospirae (14.0%), Bacteroidetes (14.0%), and Actinobacteria (4.7%) were the

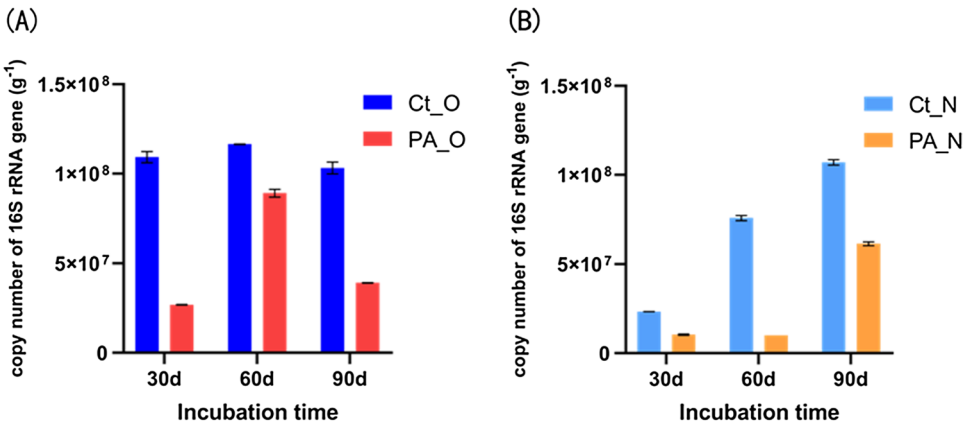

Fig. 1 The copy number of 16S rRNA gene from the sludge samples under **A** aerobic and **B** anaerobic conditions. Ct_O, without PFOA under aerobic condition; PA_O, with PFOA under aerobic condition; Ct_N, without PFOA under anaerobic condition; PA_N, with PFOA under anaerobic condition)

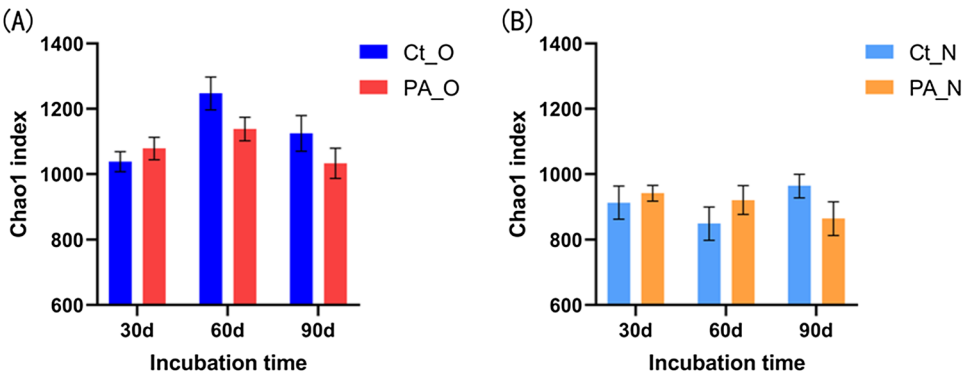

Fig. 2 Alpha-diversity of the AS microbial communities under Ct_O, PA_O, Ct_N, and PA_N treatment over 90 days of incubation. The bar chart indicates the measurements of Chao1 index under **A** aerobic condition and **B** anaerobic condition

predominant bacterial phyla. After 3 months of incubation, Firmicutes and Ignavibacteriae became the dominant phyla in control sludge.

The relative abundance of bacterial phyla of sewage sludge containing PFOA under oxic condition is shown in Fig. 3A. Proteobacteria, Bacteroidetes, Firmicutes, and Ignavibacteriae were the predominant bacterial phyla in both PFOA-added sludge and control sludge on the 90th day, accounting for more than 90% of the whole community. However, distinct structure of microbial community of the sludge amended with PFOA was observed. The long-term aerobic treatment of activated sludge with PFOA resulted in remarkable changes in microbial community composition. Proteobacteria, the most abundant phylum in the PFOA treatment (49.4%), was remarkably lower than that in the control (68.0%). In contrast, Ignavibacteriae and Bacteroidetes were more abundant in PFOA-added samples than in the control samples.

Genus level analysis of microbial community was further conducted. Top 20 bacterial genera in terms of the relative abundance are presented in Fig. 3B. Among them, *Dokdonella* and *Xanthobacter* were the abundant genera in all samples on the 90th day. But the addition of PFOA caused a decline of the abundance of *Dokdonella*, *Xanthobacter*, *Azospirillum*, and *Pseudoxanthomonas* after the 90-day incubation. Meanwhile, an obvious variation of *Azospirillum* was observed in PA_O samples. In the first 60 days, the abundance of *Azospirillum* in PA_O sludge samples was significantly higher than that in the Ct_O samples, accounting for more than 10%. However, by the 90th day, the abundance of *Azospirillum* decreased sharply and was even lower than that of the control group. Additionally, the relative abundances of *Rhodopseudomona*, *Acetobacteriums*, and *Flavobacterium* slightly increased in the samples adding with PFOA (3.6%, 3.3%, 2.4%) compared to control samples (2.6%, 0.3%, 0.1%). The proportion of *Ignavibacterium* was much higher in PFOA treatment (6.6%) than that in control samples (1.9%).

Structure of microbial community under anaerobic condition

The relative abundance of the sludge samples under anoxic condition is shown in Fig. 3C and D. Proteobacteria, Bacteroidetes, and Firmicutes were the dominant phyla in both groups under anaerobic condition. Bacteroidetes had a lower proportion in PFOA-added sludge (12.2%) than that in control treatment (24.3%) under anaerobic conditions on the 90th day. The relative abundance of Ignavibacteriae was decreased from 2.4% (Ct) to 1.0% (PFOA). The relative abundance of phylum Firmicutes remarkably increased in samples spiked with PFOA (58.8%) after 90 days of incubation, compared with the control sample (39.3%).

Genus level analysis in anaerobic samples was further performed (Fig. 3D). *Sporomusa* and *Rhodopseudomonas* consisted of a considerable proportion in the microbial community. The relative abundances of *Sporomusa* increased rapidly in sludge amended with PFOA, which was eventually 18.6% higher than that in control sludge. Additionally, *Melioribacter*, *Acetobacterium*, *Oscillibacter*, and *Candidatus_Endomicrobium* were slightly depressed over time treated with PFOA, whereas *Hyphomicrobium*, *Methyloversatilis*, and *Eubacterium* were promoted in the same group. For example, the final proportion of *Eubacterium* in PFOA-amended sludge (2.1%) was higher than that in the control sludge (0.4%).

Further genus level analysis by Welch's t test

According to the abovementioned analysis, it is observed that the addition of PFOA resulted in the variations of the composition and diversity of community in sludge. In this study, the differential species were further identified and confirmed by Welch's *t* test at the genus level (Parks et al. 2014).

Genera with significant difference under aerobic condition

Eleven significantly distinct bacteria genera were observed between the two groups under aerobic condition (Fig. 4A). The genera *Azospirillum*, *Methylobacillus*, *Acetobacterium*, *Hydrogenophaga*, *Methyloversatilis*, *Gracilibacter*, *Fluviicola*, and *Brevundimonas* demonstrated a relatively higher mean proportion in microbiota of PFOA-exposed sludge than that in Ct sludge. The mean proportions of *Sporomusa*, *Ancylobacter*, and *Anaerovorax* were lower in PFOA-amended samples.

Genera with significant difference under anaerobic condition

Genera significantly enriched in anaerobic condition are shown in Fig. 4B. A total of ten genera were identified with significant differences between PFOA-exposed sludge and Ct sludge. *Sporomusa*, of phylum Firmicutes, was the most different genus with the largest effect size, followed by *Candidatus_Endomicrobium* in Elusimicrobia. The relative abundance of *Hyphomicrobium*, *Nitrospira*, *Acetoanaerobium*, *Anaerovorax*, *Proteiniphilum*, *Sedimentibacter*, and *Gp6* decreased slightly in PFOA-contaminated sludge. This indicates a growth inhibition of these microbes brought by the addition of PFOA. All of these 10 genera with significant difference could be applied as potential biomarkers responded to PFOA under anaerobic condition.

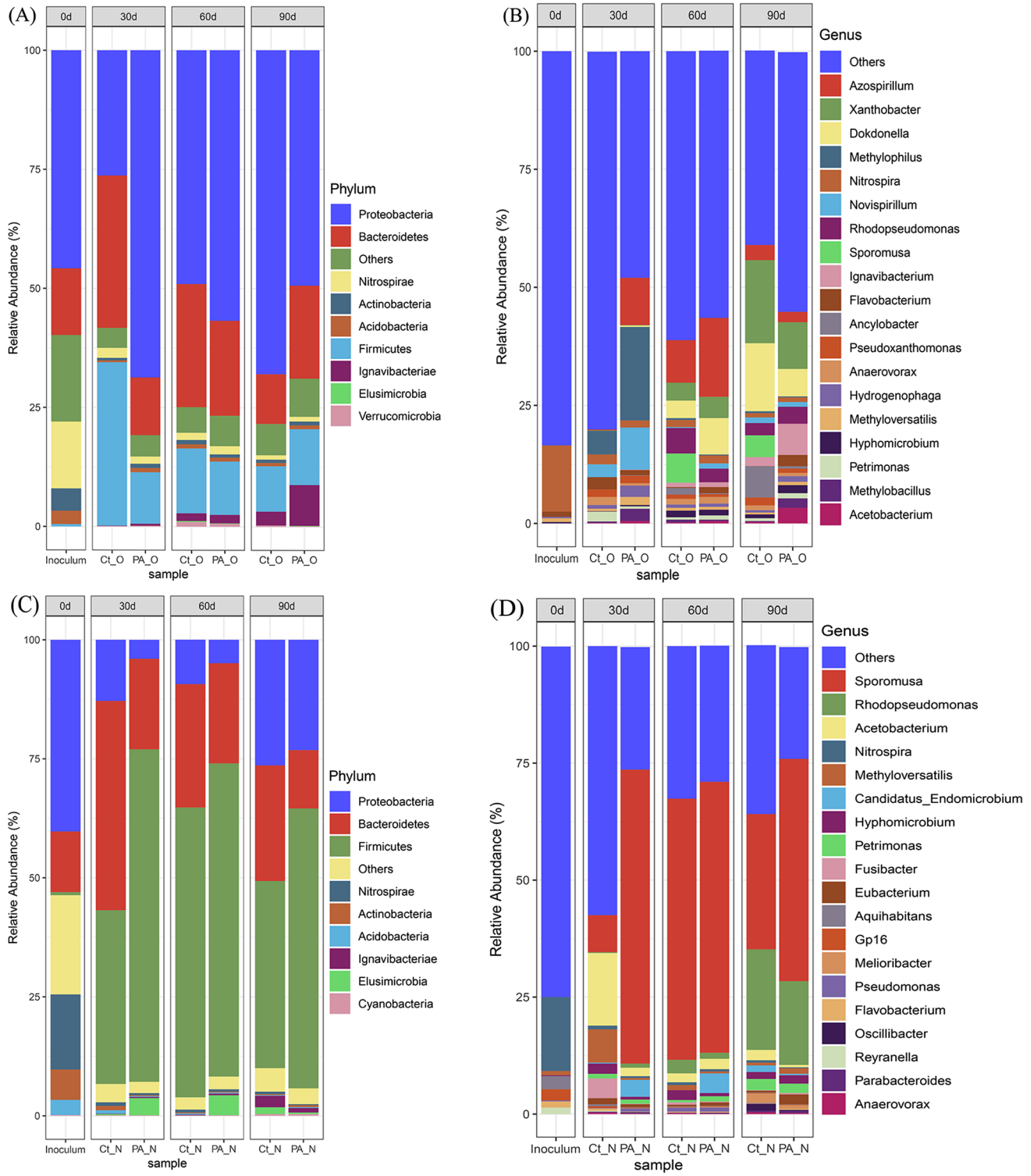

Fig. 3 Exposure of PFOA over 90 days induced a shift of compositions of AS microbial community. "Inoculum" on the X-axis represents the original sludge at the 0th day, "Ct" is the control group without PFOA, and "PA" is the treatment group with PFOA. The bar charts showed the major phyla and genera under **A**, **B** aerobic and **C**, **D** anaerobic conditions, respectively

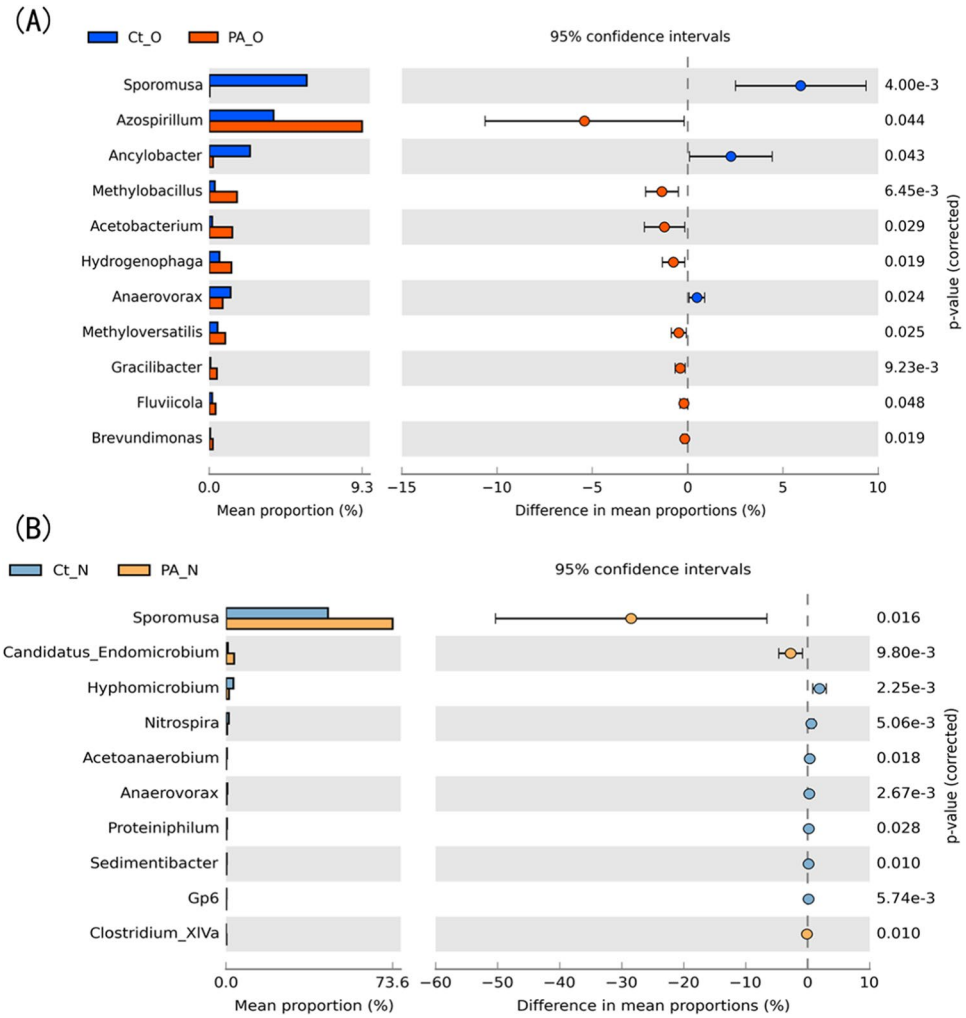

Fig. 4 The extended error bar plot of the genera with significant difference between either **A** Ct_O vs. PA_O treatment and **B** Ct_N vs. PA_N treatment

Sludge microbial interaction affected by PFOA

In this study, microbial co-occurrence networks were applied to investigate the interactions of sludge microbiota. According the experimental design, two co-occurrence networks, the "PFOA-aerobic network" and "PFOA-anaerobic network," were constructed to investigate microbial interactions in activated sludge affected by the long-term exposure of PFOA under aerobic and anaerobic conditions, respectively (Fig. 5).

Microbial interaction networks under aerobic condition

A total of 290 nodes and 6355 pair-wise correlations (3498 positive links and 2857 negative links) were obtained from the network. The majority of taxonomic nodes in PFOA-aerobic networks were mainly subordinate to Proteobacteria (58.63%), followed by Bacteroidetes (16.55%), Firmicutes (12.41%), and Nitrospirae (2.07%). The highly connected taxa at genera level can be characterized by calculating the network's topological parameters such as high average degree (Banerjee et al. 2018). Accordingly, *Azospirillum*, *Xanthobacter*, and *Methylophilus* were identified as the most connected taxonomies in PA_O community due to their high mean degree in the PFOA-aerobic network. In addition, the ratio of the number of positive and negative correlated links in the networks was also calculated. In the PFOA-aerobic network, the ratio of positive links (~55%) was close to that of negative links (~45%).

Microbial interaction networks under anaerobic condition

The PFOA-anaerobic network was generated capturing 7716 pair-wise correlations (6328 positive links and 1388 negative links in Fig. 5B) among 219ASVs (nodes in Fig. 5B). The most taxonomic nodes in the network were assigned to Firmicutes (67.56%), followed by Bacteroidetes (18.48%), Proteobacteria (6.39%), and Nitrospirae (1.37%). Genera *Sporomusa*, *Petrimonas*, and *Eubacterium* were identified as the most connected members. In the PFOA-anaerobic network, the proportion of the positive links was much larger than the negative links, which was nearly four times larger.

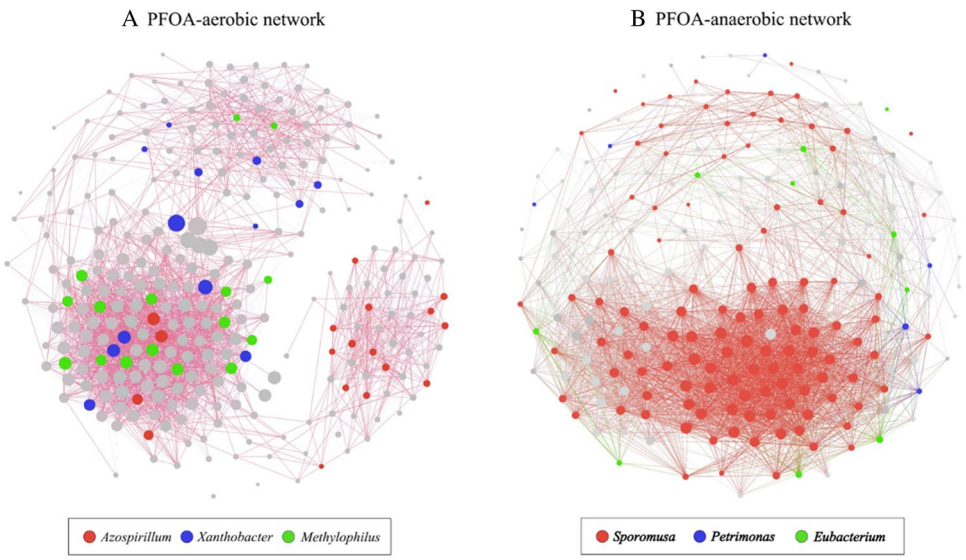

Fig. 5 The co-occurrence networks of the AS microbiomes under the **A** PA_O treatments ($n=9$) and **B** PA_N ($n=9$) treatments. The color of most connected node (top 3) is marked using the assignment at the genus level. The size of each node was proportional to its connection number (mean degree). The links represent the significant and strong Spearman's correlations ($|R|>0.85$, $p<0.05$)

Microbial functional performance in activated sludge affected by PFOA

Microbial community function analysis under aerobic condition

The prediction of functional genes of the activated sludge bacterial microbiota was performed using PICRUSt. Under aerobic condition (Fig. 6), compared with the control group, the exposure of activated sludge to PFOA for 90 days resulted in a relatively lower abundance of most functional genes, such as secondary metabolites biosynthesis transport and catabolism, transcription, amino acid transport and metabolism, chromatin structure and dynamics, energy production and conversion, inorganic ion transport and metabolism, and cell cycle control cell

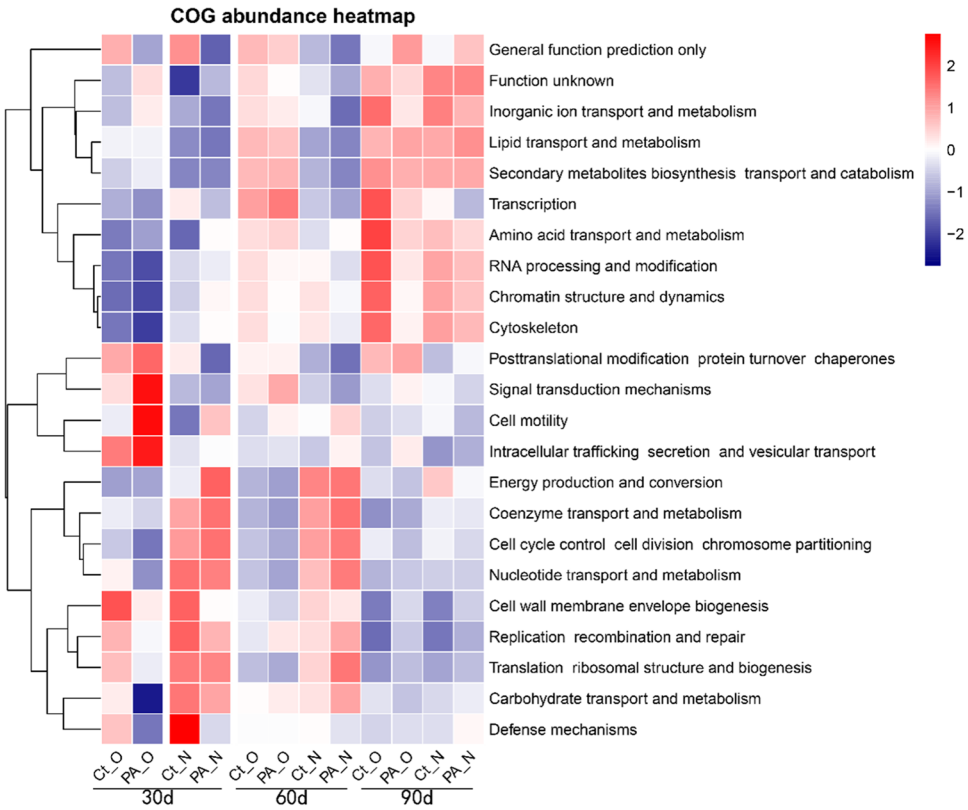

Fig. 6 Heatmap of the functional gene abundances predicted by PICRUSt in the Ct_O, PA_O, Ct_N, and PA_N treatments. The values of gene abundance were log-transformed and colored from red to blue to indicate high-to-low relative abundances

division chromosome partitioning. This lower abundance in PFOA-amended samples demonstrates that the exposure to PFOA might suppress the expression of microbial functional genes.

Microbial community function analysis under anaerobic condition

Similar to the samples under aerobic condition, PFOA also presented a certain inhibitory effect on some functional genes of the microbial community under anaerobic conditions (Fig. 6). But the abundance of many functional genes that was reduced at the end of the incubation with PFOA was different from that in aerobic condition in terms of metabolic pathways. The affected genes involved transcription, cell motility, energy production and conversion, inorganic ion transport and metabolism, signal transduction mechanisms, and chromatin structure and dynamics. Remarkably, at the 30th day, functional genes involved in cell motility, energy production and conversion, and amino acid transport and metabolism were facilitated, while, over the coming 60 days until the 90th day, abundance of these genes in PA_N gradually reduced and eventually lower than that in Ct_N. It might be due to the long-term accumulation of PFOA in activated sludge.

Discussion

Effect of PFOA on activated sludge bacterial richness

In our study, prolonged exposure to high concentrations of PFOA led to a decrease of activated sludge community richness under both aerobic and anaerobic conditions (Fig. 2). Similarly, Yu et al. (2018) also found a remarkable decrease of community richness in sludge exposed to PFOA. In soil environments, Senevirathna et al. (2022) observed similar patterns that high concentration of PFAS caused a reduction of the richness of microbial community. Bao et al. (2018) also indicated that perfluorooctanesulfonate (PFOS) lowered the microbial richness and diversity. These results were consistent with the current study.

However, the above related studies were all carried out under aerobic conditions. Few published results reveal the effects of PFOA on activated sludge microbial community richness under anaerobic condition. Our study found that the richness of PFOA-amended activated sludge decreased under anaerobic conditions. The anaerobic PFOA-treated samples had fewer copy numbers of the 16S rRNA gene than the control group, also supporting the current finding of reduced richness of activated sludge exposed to PFOA (Fig. 1).

In general, microbiota with higher richness are able to maintain a more stable ecology (Hooper and Macpherson 2010). And high richness implies a greater probability of functional redundancy, which can increase the community's resilience to disturbance (Zhang et al. 2019). Therefore, our results indicate exposure to PFOA aerobically and anaerobically might both restrain the biomass growth and decrease the richness of activated sludge due to its cumulative toxicity, and might further influence the stableness of activated sludge bacteria consortia.

Effect of PFOA on bacterial community composition and structure in activated sludge

The addition of PFOA induced instinct changes of the composition of sludge microbial community under both aerobic and anaerobic conditions (Fig. 3). In our study, the high abundance of Proteobacteria, Bacteroidetes, and Firmicutes maintained over time of all groups regardless of aerobic and anaerobic conditions, indicating their tolerance to PFOA. Proteobacteria and Bacteroidetes have been found as the most abundant phyla in the aerobic sequencing batch reactor (SBR) continuously exposed to PFOA (Yu et al. 2018).

Under aerobic condition, Proteobacteria was the most abundant phylum in PFOA-spiked sludge, although its relative abundance was lower than in the control sludge. However, Senevirathna et al. (2022) reported that the relative abundance of Proteobacteria in PFAS-rich contaminated soil was higher than that in uncontaminated soil, which could be the result of different experimental subject and physicochemical parameters. Moreover, the abundance of some genus in PFOA-amended treatment increased with a variety of degrees, such as *Ignavibacterium*, *Rhodopseudomona*, and *Flavobacterium.* Enrichment of these taxonomies might be attributed to their preference, tolerance, or degradation of PFOA. Previous studies draw similar conclusions. *Fluoroacetate dehalogenase* (FAcD) RPA1163, a defluorinase, was already discovered from *Rhodopseudomonas palustris CGA009*, indicating that *Rhodopseudomona* might have the potential to defluorinate PFOA (Chan et al. 2011). Both *Ignavibacterium* and *Flavobacterium* possess dechlorinated potential. *Ignavibacterium* could enhance dechlorination of polychlorinated biphenyl (Yu et al. 2016). *Flavobacterium* was the classical aerobic pentachlorophenol (PCP)–degrading bacteria (Bosso and Cristinzio 2014). Since both fluorine and chlorine are halogen elements, they share similar physical and chemical properties, so it can be speculated that their metabolic pathways are likely to be similar (Neufeld and Wolfire 2009). Hence, *Ignavibacterium* and *Flavobacterium* might be resistant to PFOA, and more laboratory studies are needed in the future to investigate their potential for defluorination.

Under anaerobic condition (Fig. 3A), Firmicutes increased to be the most abundant phylum in PFOA-spiked sludge, suggesting that they have higher tolerance to the high concentration of PFOA. Firmicutes widely exists in the environment and has the ability to degrade a variety of organic pollutants (Li et al. 2021c; Wolfe et al. 2014; Zhang et al. 2018). The enrichment of genera *Sporomusa*, *Hyphomicrobium*, and *Eubacterium* also indicated their potential to tolerate or degrade PFOA. *Sporomusa* is known as an anaerobic methanol-utilizing bacterium (Ammam et al. 2016), and has been isolated from varied environments such as rice paddy soil, forest soil, sediment of river, and gut of termites (Balk et al. 2010). The high abundance of *Sporomusa* in sludge either with or without PFOA might be as a result of the addition of methanol in both groups. Besides, *Sporomusa* was reported as an acetogenic bacterium with the capacity to oxidize a wide range of organic substrates (Balk et al. 2010). Previous studies reported that *Eubacterium* was resistant to high concentrations of hexadecyltrimethylammonium bromide (CTAB) under anaerobic conditions (Keith and John 2001). *Eubacterium* processes the capacity to adapt to complex pollutant environments (Zhang et al. 2017a). The increasing number of *Eubacterium* in PA_N suggested their potential capability to resist the toxicity of PFOA under anaerobic condition.

PFOA usually played a dual role in the sewage sludge microbial community. At the same time, the inhibitory effect of PFOA on certain bacteria in the sludge community was also observed. Under aerobic condition, the relative abundance of genera *Dokdonella*, *Azospirillum*, *Pseudoxanthomonas*, and *Xanthobacter* was decreased with PFOA treatment compared with the Ct group. Among them, *Dokdonella*, *Pseudoxanthomonas*, and *Xanthobacter* show a substantial denitrification capacity during wastewater biological treatment (Long et al. 2019; Wang et al. 2018a; Zhang et al. 2020). Under anaerobic condition, *Melioribacter*, *Acetobacterium*, *Oscillibacter*, and *Candidatus_Endomicrobium* were suppressed over the incubation course with the presence of PFOA. Notably, *Melioribacter* is also a denitrifier in sludge (Han et al. 2021). Additionally, *Acetobacterium* and *Oscillibacter* are acid-producing bacteria. They play important roles in acidification process of activated sludge, which are capable of improving the degradation of organic matter in wastewater (Zhou et al. 2015). Thus, PFOA might affect the sludge performance of biological treatment due to its inhibitory effect on denitrification and acidification processes under both aerobic and anaerobic conditions.

The decline of these microorganisms suggests that they are vulnerable to PFOA's toxicity. PFASs with long carbon chain may induce expression of several stress genes, resulting in membrane damage, oxidative damage, and DNA damage (Nobels et al. 2010). PICRUSt results in this study also supported that the majority of metabolic functions and gene expression was repressed in the PFOA group, such as nucleotide transport and metabolism, and secondary metabolite biosynthesis transport and catabolism (Fig. 6). These evidences could explain the decrease of this taxonomic richness. The community profiling results suggest the necessity of further investigations on physiology of the above taxonomies, which will help better assess their exact role in sludge communities exposed to PFOA.

Highly connected microbial taxonomies as responses to PFOA

Highly connected taxonomies play important roles for community structure and function (Banerjee et al. 2018). *Azospirillum*, *Xanthobacter*, and *Methylophilus* were identified as the most connected taxonomies in the PA_O community. The abundance variation of all these three microbes showed a rising trend at the first 60 days and then decreasing until the 90th day, which may be related to the overall gradual increase of PFOA concentration, ultimately exceeding their PFOA tolerance threshold. *Azospirillum* is known as typical associative N_2-fixing bacteria (Steenhoudt and Vanderleyden 2000). It also has been reported to degrade a wide variety of organic pollutant such as polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs) (Godini et al. 2019; Grobelak et al. 2020). In this study, *Azospirillum* was the genus with significant difference between PFOA-amended and unamended sludge samples; thus, the *Azospirillum* could be used as biomarkers to indicate the effect of PFOA. *Xanthobacter* was also recognized as nitrogen-fixing bacteria (Arzumanyan et al. 1997). *Xanthobacter* and *Methylophilus* processed the potential of dehalogenation and might play a key role in the PA_O community. *Xanthobacter* was reported to catalyze the conversion of halogenated hydrocarbons to their corresponding alcohols through hydrolytic haloalkane dehalogenase (Erable et al. 2005). *Methylophilus* was characterized as methylotrophic bacteria (Babbitt et al. 2009). Members of *Methylophilus* were identified to utilize dichloromethane as the sole carbon source with dichloromethane dehalogenase (Chaignaud et al. 2017).

Genera *Sporomusa*, *Petrimonas*, and *Eubacterium* were identified as the most connected taxonomies in the PFOA-anaerobic network (PA_N) by screening the bacteria with high average degree. Experiment results showed that the abundances of *Sporomusa* and *Eubacterium* were enriched in PFOA-exposed sludge. The enrichment of *Sporomusa* and *Eubacterium* was probably due to their potential to resist PFOA, which was discussed above. The abundance of *Petrimonas* was slightly enriched with PA_N treatment. As *Petrimonas*, a popular fermentative bacterium, was able to provide electron donor by degrading glucose for dichlorination (Wang et al. 2018b), *Petrimonas* in PA_N microbial

consortium might exert assistant effects by providing extra nutrients.

When a large proportion of the members of a microbiome community are linked together by positive correlation, it tends to be unstable. This is because in such a community, positively correlated species respond to environmental fluctuations in the same way, leading to positive feedback. On the contrary, negative links representing competitive relationship may stabilize the community by suppressing the positive feedback loop (Coyte et al. 2015). In the PFOA-aerobic network (PA_O), the proportion of positive and negative correlation lines is closer, while in the PFOA-anaerobic network (PA_N), the ratio of negative correlations was much lower than that of positive one. Therefore, it can be inferred that the influence of PFOA on the stability of sludge microbial community under aerobic condition is relatively smaller than that under anaerobic condition.

The impact of PFOA on sludge function

Through functional prediction in the present study, it was found that PFOA exerted negative effects on the abundance of some specific genes at different oxygen levels. Similarly, previous studies had shown that PFASs with long carbon chain length inhibited the expression of genes responsible for defense mechanisms and cell wall membrane envelope biogenesis and also inhibited the enzyme activities of soil as well (Qiao et al. 2018). PFOA declined the translation of some mRNA and then further inhibited the transcription of the proteins (Choi et al. 2013).

In the current study, genes involved in transcription, amino acid transport and metabolism, energy production and conversion, chromatin structure and dynamics, inorganic ion transport and metabolism, and cell cycle control cell division chromosome partitioning were decreased in both aerobic and anaerobic conditions. Amino acid not only acts as the substrate to synthesize protein but also plays a regulatory role as signaling molecules (Sturme et al. 2002). Hence, it could be further speculated that cellular structure of bacterial cells may be damaged, resulting in the inactivation or death of some bacteria. In addition, the inhibition of amino acid transport and metabolism might further interrupt the synthesis and function of protein. The declined abundance of genes linked to energy production and conversion demonstrates that a wide range of microorganisms in activated sludge are susceptible to the selective pressure of PFOA and their ability to metabolize different energy sources was restrained (Milojevic and Weckwerth 2020). The results indicated that PFOA posed a potential threat to sludge function under both aerobic and anaerobic conditions. More evidences at microbial genomic and environmental metagenomic level are expected to uncover the effects of PFOA on AS microorganisms.

Conclusions

Both aerobic and anaerobic microcosm experiments were conducted to study the PFOA stress on microbiome in activated sludge. Substantial shifts in microbial community of activated sludge were observed, supported by the results of the appreciable variations of the structure, richness, and interaction of the bacterial community. After 90 days of coexistence with PFOA, sludge microbiota richness eventually decreased under both aerobic and anaerobic conditions compared with their counterparts amended with methanol only. Functional gene prediction indicated that the long-term exposure of PFOA may inhibit some sludge microbial metabolism processes in activated sludge microbiota including transcription, amino acid transport and metabolism, and energy production and conversion.

However, PFOA additions led to enrichment of potential PFOA-degrader or PFOA-resistant bacteria, such as *Rhodopseudomonas*, *Flavobacterium*, and *Ignavibacterium* under aerobic condition, while under anaerobic condition, the abundance of *Eubacterium*, *Hyphomicrobium*, and *Methyloversatilis* increased. The genera *Azospirillum* and *Sporomusa* were identified as the most connected nodes in the PFOA-aerobic and PFOA-anaerobic co-occurrence network, respectively.

Further studies are needed to investigate the potential risk of PFOA and identify the PFOA-degraders and their corresponding degrading mechanism. The bacteria with significant variation responding to the exposure of PFOA in this study are worthy studying as environmental biomarkers to PFOA and further to the PFAS group.

Author contribution Duanyi Huang wrote the original draft, and Zhaohui Yang, Hanzhi Lin, and Weimin Sun contributed to the conception of the study. Rui Xu, Xiaoxu Sun, Yongbin Li, and Duanyi Huang performed the experiment and data analysis. Rui Xu, Hanzhi Lin, Enzong Xiao, Zhimin Xu, Qi Wang, and Gao Pin offered comments for revisions to the manuscript. All authors read and approved the final manuscript.

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Data availability The nucleic acid reads of 16S rRNA genes in the current study are available in the NCBI database with the project number PRJNA755805.

Declarations

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Authors and Affiliations

Duanyi Huang[1,2] · Rui Xu[3] · Xiaoxu Sun[3] · Yongbin Li[3] · Enzong Xiao[4] · Zhimin Xu[5] · Qi Wang[3] · Pin Gao[3] · Zhaohui Yang[1,2] · Hanzhi Lin[3] · Weimin Sun[3,6,7]

[1] College of Environmental Science and Engineering, Hunan University, Changsha 410082, People's Republic of China

[2] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, People's Republic of China

[3] National-Regional Joint Engineering Research Center for Soil Pollution Control and Remediation in South China, Guangdong Key Laboratory of Integrated Agro-Environmental Pollution Control and Management, Institute of Eco-Environmental and Soil Sciences, Guangdong Academy of Sciences, 808 Tianyuan Road, Guangzhou 510650, China

[4] Key Laboratory of Water Quality and Conservation in the Pearl River Delta, Ministry of Education, School of Environmental Science and Engineering, Guangzhou University, Guangzhou 510006, China

[5] College of Resources and Environment, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China

[6] School of Environment, Henan Normal University, Xinxiang, China

[7] Key Laboratory of Yellow River and Huai River Water Environment and Pollution Control, Ministry of Education, Xinxiang, China