Effect and mechanism of perfluorooctanoic acid on extracellular polymeric substances of microorganisms during biological wastewater treatment

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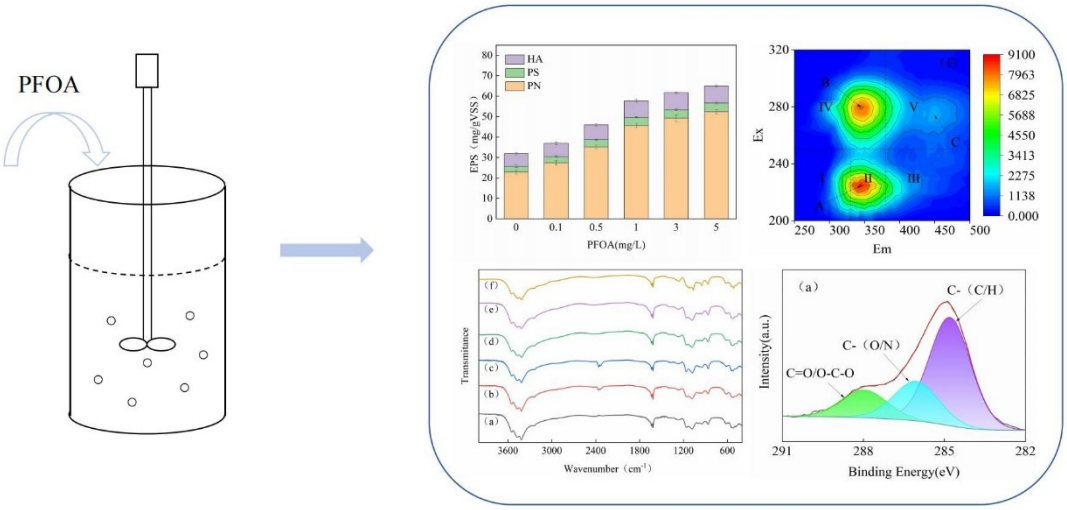
Graphical Abstract



Abstract

Perfluorooctanoic acid is ubiquitous in wastewater, bioaccumulative and biotoxic, and interferes with biological wastewater treatment. Extracellular polymeric substances are important components of microbial cells. The effects of perfluorooctanoic acid (0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 3.0 mg L^{-1}, and 5.0 mg L^{-1}) on extracellular polymeric substances were researched and the mechanisms were uncovered. The results indicated that the tightly bound extracellular polymeric substances (TB-EPS) initially increased and then decreased with the increase of perfluorooctanoic acid concentration, whereas the loosely bound extracellular polymeric substances (LB-EPS) consistently increased. Three-dimensional fluorescence spectroscopy revealed that the fluorescence intensity of TB-EPS components decreased, while that of LB-EPS components increased with the increase of perfluorooctanoic acid concentration. Fourier-transform infrared spectroscopy indicated the absorption peaks of functional groups, C=O, C-OH, C-O-C, C-N, or N-H in both TB-EPS and LB-EPS, shifted with the increase of perfluorooctanoic acid concentration. Protein secondary structure analysis demonstrated that perfluorooctanoic acid reduced the proportion of α-helices, leading to loose protein structures. Additionally, X-ray photoelectron spectroscopy showed that, as

the concentration of perfluorooctanoic acid increased, the amount of C=O and O-C-O groups in LB-EPS increased and the proportion of C-(C/H) groups in TB-EPS decreased.

Keywords: Perfluorooctanoic acid, Biological wastewater treatment, Extracellular polymeric substances, Functional groups, Protein secondary structure

1. Introduction

Perfluorooctanoic acid is widely used in textile, semiconductor, food packaging, and fire apparatus manufacturing industries(Forster et al., 2024). Current research has found that perfluorooctanoic acid is highly bioaccumulative and biotoxic, and prevalent in wastewater, drinking water(Sinkway et al., 2024), surface water(Zhu et al., 2024), sediment(T. Li et al., 2024), and soil(Lv et al., 2023). Studies have shown that perfluorooctanoic acid has an effect on biological wastewater treatment. Yu(X. Yu et al., 2018) et al. found that perfluorooctanoic acid inhibited the growth of microorganisms and affected the removal of dissolved organic carbon when perfluorooctanoic acid was 20 mg L^{-1}. In the sequence batch reactor, perfluorooctanoic acid inhibited the activities of nitrate reductase, nitrite reductase, exophosphatase, polyphosphate kinase, and other major functional enzymes for denitrogenation and phosphorus removal, thus affecting the treatment effect of the reactor(X. Zheng et al., 2023). Li(W. Li et al., 2023) et al. demonstrated experimentally that the dewatering performance of sludge was significantly reduced when the concentration of perfluorooctanoic acid ranged from 1 mg L^{-1} to 100 mg L^{-1}. In addition, in the anammox system, perfluorooctanoic acid at concentrations ranging from 5 mg L^{-1} to 50 mg L^{-1} stimulated the production of reactive oxygen species in microorganisms and down-regulated the expression of genes involved in anammox and nitrification(Tang et al., 2022).

Extracellular polymeric substances in activated sludge play a crucial role in biological wastewater treatment(Y. Ma et al., 2023). Li(Z. Li et al., 2016) et al. showed that the flocculation of activated sludge is more effective and the average floc size is larger with the increase of extracellular polymeric substances. In addition, the research showed that the higher the ratio of extracellular proteins to extracellular polysaccharides, the better settling performance of the activited sludge. This is mainly due to the hydrophobicity of extracellular proteins(Yang et al., 2022). Additionally, various functional

groups in extracellular polymeric substances, such as hydroxyl, carboxyl, and amide groups, provide binding sites for the adsorption of organic and inorganic pollutants(Vandana et al., 2023). As a new pollutant, there are fewer studies related to the effects of perfluorooctanoic acid on microbial extracellular polymeric substances in biological wastewater treatment. Therefore, it is essential to study the effect and mechanism of perfluorooctanoic acid on extracellular polymeric substances to reveal the ecological hazards and mechanisms of perfluorooctanoic acid in biological wastewater treatment.

So this study aimed to investigate the effect of perfluorooctanoic acid on microbial extracellular polymeric substances in biological wastewater treatment and to reveal the effect mechanisms. First, the effects of different concentrations of perfluorooctanoic acid on the content and composition of microbial tightly bound extracellular polymeric substances (TB-EPS) and loosely bound extracellular polymeric substances (LB-EPS) were analyzed. Secondly, three-dimensional fluorescence spectroscopy and Fourier-transform infrared spectroscopy were used to study the effects of different concentrations of perfluorooctanoic acid on the composition and structure of extracellular polymeric substances. Finally, the potential effect mechanisms of perfluorooctanoic acid on the structure of extracellular polymeric substances was explored by protein secondary structure analysis, and X-ray photoelectron spectroscopy was used to determine the correlation between different concentrations of perfluorooctanoic acid and the proportion of functional groups in extracellular polymeric substances. This study provides further insight into the effects of new contaminants.

2. Materials and methods

2.1 Experimental materials and design

The perfluorooctanoic acid was purchased from Shanghai Aladdin Biotechnology Co. The stock solution of 1 g L^{-1} perfluorooctanoic acid was configured by dissolving a gram of perfluorooctanoic

acid in a Liter of ultrapure water. The sludge used in the experiment was sourced from the aerobic end of a municipal wastewater treatment plant in Hefei, China. Synthetic wastewater was conducted in the experiment with CH_3COONa, NH_4Cl, and KH_2PO_4. The concentration of chemical oxygen demand (COD), ammonia nitrogen and soluble phosphorus was 150 mg L^{-1}, 15 mg L^{-1} and 6 mg L^{-1}, respectively. The other compounds in the synthetic wastewater are listed in Table 1.

Table 1. Content of trace elements in synthetic wastewater

ingredient	**concentration (mg L^{-1})**	**ingredient**	**concentration (mg L^{-1})**
Boric acid	0.10	Copper sulfate	0.03
Potassium iodide	0.18	Manganese chloride	0.12
Sodium Molybdate	0.06	Ferric chloride	1.50
Zinc sulfate	0.12	Cobalt chloride hexahydrate	0.08

The six sequence batch reactors (SBRs)were utilized in the experiment. Each reactor operated for six cycles per day. Each cycle included a 15 minute influent period, a 60 minute anaerobic period, a 105 minute aerobic period, a 40 minute settling period, a 15 minute effluent period, and a 5 minute idle period. The concentrations of perfluorooctanoic acid in each reactor were 0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 3.0 mg L^{-1}, and 5.0 mg L^{-1}, respectively. When these reactors were stable, perfluorooctanoic acid stock solution was added in each reactor. The extracellular polymeric substances were extracted for detection and analysis. All tests were conducted in triplicate from three parallel experiments, with values expressed as mean ± standard deviation.

2.2 Extraction and content determination of extracellular polymeric substances

Extraction of extracellular polymeric substances was conducted using thermal extraction as follows:

A 50 mL homogeneous sludge mixture was sampled from the aerobic end of the reactor. The sludge was washed three times with phosphate buffer, then centrifuged at 4°C and 4000 rpm for 5 minutes. The supernatant was removed. The phosphate buffer was preheated to 80°C and mixed with the remaining sludge for 1 min, then centrifuged at 4°C and 4000 rpm for 10 min. The supernatant was filtered using a 0.45 μm filter membrane to obtain the LB-EPS solution. The remaining sludge was resuspended in phosphate buffer, and heated in a water bath to 60°C. After 60 min, the mixture was centrifuged at 4°C and 4000 rpm for 15 min, and the supernatant was filtered using a 0.45 μm filter membrane to obtain the TB-EPS solution. Each extract was stored at 4°C for later use. The protein and humic acid content of extracellular polymeric substances were determined using the modified Lowry method(D. Ma et al., 2024), and the polysaccharide content was determined using the anthrone-sulfuric acid method(Peng et al., 2021).

2.3 Three-dimensional fluorescence spectroscopy

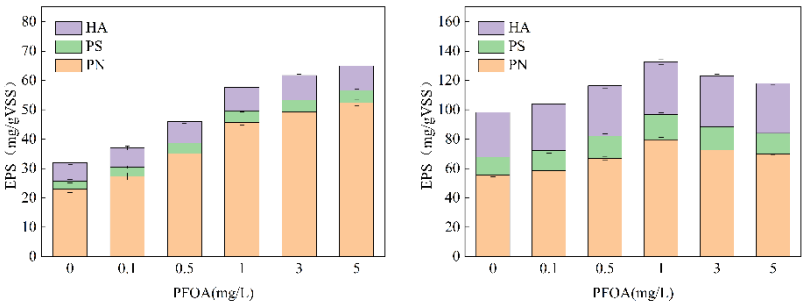
Three-dimensional fluorescence spectra of LB-EPS and TB-EPS samples were measured using a luminescence spectrometer. In the measurements, the excitation and emission wavelengths were set to 200-320 nm and 250-500 nm, respectively, and the scanning speed was 2400 nm min^{-1}, using an excitation and emission interval of 10 nm.The data were processed using Origin 8.0 software.

2.4 Fourier-transform infrared spectroscopy and protein secondary structure analysis

The Fourier-transform infrared spectroscopy of the LB-EPS and TB-EPS samples were measured using an FTIR instrument. In the measurement, the spectral range was set to 4000-400 cm^{-1} with a resolution of 4 cm^{-1}. The overlapping peaks in the amide I region were delineated using the deconvolution method through Peakfit software to obtain the proportion of protein secondary structure.The data were processed using Origin 8.0 software.

2.5 X-ray photoelectron spectroscopy

The C, O, and N elements in the extracellular polymeric substances were analyzed using X-ray photoelectron spectroscopy, and all binding energies were based on the neutral C 1s peak at 284.6 eV to compensate for surface charging effects. The X-ray photoelectron spectroscopy data were peak-split using Avantage software.



(a)LB-EPS (b)TB-EPS

Figure 1. Changes of LB-EPS and TB-EPS under different concentrations of perfluorooctanoic acid

3. Results and analysis

3.1 Effect of perfluorooctanoic acid extracellular polymeric substances

The changes in LB-EPS and TB-EPS components under different concentrations of perfluorooctanoic acid are showed in Figure 1. Figure 1 showed that low concentrations of perfluorooctanoic acid enhanced the secretion of extracellular polymeric substances in activated sludge. Conversely, high concentrations of perfluorooctanoic acid exhibit different effects on extracellular polymeric substances.

In Figure 1(a), the secretion of LB-EPS increases steadily with the increase of perfluorooctanoic acid concentrations. Specifically, when the perfluorooctanoic acid concentration was 5.0 mg L^{-1}, the protein (PN), polysaccharide (PS), and humic acid (HA) contents in LB-EPS increased by 29.53 mg $gVSS^{-1}$, 1.38 mg $gVSS^{-1}$, and 2.19 mg $gVSS^{-1}$, respectively, compared with the control group.

In Figure 1(b), In the control group, the content of PN, PS, and HA are 55.43 mg $gVSS^{-1}$, 12.27 mg $gVSS^{-1}$, and 30.52 mg $gVSS^{-1}$, respectively. When the perfluorooctanoic acid concentration was 1.0 mg L^{-1}, the PN, PS, and HA contents increased to 79.55 mg $gVSS^{-1}$, 17.24 mg $gVSS^{-1}$, and 35.75 mg $gVSS^{-1}$, respectively. Subsequently, As the concentration of perfluorooctanoic acid increased, the content of TB-EPS gradually decreased. Studies have shown that perfluorooctanoic acid a long-chain fluoride with strong hydrophobicity, readily binds to the internal structure of the phospholipid bilayer on cell membranes, leading to changes in cell membrane permeability(D. Li et al., 2023). Changes in the structure of the cell membrane will in turn disrupt the metabolism of the microorganisms, generating large amounts of reactive oxygen species that oxidize the fatty acids in the cell membrane, ultimately leading to cellular breakdown and inactivation of the microorganisms(T. Zheng et al., 2021). This may account for the reduced secretion of TB-EPS under high perfluorooctanoic acid conditions.

Under different concentrations of perfluorooctanoic acid concentration, PN accounted for 56%-81% of the sum of PN, PS and HA in LB-EPS and TB-EPS. Obviously, among the components of the extracellular polymers, the PN content accounted for the highest percentage. For example, when the perfluorooctanoic acid concentration was 5.0 mg L^{-1}, the growth rates of PN, PS, and HA contents were 129%, 49%, and 36%, respectively, in LB-EPS, and the growth rates of PN, PS, and HA contents were 26%, 14%, and 11%, respectively, in TB-EPS. And the PN content was most significantly affected by perfluorooctanoic acid concentration. It has been shown that the changes in PN content are mainly related to the enzyme activities in microorganisms(Corsino et al., 2017). The perfluorooctanoic acid can reduce PN content by combining with enzymes to form aggregates, reducing enzyme activity and inhibiting their functioning(Xu et al., 2020).

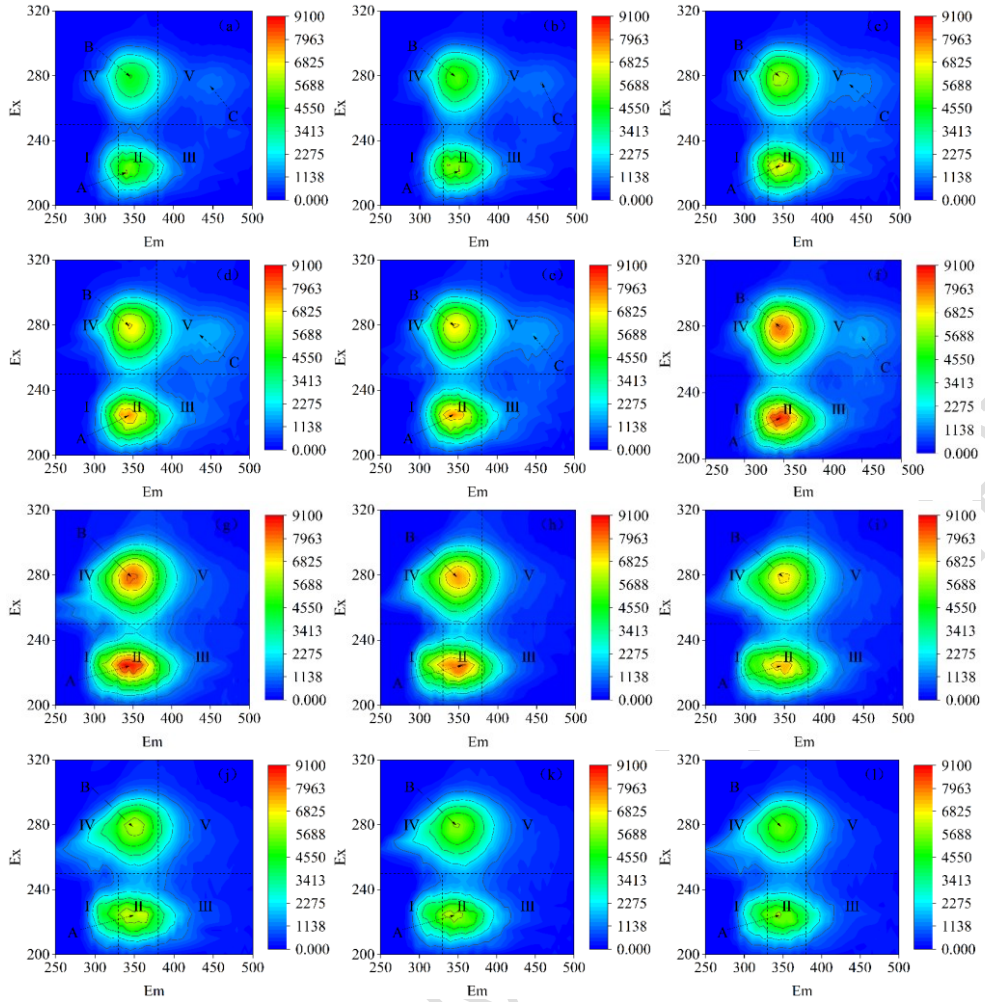


Figure 2. Three-dimensional fluorescence spectroscopy of (a-f) LB-EPS and (g-l) TB-EPS at different concentrations of perfluorooctanoic acid (where a-f and g-l characterize the concentrations of perfluorooctanoic acid as 0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 3.0 mg L^{-1}, 5.0 mg L^{-1})

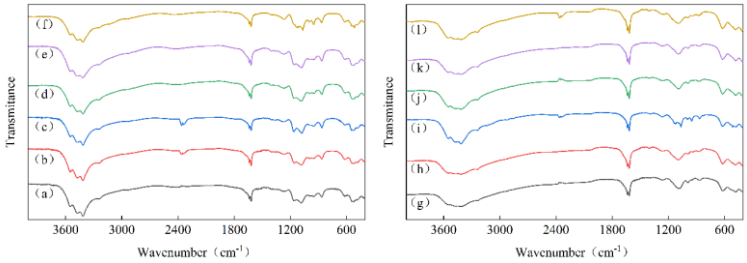
3.2 Effect of perfluorooctanoic acid on fluorescent compounds in extracellular polymeric substances

Figure 2 displays the three-dimensional fluorescence spectroscopy of LB-EPS and TB-EPS at different concentrations of perfluorooctanoic acid. The spectrogram are segmented into five regions based on excitation and emission wavelengths: fluorescent aromatic-like proteins I (200-250/250-330 nm), fluorescent aromatic-like proteins II (200-250/330-380 nm), fulvic acid-like substances (200-250/380-500 nm), soluble microbial metabolites (250-320/250-380 nm), and humic acid-like substances (250-320/380-500 nm). This study identified three prominent fluorescence peaks in

extracellular polymeric substances: Peak A (220-225/335-355 nm) for tryptophan-like substances, Peak B (280/345-350 nm) for soluble microbial products-like substances, and Peak C (270-275/435-455 nm) for humic acid-like substances(Qian et al., 2021).

When the concentration of perfluorooctanoic acid increased from 0 mg L^{-1} to 5.0 mg L^{-1}, the fluorescence intensities of peak A, peak B, and peakC in the spectra of LB-EPS increased from 5968, 4642, and 1046 to 9016, 8091, and 1615, respectively. Conversely, the fluorescence intensities of peak A and peak B in the spectra of TB-EPS decreased from 9062 and 8057 to 6176 and 5435, respectively. The results indicate a significant increase in protein-like substances in LB-EPS with higher perfluorooctanoic acid concentrations, whereas the change in humic acid-like substances was more modest, consistent with the component analysis of LB-EPS. In TB-EPS, the fluorescence intensity of tryptophan-like substances and soluble microbial products-like substances decreased significantly with increasing concentrations of perfluorooctanoic acid. Analysis of TB-EPS composition suggests that the substantial fluorescence quenching observed in its spectra may be attributed to the higher binding affinity of its fluorescent organics for perfluorooctanoic acid. Guo(Guo et al., 2016) et al. demonstrated a prevalence of hydrophobic functional groups in TB-EPS compared to LB-EPS, facilitating binding with perfluorooctanoic acid. Regarding the position of fluorescence peaks, peak A, characteristic of tryptophan-like proteins in both LB-EPS and TB-EPS, exhibited a red-shift in the emission direction. This shift suggests an increase in carbonyl, carboxyl, and hydroxyl functional groups within the fluorescent moiety(Z. Liu et al., 2023), attributed to the presence of perfluorooctanoic acid. The experiment results also indicated that the binding of extracellular polymeric substances and perfluorooctanoic acid was closely related to the proteinaceous substances in the extracellular polymeric substances, mirroring the findings of Yan et al.(Yan et al., 2023).

Compared with TB-EPS, LB-EPS has a closer relationship with the dewatering performance of sludge(X. Y. Li & Yang, 2007); Liu(J. Liu et al., 2016) et al. showed that tryptophan and complex amino acids in sludge increase the difficulty of sludge-water separation, while humic acid has less effect on it. In addition, there are a large number of hydrophilic acids in the microbial metabolites, which will allow the extracellular polymers to fully absorb water. In conclusion, perfluorooctanoic acid promoted the secretion of tryptophan and microbial metabolites in LB-EPS, which adversely affected the sludge dewatering performance.



（a-f）LB-EPS　　（g-l）TB-EPS

Figure 3. Fourier-transform infrared spectroscopy of LB-EPS and TB-EPS at different concentrations of perfluorooctanoic acid (where a-f and g-l characterize the concentrations of perfluorooctanoic acid as 0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 3.0 mg L^{-1}, 5.0 mg L^{-1})

3.3 Effects of perfluorooctanoic acid on functional groups and protein secondary structure in extracellular polymeric substances

The effects of perfluorooctanoic acid on functional groups in extracellular polymeric substances are shown in Figure 3. As seen in Figure 3, Fourier-transform infrared spectroscopy can be divided into six regions: 1700 cm^{-1}-1600 cm^{-1} (amide I region), 1600 cm^{-1}-1500 cm^{-1} (amide II region), 1500 cm^{-1}-1300 cm^{-1} (carboxylic group-containing and hydrocarbon-like compounds), 1300 cm^{-1}-1200 cm^{-1} (amide III region), 1200 cm^{-1}-900 cm^{-1} (polysaccharides or nucleic acids), and 900-600 cm^{-1}

(fingerprint region)(Y. Li et al., 2023). The presence of absorption peaks in all regions indicates that the extracellular polymers have a complex composition.

In Fourier-transform infrared spectroscopy, the absorption peak near 3413 cm^{-1} characterizes the stretching vibration of -OH; the absorption peak near 1617 cm^{-1} belongs to the stretching vibration of C=C and C=O in the protein-associated amide I region; the absorption peak near 1540 cm^{-1} characterizes the stretching vibration of C-N and N-H in the protein-associated amide II region. The absorption peak near 1400 cm^{-1} characterizes the C=O stretching vibration in the carboxyl group; the absorption peak near 1263 cm^{-1} belongs to the C-N and N-H stretching vibration in the protein-associated amide III region; and the absorption peak near 1159 cm^{-1} characterizes the C-OH stretching vibration in polysaccharides. The absorption peak near 1081 cm^{-1} corresponds to the C-O-C stretching vibration in polysaccharides.

The shift in the positions of the absorption peaks in the spectra characterizes the structure change of the extracellular polymeric substances. With the increase of perfluorooctanoic acid concentrations, the positions of the absorption peaks at 1400 cm^{-1}, 1263 cm^{-1}, 1159 cm^{-1}, and 1081 cm^{-1} shift. The effects of perfluorooctanoic acid on functional groups in extracellular polymeric substances are mainly focused on carboxyl groups, protein-associated amide groups, and hydroxyl and ether bonds in polysaccharides. Yan et al.(Yan et al., 2021) concluded that the protonated amine in the amide group can interact electrostatically with the carboxyl head of perfluorooctanoic acid, facilitating its adsorption. Simultaneously, the hydroxyl group can form a hydrogen bond with the carboxyl head of the carboxyl group in perfluorooctanoic acid, stabilizing the hydrogen atom within an energy shell layer. These functional groups are also important constituents of enzymes in sludge, and their structural changes provide evidence for the previous speculation that perfluorooctanoic acid inhibits PN secretion by binding to related enzymes.

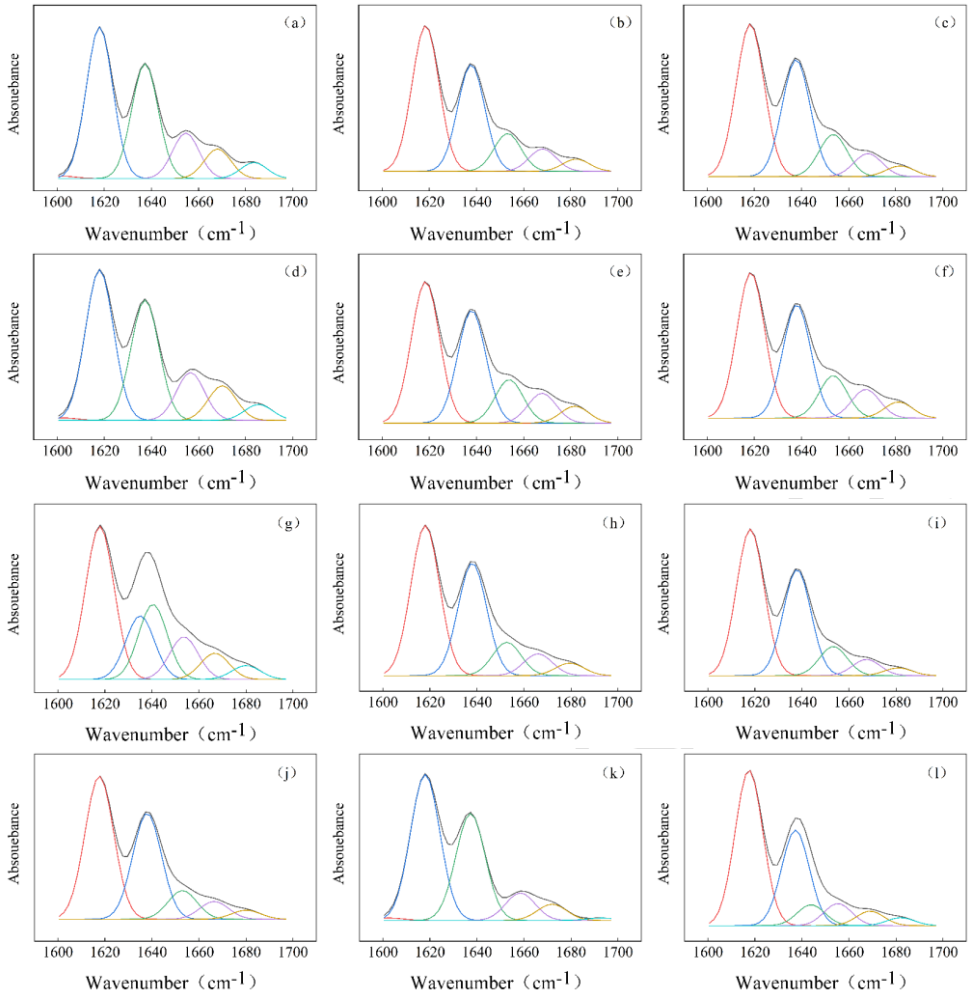
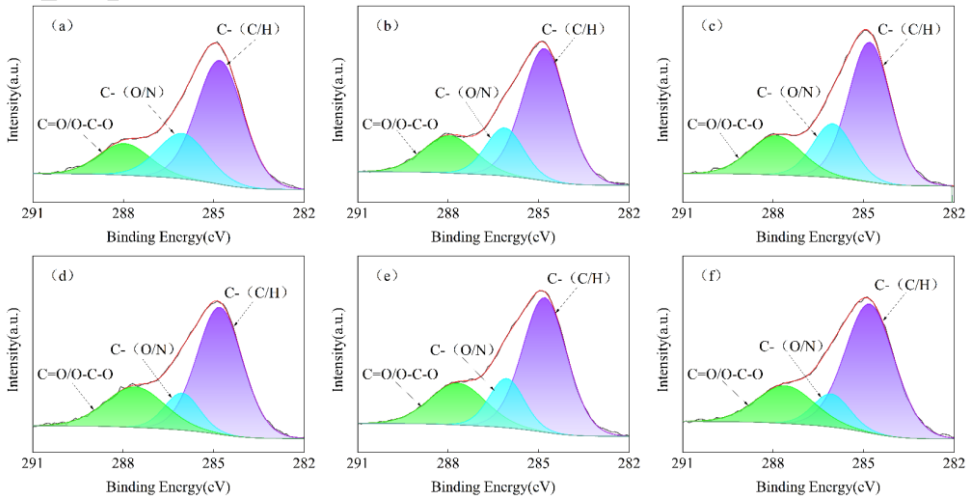


Figure 4. Fourier-transform infrared spectroscopy of (a-f) LB-EPS and (g-l) TB-EPS in the amide I region (where a-f and g-l characterize the concentration of perfluorooctanoic acid as 0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 3.0 mg L^{-1}, 5.0 mg L^{-1})

The effects of different concentrations of perfluorooctanoic acid on the secondary structure of LB-EPS and TB-EPS proteins in extracellular polymeric substances are shown in Figure 4. As seen in Figure 4, the protein secondary structures are mainly categorized into four groups: β-sheet (1630 cm^{-1}-1640 cm^{-1}), random coil (1640 cm^{-1}-1650 cm^{-1}), α-helix (1650 cm^{-1}-1660 cm^{-1}), and antiparallel β-sheet (1680 cm^{-1}-1690 cm^{-1}). When the concentration of perfluorooctanoic acid increased from 0 mg L^{-1} to 5.0 mg L^{-1}, the α-helix in LB-EPS and TB-EPS decreased from 13% and 11% to 12% and 7%,

respectively. In contrast, the β-sheets in LB-EPS and TB-EPS increased from 32% and 17% to 33% and 30%, respectively. The results indicated that perfluorooctanoic acid increased the proportion of β-sheet structures and decreased the proportion of α-helix structures in the amide I region of the protein. It has been demonstrated that perfluorooctanoic acid can alter proteins' secondary structure by reducing the energy required for α-helix unfolding. Meanwhile, perfluorooctanoic acid binds to the unfolded body of the α-helix through hydrogen bonding and hydrophobic interactions to form a more stable bound state(Yadav et al., 2024).

It has been shown that the peptide bonds in each peptide chain can form hydrogen bonds, therefore, the α-helix has high stability, while the β-fold is mainly formed by hydrogen bonds between carbonyl oxygen and amide hydrogen in the same or adjacent peptide chains(Wu et al., 2017). α-helix/(β-fold + irregular curls) is often used to characterize the compactness of the protein structure(E. Li et al., 2021). In this study, the proportion of α-helix/(β-folding + irregular curling) in LB-EPS and TB-EPS proteins was reduced from 40% and 31% to 37% and 18%, respectively, as the concentration of perfluorooctanoic acid increased. This result indicates that the structure of LB-EPS and TB-EPS became looser with increasing perfluorooctanoic acid concentration, which is unfavorable for sludge flocculation(J. Yu et al., 2023).



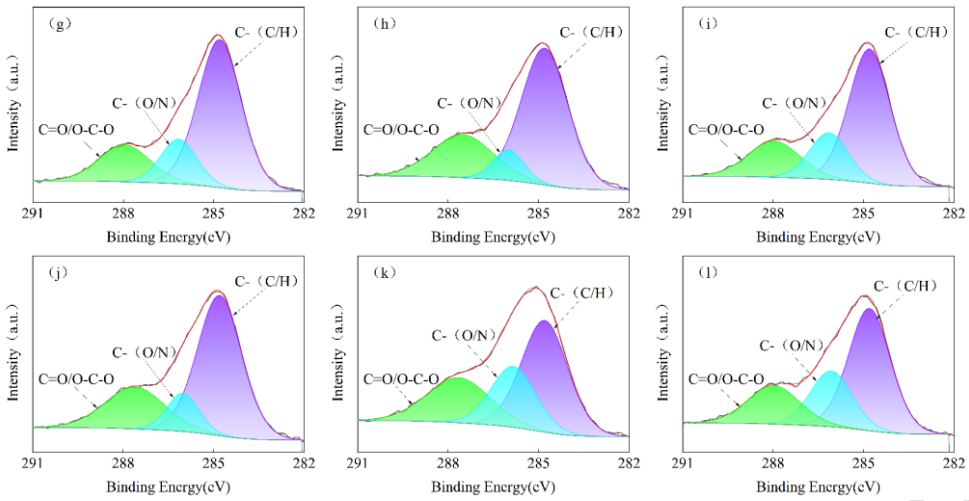


Figure 5. High-resolution C 1s spectra in the X-ray photoelectron spectroscopy of (a-f) LB-EPS and (g-l) TB-EPS (where a-f vs. g-l characterize the concentration of perfluorooctanoic acid as 0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 3.0 mg L^{-1}, 5.0 mg L^{-1})

3.4 Effects of perfluorooctanoic acid on the elemental composition and functional groups of extracellular polymeric substances

X-ray photoelectron spectroscopy was performed on the extracellular polymeric substances to further investigate the effects of perfluorooctanoic acid on the elemental composition and characteristic functional groups in LB-EPS and TB-EPS. The analytical results showed that the atomic ratios of carbon (C) and oxygen (O) in the extracellular polymeric substances were 52%-57% and 37%-44%, respectively, indicating high elemental abundances of C and O in LB-EPS and TB-EPS at various concentrations of perfluorooctanoic acid. As the increase of perfluorooctanoic acid concentration, the O/C molar ratios in LB-EPS and TB-EPS increased from 0.70 and 0.72 to 0.73 and 0.77, respectively. Additionally, the N/C molar ratios increased from 0.10 and 0.12 to 0.12 and 0.14, respectively, when the concentration of perfluorooctanoic acid was 5.0 mg L^{-1}. This suggested that perfluorooctanoic acid promoted the secretion of nitrogenous and oxygenated compounds by microorganisms. Since carbon (C 1s) is the central element in extracellular polymeric substances, this study further analyzes

high-resolution C 1s spectra to quantify each functional group as a molar ratio to the total carbon. This approach allows for comparisons of the various functional groups associated with carbon.

The high-resolution C 1s spectra in the X-ray photoelectron spectroscopy of LB-EPS and TB-EPS are shown in Figure 5. As shown in Figure 5, three subpeaks can be decomposed from the C 1s peak: the C-(C/H) group near 284.8 eV, the C-(O/N) group near 286.3 eV, and the C=O or O-C-O group near 288.0 eV(An et al., 2023). When the concentration of perfluorooctanoic acid increased from 0 mg L^{-1} to mg L^{-1}, the molar ratios of C=O or O-C-O in LB-EPS rose from 11.81% to 15.03%, while the molar ratio of C-(C/H) in TB-EPS decreased from 41.55% to 34.27%. The C-(C/H) groups are primarily derived from hydrocarbons, including the side chains of polysaccharides, amino acids, and lipids, and represent the main hydrophobic groups in extracellular polymeric substances. The less hydrophobic the extracellular polymer is, the higher the surface charge will be on the cell surface, which will be unfavorable for intercellular interactions. This will have an effect on the aggregation and stability of the activated sludge(Sun et al., 2024). The C=O or O-C-O groups are mainly present in extracellular polymers in the form of carboxylates, carbonyl groups, etc., which are negatively correlated with bioflocculation in wastewater biological treatment(Qian et al., 2021). The X-ray photoelectron spectroscopy analysis revealed that perfluorooctanoic acid changed the group occupancy of extracellular polymers, which adversely affected the flocculation and sedimentation effect and stability of activated sludge.

4. Conclusion

The secretion of microbial extracellular polymers LB-EPS and TB-EPS was significantly promoted by low concentrations of perfluorooctanoic acid, but the promotion effect of TB-EPS secretion was weakened at higher concentrations of perfluorooctanoic acid. Perfluorooctanoic acid had different effects on the fluorescence intensity of LB-EPS and TB-EPS. With the increase of perfluorooctanoic

acid concentration, the intensity of microbial metabolite fluorescence peaks and tryptophan fluorescence peaks in TB-EPS decreased. However, the intensities of microbial metabolite fluorescence peaks, tryptophan fluorescence peaks and humic acid fluorescence peaks increased in LB-EPS, which implied that the dewatering performance of sludge became worse. Perfluorooctanoic acid affected the carboxyl groups, amide groups, ether bonds, and hydroxyl groups in the extracellular polymers, and the main binding modes of such functional groups to perfluorooctanoic acid included electrostatic interactions and hydrogen bonding. Perfluorooctanoic acid also affected the secondary structure of proteins in the extracellular polymers, decreasing the ratio of α-helices/(β-folds + irregular curls), which was detrimental to the settling performance of sludge. The relative content of C=O or O-C-O groups in LB-EPS increased with increasing concentrations of perfluorooctanoic acid, while the relative content of C-(C/H) groups in TB-EPS decreased, which had an effect on sludge stability. The experimental results show that the presence of perfluorooctanoic acid in wastewater should be given further attention. In addition, in order to fully describe the impact of perfluorooctanoic acid on biological wastewater treatment, the response of microbial communities and intracellular polymer substances to perfluorooctanoic acid needs to be further studied.

Acknowledgments

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Dear Editor,

We have carefully revised the manuscript according to all suggestions made by the editor and the reviewers. We have carefully checked all author names and corrected exactly the style of the references and formatting according to the journal instructions.The revised manuscript looks much better than the original one, and we would like to take this opportunity to thank the editors for their valuable comments. We have included the e-mail addresses of all the co-authors at the end of this post

Thank you for your time and consideration.

Sincerely,

Hua Zhang

Response to editor and reviewers

Reviewer #A:

I recommend acceptance of the manuscript after the authors successfully considered the reviewers comments.

Reply: Thank you for the valuable comments. We have made changes based on your comments.

Reviewer #C:

Your work has laid a solid foundation for future research, particularly in exploring the roles of ROS production, protein conformational changes, and enzymatic inhibition in microbial activity and extracellular polymer interactions. I encourage you to consider these factors in subsequent studies, as they could provide a more comprehensive understanding of how emerging contaminants affect microbial ecosystems in wastewater treatment.

Reply: Thank you for the valuable comments. Next, we will conduct further experiments to explore ROS generation, protein conformational changes and enzyme inhibition. Your suggestions allowed us to revise the manuscript in a reasonable way, so thank you again.

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