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Purification capacity of natural biofilms and their physiochemical and biological properties: a case study in the Jishan River, a heavily polluted river

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ABSTRACT

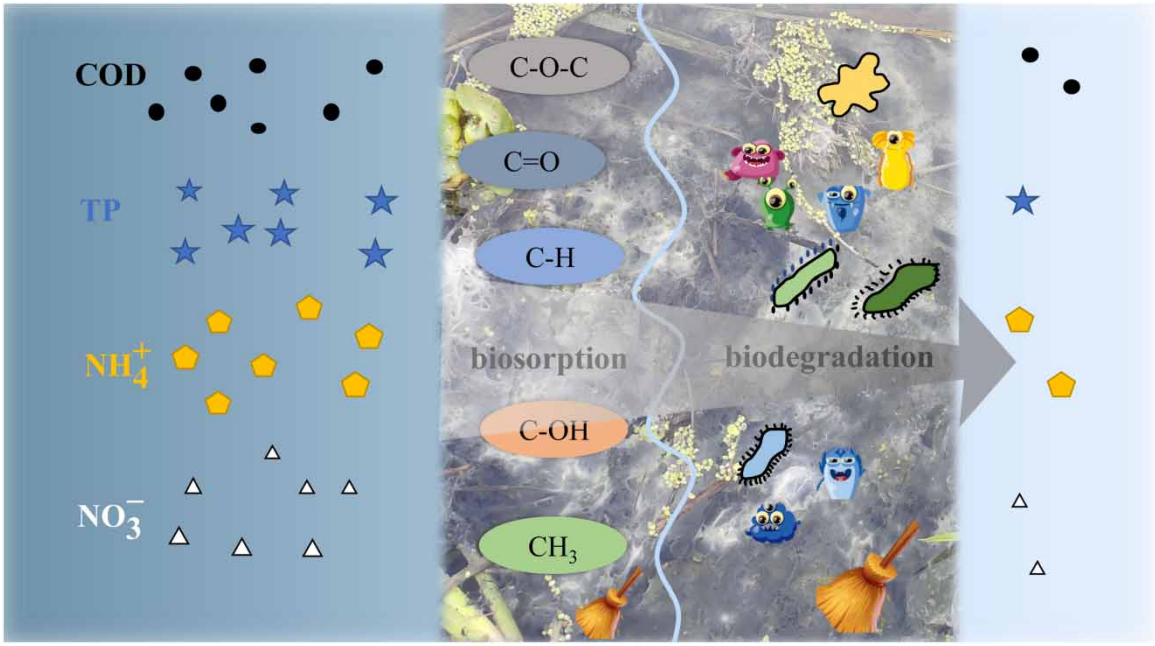
Natural biofilms, which are widely distributed in various aquatic environments, can not only serve as bioindicators of various anthropogenic contaminants but also participate in the purification and degradation of various pollutants. However, the inherent purification capacity of natural biofilms and their physiochemical and biological properties are still poorly understood. In this study, outdoor sampling and indoor experiments were used to explore the purification abilities of natural biofilms. The physiochemical and biological properties of natural biofilms were further investigated to reveal their purification mechanism. The results demonstrated that natural biofilms had an excellent purification effect on heavily polluted water. Indoor experiments showed that the purification capacity of natural biofilms was dominated by microbial biodegradation rather than physical biosorption, and after 14.0 days of incubation, the removal rates of COD, TP, NH_4^+-N, and NO_3^--N could reach 93.6, 80.83, 85.93, and 81.03%, respectively. The SEM, FTIR spectra, and component analyses revealed that natural biofilms were mainly composed of polysaccharides and proteins. The dominant phyla in the bacterial community structure were *Campilobacterota, Proteobacteria, Bacteroidota, Firmicutes*, and *Desulfobacterota*, and the major phyla in the fungal community structure were *Chytridiomycota* and *Ascomycota*. These microorganisms might be the main degraders of riverine pollutants.

Key words: natural biofilms, microbial community structure, physiochemical and biological properties, purification capacity

HIGHLIGHTS

- Natural biofilms had an excellent purification effect on heavily polluted water.
- The purification capacity of natural biofilms is dominated by biodegradation.
- The SEM and FTIR spectra were used to reveal the physiochemical properties.
- Natural biofilms were mainly composed of polysaccharides and proteins.
- Microbial community structures were utilized to show the biological properties.

GRAPHICAL ABSTRACT



INTRODUCTION

Although the river is the sink for the discharge of terrestrial pollutants, it has certain self-purification which can be used to maintain the ecological health and balance of the river. Self-purification of water refers to the reduction of pollutant concentration and partial or complete restoration to original river levels through natural processes, including physical, chemical, and biological purification and their interactions (McColl 1974; Vagnetti *et al.* 2003; Semenov *et al.* 2019; Cai *et al.* 2021). Among them, the physical self-purification mainly includes dilution, adsorption, sedimentation, volatilization, etc. Chemical self-purification contains neutralization reactions, REDOX reactions, precipitation reactions, adsorption, and condensation reactions. Notably, biological self-purification includes plant absorption, enrichment, and transformation as well as microbial assimilation and dissimilation, microorganism secretion of extracellular polymer flocculation, sedimentation, polymerization, etc. (Wu *et al.* 2012; Han *et al.* 2015; Li *et al.* 2021b). Since physical and chemical processes are affected by biological factors, biological self-purification is the core of the overall self-purification process (Ostroumov 2017). The participation of microorganisms in the process of biological self-purification is the main reason for the removal of pollutants (Battin *et al.* 2016).

Approximately 95% of all aquatic microorganisms can adhere to the inner surface of pipe materials, forming a biofilm, and only 5% are floating in the water column (Hemdan *et al.* 2019). Natural biofilms, which are widely distributed in various aquatic environments, are microbial assemblages of algae, fungi, bacteria, protozoa, metazoans, and other micro-biotic and abiotic matters in which cells are frequently embedded in their self-produced matrix of extracellular polymeric substances (EPSs) (Costello *et al.* 2016; Zhang *et al.* 2022). As a vital component of metabolic products, EPSs have been considered to be representative of a biofilm structure and function (Felz *et al.* 2019). Organic matter biodegradation, primary production, and cycling of nitrogen, phosphorus, and sulfur are directly regulated by processes driven by biofilms (Costello *et al.* 2016; Zhang *et al.* 2022). A previous study has demonstrated that contaminants could be adsorbed and degraded by biofilms through different mechanisms, including electrostatic, cation exchange, complexation, hydrophobic, and micropore filling interactions (Huang *et al.* 2018). Hence, biofilms have been proposed as natural bioindicators to reflect the spatial distribution of anthropogenic pesticides (Tien *et al.* 2013; Zhang *et al.* 2022). Furthermore, biofilms provide major food sources that fuel the secondary production of invertebrates and fishes, thus making the related pollutants trophically transferable to the food chain (Costello *et al.* 2016; Zhang *et al.* 2022). More importantly, biological contact oxidation technology based on natural biofilm technology has been used for heavily polluted water treatment with excellent results (Wu *et al.* 2012). These suggest that biofilms play important roles in the transport and transformation of pollutants in aquatic environments.

Previous studies have proved that river biofilms contribute significantly to the self-purification of the river (Tien *et al.* 2013; Hou *et al.* 2022). Moreover, several studies have paid attention to the variation in the microbial community structure of river biofilms during the biodegradation processes and the effects of hydrodynamics on biofilm purification abilities (Tien *et al.* 2013; Hou *et al.* 2022). However, little was known about the inherent purification capacity of natural biofilms for different pollution indicators of heavily polluted water bodies and their physiochemical and biological properties. We hypothesized that natural biofilms had strong wastewater purification capacity. Herein, we explored the purification effects of natural biofilms by outdoor sampling and indoor experiments and further investigated the physiochemical and biological properties of natural biofilms to reveal their purification mechanism (Supplementary material, Figure S2). The main goals of this study were (1) the inherent purification capacity of natural biofilms; (2) the microbial community structure and function of natural biofilms; and (3) the physiochemical properties of natural biofilms. These results provide a new perspective on the in situ remediation function of natural biofilms and reveal the potential biological mechanisms of natural biofilms for the remediation of polluted water bodies.

MATERIALS AND METHODS

Site description and sampling

The experiment described in this paper was conducted in the Jishan River (Supplementary material, Figure S1) of Jinmen, Hubei province, China (Zhu *et al.* 2022b). The length of the river is 4,150 m (latitude 30°29′N, longitude 112°10′E). With several chemical factories and soybean processing factories, residential areas, and rapeseed fields along its banks, this river is an important source of drinking and irrigation for local residents, as well as a major discharge area for industrial effluent, which plays an important role in the normal life and economic development of local residents. There are a lot of aquatic plants downstream of the river. A large number of clearly visible loose porous, mature white flocculent biofilms were attached to the plant's surface and between the plants and the substrate above the water surface. Five representative surface water samples were collected along the river in November 2020 (Supplementary material, Figure S1). The collection, preservation, and transportation of water samples were carried out in accordance with the Standard Method for Water and Wastewater Monitoring (Ministry of environmental protection of China 2002). Duplicate natural biofilm samples were scraped from aquatic plants and collected in sterile centrifuge tubes. All samples were refrigerated and brought back to the laboratory. The water samples were divided into two parts: one was stored in a refrigerator at 4 °C for the determination of physiochemical parameters, and the other was filtered through a 0.22-μm membrane and then stored at −20 °C for molecular analysis.

Experimental setup

The laboratory experiments on the self-purification ability of natural biofilms were constructed in duplicate in 500-mL conical flasks containing 250 mL of synthetic black-odor water and incubated at 30 °C with moderate shaking. Four treatments were conducted: sterilized black-odor water (SW), black-odor water (W), black-odor water + 0.1 g biofilms (BW), black-odor water + 0.1 g sterilized biofilms (SBW). Approximately 4.0 mL of mixtures was taken out at a flexible interval of 1.0–5.0 days for detecting physiochemical parameters. To ensure the reproducibility of the experimental results, the experiments were conducted with synthetic black-odor water. Working guidelines for the treatment of urban black-odor water were issued by the Chinese Ministry of Housing and Urban-Rural Development in 2015 (Chinese Ministry of Housing and Urban-rural Development 2015), defining black-odor waterbodies as those that present with unpleasant colors and/or emit stinky odor. Synthetic black-odor water formulation was modified based on He *et al.* (2021), and its specific protocol was described in the Appendix. The physiochemical characteristics of this black-odor water are shown in Supplementary material, Table S1. The sterilized biofilms were prepared by autoclaving at 121 °C for 25 min.

Physiochemical analysis

Physiochemical parameters were analyzed after filtering the water samples through 0.45-μm membranes. The concentrations of ammonia nitrogen NH_4^+-N), nitrite nitrogen (NO_2^--N), and nitrate nitrogen (NO_3^--N) were determined using Nadler's reagent colorimetry (GB/T 7479-87), sulfanilamide coupled with N-(1-naphthyl)-ethylenediamine (GB/T 7493-87), phenoldisulfonic acid spectrophotometry (GB/T 7480-87), respectively. Total phosphorus (TP) and Chemical oxygen demand (COD) were measured using the ammonium molybdate spectrophotometry method (GB 11893-89) and the potassium dichromate method (GB/T 34500.2-2017).

Microbial community analysis

To explore the community structures and diversities of natural biofilms, high-throughput sequencing of the 16S rRNA and internal transcribed spacer-1 (ITS1) gene of biofilms were conducted (Zhu *et al.* 2022a). The total DNA was extracted from the biofilms with the DNeasy® PowerSoil® Kit (100 T, Qiagen, Germany) following the manufacturer's instructions. Electrophoresis and NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, USA) were used to quantify the DNA quality and concentration. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified to profile bacterial community structures of natural biofilms with primers (338F: 5′-ACTCC-TACGGGAGGCAGCAG-3′, 806R: 5′-GGAC-TACHVGGGTWTCTAAT- 3′). The fungal ITS1 was amplified with primers (ITS1F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′, ITS2R: 5′-GCTGCGTTCTTCATCGATGC-3′). After qualified library preparation, DNA sequencing was performed on the Illumina HiSeq PE300 platform at Shanghai Meiji Biomedical Technology Co., Ltd. Raw reads of gene amplicons were quality-controlled and analyzed using QIIME2 0.

EPSs extraction and determination

The total structural EPSs was extracted from the biofilms with a mild temperature Na_2CO_3 extraction method as described previously (Felz *et al.* 2016). In brief: a biofilm sample of 6.0 g (wet weight) was added into a 0.5% (w/v) Na_2CO_3 solution up to 100 mL and subsequently stirred for 35 min at 80 °C in a water bath., followed by centrifuge at 4,000 × *g* and 4 °C for 20 min. The organics in the supernatant comprised the total extractable EPSs. The protein contents (PN) were measured by a modified Lowry method with bovine serum albumin (Sigma, USA) as standard (Griebe & Nielsen 1995; Frølund *et al.* 1996). The polysaccharide concentration (PS) was determined by phenolsulfuric acid assay using glucose (Sigma, USA) as standard. The DNA content was calculated by a diphenylamine colorimetric method (Zhao *et al.* 2013).

Scanning electron microscope

The microstructure of biofilm was observed and analyzed by scanning electron microscope (SEM; TESCAN MIRA). 5.0 g biofilm samples were taken out and washed with phosphate buffer solution (pH 8.0) for three times. The supernatant was removed to retain the precipitation. The precipitated samples were immersed in 2.5% glutaraldehyde solution at 4 °C overnight. The samples were then washed three times with phosphate buffer solution (pH 8.0) to remove glutaraldehyde for 10 min each time. The biofilm samples were dehydrated in a gradient of 30, 50, 70, 90, and 100% ethanol solutions (v/v) with a concentration gradient of 15–20 min each, adding a 1:1 solution of anhydrous ethanol: isoamyl acetate, then removing the supernatant and adding isoamyl acetate for 15 min to remove the precipitates from the supernatant. The samples were dried in a vacuum freeze dryer, followed by gold spraying in an ion sputtering apparatus (SC 7620), and finally, the samples were observed and photographed using SEM.

Fourier transform infrared spectroscopy

The main functional groups of natural biofilms were conducted by using Fourier transform infrared spectrometer (FTIR, Cary 630, Agilent, USA). Lyophilization of natural biofilms was compressed with potassium bromide (KBr) in the mass ratio of 1:200 into tablets. FTIR measurements consisted of 16 scans with a resolution of 4 cm^{-1} from 4,000 to 400 cm^{-1} (Li *et al.* 2020; Ugya *et al.* 2021).

Statistical analysis

Experiment data were presented statistically as mean and standard deviation. Excel 2016 (Microsoft, USA), OriginPro 2022 (OriginLab, USA) were used for data processing and mapping. The sequences obtained by Miseq sequencing were first splintered according to FLASH (1.2.11), and the quality of the sequences was controlled and filtered. UPARSE (7.0.109) was used for OUT clustering and annotation. MOTHUR software was used for bacterial Alpha diversity analysis, Uparse is used to process OUT data, and the community structure of the sample was analyzed by R software. FAPROTAX and was used to predict bacterial metabolic functions. FUNGuild was used to distinguish communities and predict fungi functions.

RESULTS

Water quality physiochemical characteristics

Table 1 shows the detailed water quality physiochemical characteristics, such as COD, NH_4^+-N, NO_2^--N, NO_3^--N, and TP at 6 different sampling sites along the Jishan River. Results revealed that the water quality of the Jishan River is worse than Grade V based on the Environmental Quality Standards for China (GB3838-2002). The concentrations of COD, NH_4^+-N,

Table 1 | Water quality physiochemical characteristics of six different sampling sites along the Jishan River

	A	B	C	D	E	F
COD (mg/L)	1,020 ± 16	222 ± 24	187.60 ± 8	127.79 ± 16	108 ± 0	52.4 ± 0
NH_4^+-N (mg/L)	13.6 ± 0.12	7.08 ± 0.34	5.24 ± 0.20	6.63 ± 0.37	4.94 ± 0.14	2.10 ± 0.76
NO_2^--N (mg/L)	0.38 ± 0	0.01 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
NO_3^--N (mg/L)	2.50 ± 0.07	1.21 ± 0.04	1.27 ± 0.02	1.30 ± 0.01	0.54 ± 0.03	0.47 ± 0.02
TP (mg/L)	0.44 ± 0.01	0.84 ± 0.24	0.78 ± 0.01	0.80 ± 0	0.47 ± 0.01	0.34 ± 0.03

NO_2^--N, NO_3^--N, and TP ranged from 52.4 to 1,020 mg/L, 2.10 to 13.60 mg/L, 0 to 0.38 mg/L, 0.47 to 2.50 mg/L, and 0.34 to 0.84 mg/L, respectively. Site A, as the source of this river, showed the highest concentrations of COD, NH_4^+-N, NO_2^--N, and NO_3^--N. Site B, as the second sampling site next to site A had the highest concentration of TP. It was worth mentioning that the content of these physiochemical characteristics generally showed a decreasing trend with the course of this river. Therefore, it could be presumed that this river had natural self-purifying capacities. Moreover, site F had the lowest levels of COD, NO_2^--N, NO_3^--N, and TP. Observation in the field also revealed a large amount of white flocculent biofilms attached to aquatic plants at site F (Supplementary material, Figure S2). We initially hypothesize that these biofilms have a strong connection with the natural self-purification ability of the river. Biofilm samples were taken for indoor test verification.

Natural biofilms purification ability for black-odor water

We verified whether natural biofilms have the function of purifying synthetic black-odor water by indoor experiments (Figure 1). After 14.0 days of indoor incubation, COD, TP, NH_4^+-N, and NO_3^--N were significantly decreased in the two groups with black-odor water alone and with the addition of black-odor water and natural biofilms; in comparison, COD, TP, NH_4^+-N, and NO_3^--N showed little changes in the two groups of sterilized black-odor water and sterilized black-odor water plus biofilms. This implied that the microbial communities significantly contributed to the removal and degradation of these nutrient parameters. To address how natural biofilms affect the microbial communities-catalyzed removal of different nutrient parameters in synthetic black-odor water, we added an additional 0.1 g of biofilms in biotic black-odor water. After 14.0 days of indoor incubation, in contrast to the experimental group with black-odor water only, the experimental group with additional biofilms had 93.6, 80.83, 85.93, and 81.03% reduction in COD, TP, NH_4^+-N, and NO_3^--N, respectively. The indoor experiments confirmed that the natural biofilms could facilitate the degradation of COD, TP, NH_4^+-N, and NO_3^--N and its biological purification ability dominated. We need to further investigate the microbial structure and function of natural biofilms.

Microbial community structure and function of natural biofilms

As shown in Figure 2, the Illumina high-throughput sequencing technique was used to determine the microbial community structure of natural biofilms. For the bacteria, we obtained a total of 43,336 valid sequences that were classified into 699 bacterial OTUs from the natural biofilms. For the fungi, we obtained 61,093 valid sequences and 515 OTUs. Table 2 shows the results of the α-diversity of natural biofilms using OTUs, Chao1 index, Coverage, ACE, and the Shannon and Simpson indices to characterize the abundance, diversity, and coverage of bacterial and fungal communities, respectively. For the bacteria, the Chao1 index, Coverage, ACE, and the Shannon and Simpson indices were 974.82, 99.48%, 961.26, 3.84, and 0.06, respectively. For the fungi, the Chao1 index, Coverage, ACE, and the Shannon and Simpson indices were 564.64, 99.83%, 586.46, 2.23, and 0.27, respectively. The abundance and diversity of the microbial community structure reflect the integrity of the ecosystem to some extent. The bacterial OTUs were assigned to 33 phyla, 254 families, and 537 genera. Bacterial communities at the phylum level were dominated by *Campilobacterota* (52.65%), followed by *Proteobacteria* (28.45%), *Bacteroidota* (9.51%), *Firmicutes* (4.56%), and *Desulfobacterota* (1.99%), respectively. At the genus level, *Pseudarcobacter* (16.75%), *unclassified_f_Arcobacteraceae* (15.80%), *Sulfuricurvum* (12.24%), *unclassified_f_Comamonadaceae* (8.68%), *Sulfurimonas* (4.77%), *Acinetobacter* (4.34%), *Maikia* (3.65%), *Paludibacter* (2.53%), and *Flacobacterium* (2.20%) were the most abundant genera in natural biofilms. The fungal OTUs were assigned to 9 phyla, 81 families, and 183 genera. The *Chytridiomycota*, *Ascomycota*, and *unclassified_k_Fungi* were the most dominant fungal phyla, accounting for 49.42, 37.69, and 11.65% of the total fungal sequences, respectively. At the genus level, *unclassified_p_Chytridiomycota* (49.42%),

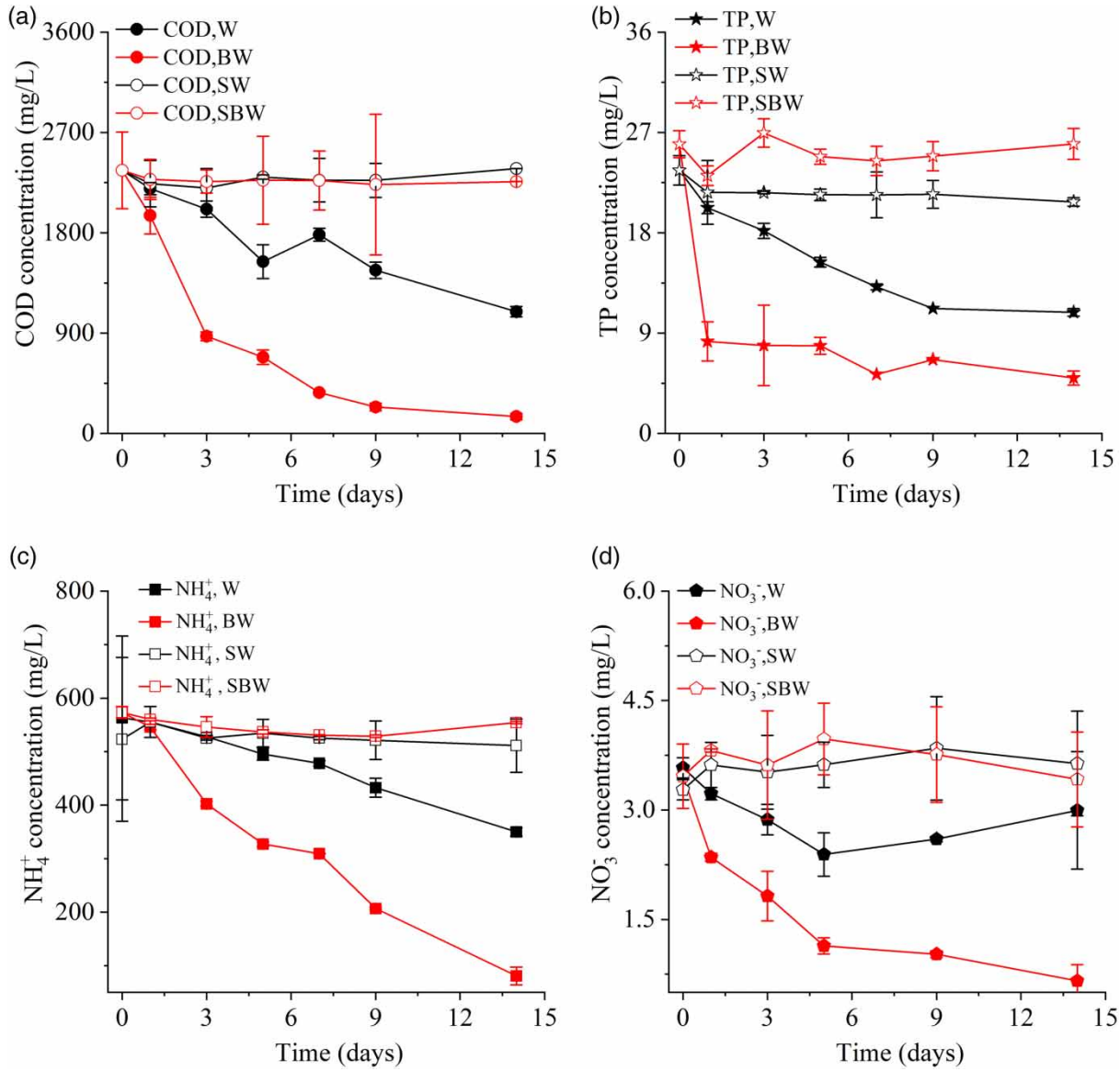


Figure 1 | Natural biofilm purification ability for black-odor water. Variations in (a) COD; (b) TP; (c) NH_4^+-N; and (d) NO_3^--N.

Kazachstania (13.31%), *unclassified_k__Fungi* (11.65%), *Plectosphaerella* (9.83%), *Curvularia* (1.84%), *Cladosporium* (1.75%), *Gibberella* (1.36%), *Nigrospora* (1.30%), *unclassified_o_Pleosporales* (1.24%), and *Lectera* (1.11%) were the major genera of natural biofilms.

The FAPROTAX annotation was used to predict the potential metabolic functions of the microbial communities in natural biofilms. Results (Figure 3(a)) revealed that the cyclic metabolism of carbon (C), nitrogen (N), and sulfur (S), Animal parasites or symbionts, and human pathogens were the prominent microbial functions in natural biofilms. Notably, the microorganisms related to C cycle metabolism are mainly chemo heterotrophy (20.90%), aerobic chemo heterotrophy (11.68%), fermentation (8.78%), and aromatic compound degradation (4.34%). The main microorganisms associated with nitrogen metabolism are nitrate reduction (3.94%), nitrate respiration (2.86%), nitrite respiration (1.49%), and nitrate denitrification (1.20%). The dominant microorganisms involved in the sulfur cycle consist of dark oxidation of sulfur compounds (4.03%), dark sulfide oxidation (3.95%), dark sulfur oxidation (3.93%), and respiration of sulfur compounds (1.96%). Animal pathogenic microorganisms are based on animal parasites or symbionts (11.06%), human pathogens (10.50%), and human pathogens pneumonia (5.88%). FUNGuild was used to predict the trophic and functional groups of fungal communities in natural biofilms. The

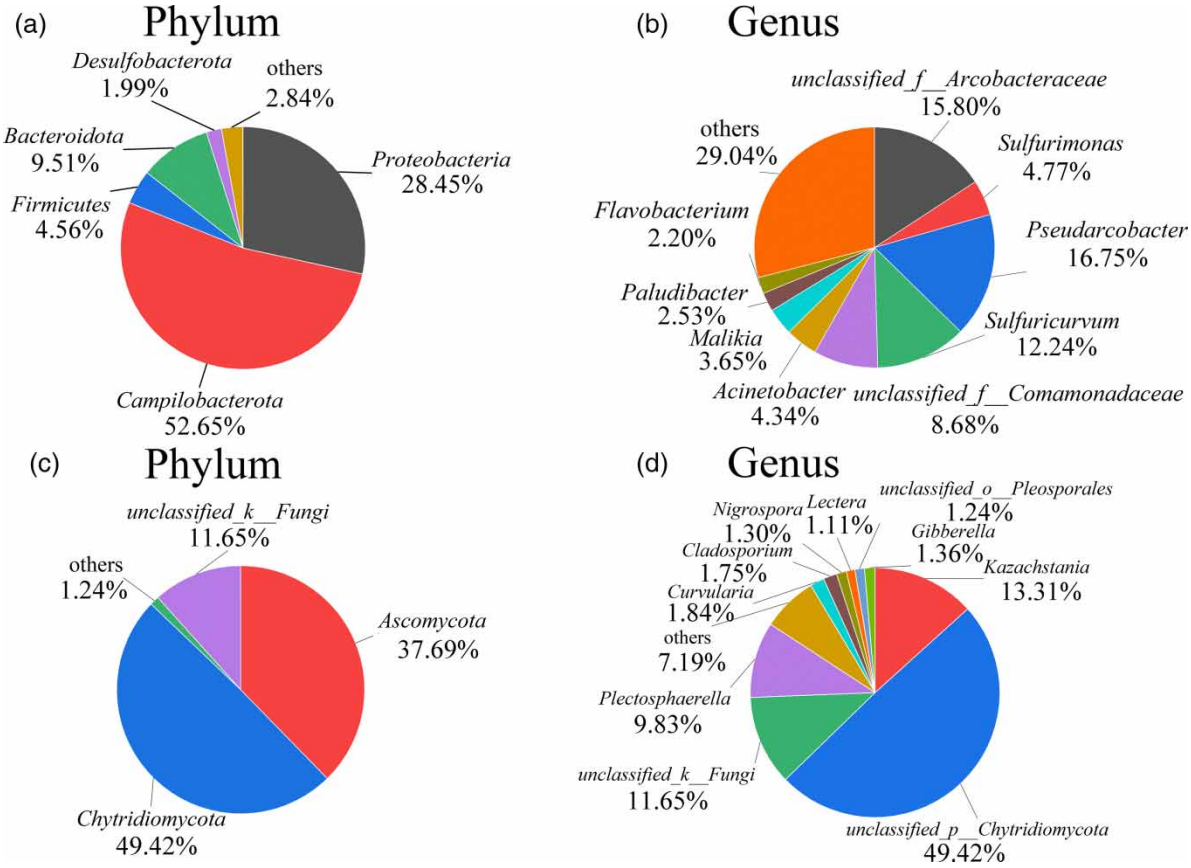


Figure 2 | The microbial community structure of natural biofilms. (a) bacterial community at the phylum level; (b) bacterial community at the genus level; (c) fungal community at the phylum level; and (d) fungal community at the phylum level.

Table 2 | The results of the α-diversity of natural biofilms

	OTUs	Shannon	Simpson	ACE	Chao1	Coverage
Bacteria	699	3.84	0.06	961.26	974.82	99.48%
Fungi	515	2.23	0.27	586.46	564.64	99.83%

identification result was shown in Figure 3(b). The trophic type and functional groups of most fungal microorganisms in natural biofilms are unknown (63.38%). Among the known fungal taxa, in general, natural biofilms were dominated by saprotroph and pathotroph, containing 32 Guilds. The leading Guilds include undefined saprotroph, plant pathogen, animal pathogen–endophyte–lichen parasite–plant pathogen–wood saprotroph, and animal pathogen–plant pathogen–undefined saprotroph.

The physiochemical properties of natural biofilms

Determination of EPSs components and their contents in biofilms

Microbial EPSs of natural biofilms consisted of protein, polysaccharides, and DNA. As shown in Figure 4(a), protein accounted for the largest proportion of the extracted EPSs, while the concentration of polysaccharides was significantly lower than that of protein. DNA only constituted a minor portion of EPSs. Hence, protein and polysaccharides were the predominated part of the EPSs components in natural biofilms. This result was similar to the previous results of EPSs extracted from aerobic granular sludge using NaOH–formaldehyde methods, heating and cation exchange resin methods (Li *et al.* 2020).

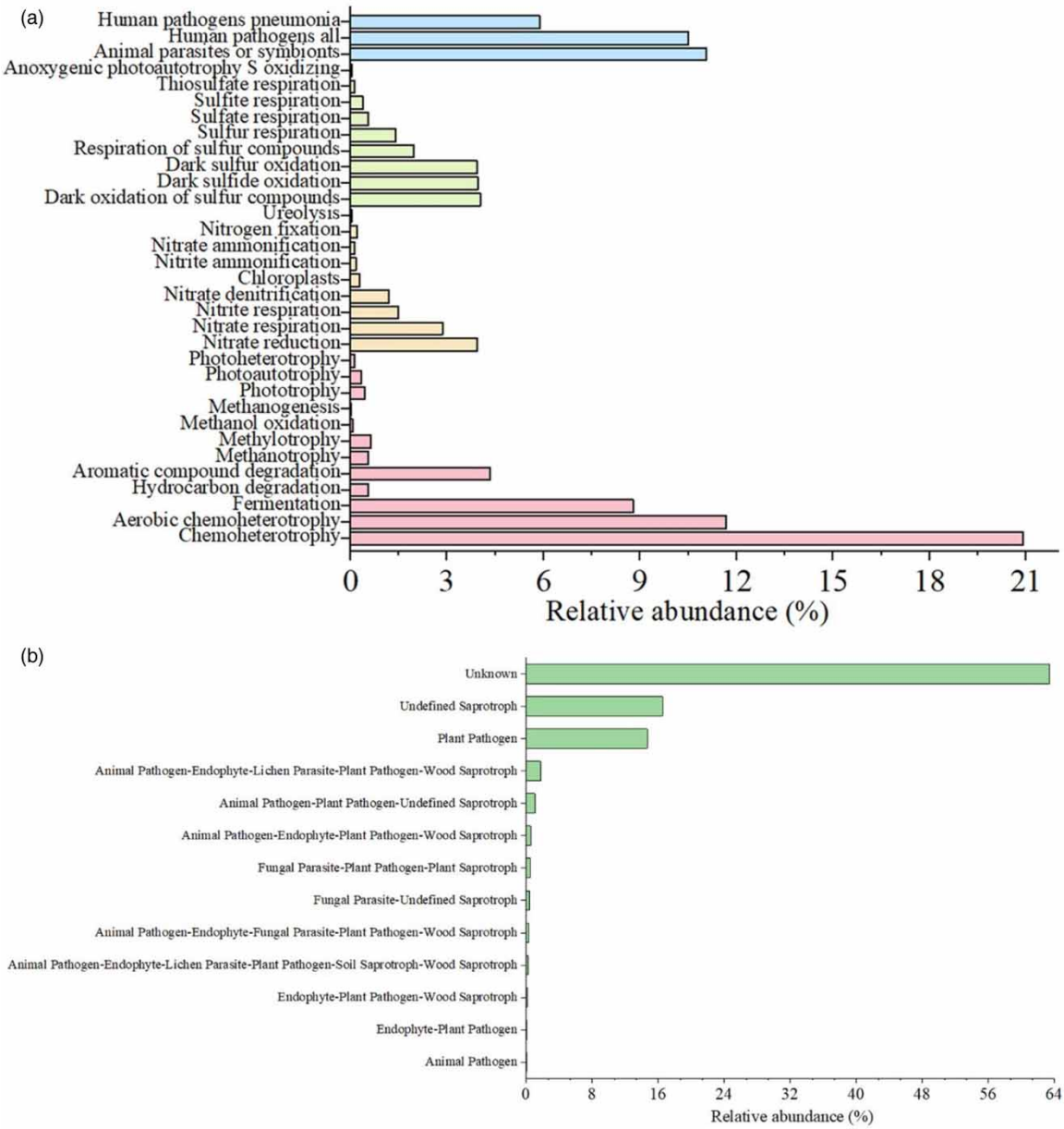


Figure 3 | Functional predictions of natural biofilms in (a) bacterial community using the FAPROTAX annotation and (b) fungal communities using FUNGuild.

SEM observation of biofilms

The observation of SEM (Figure 4(b)–4(d)) showed the microscopic morphology and the formation of natural biofilms. The lyophilized biofilm samples were white sponge-like with uniform and elastic textures. The morphology of the biofilms at 100 μm was shown in Figure 4(b), which can be seen in the form of bands and blocks, with an overall loose and porous appearance. When this biofilm sample was magnified to 10 μm, the presence of a large number of rod-shaped microorganisms, more discontinuous pores, and complex embedding of extracellular polymers with bacterial colonies to form a dense layer of colonies in various forms such as blocks or long strips could be observed. Continuing to zoom in to 2 μm, the different sizes of bacilli can be seen more clearly attached to the extracellular polymer in a lumpy form, and it can also be seen that the extracellular polymer is the backbone of the entire biofilm, providing a habitat for microorganisms.

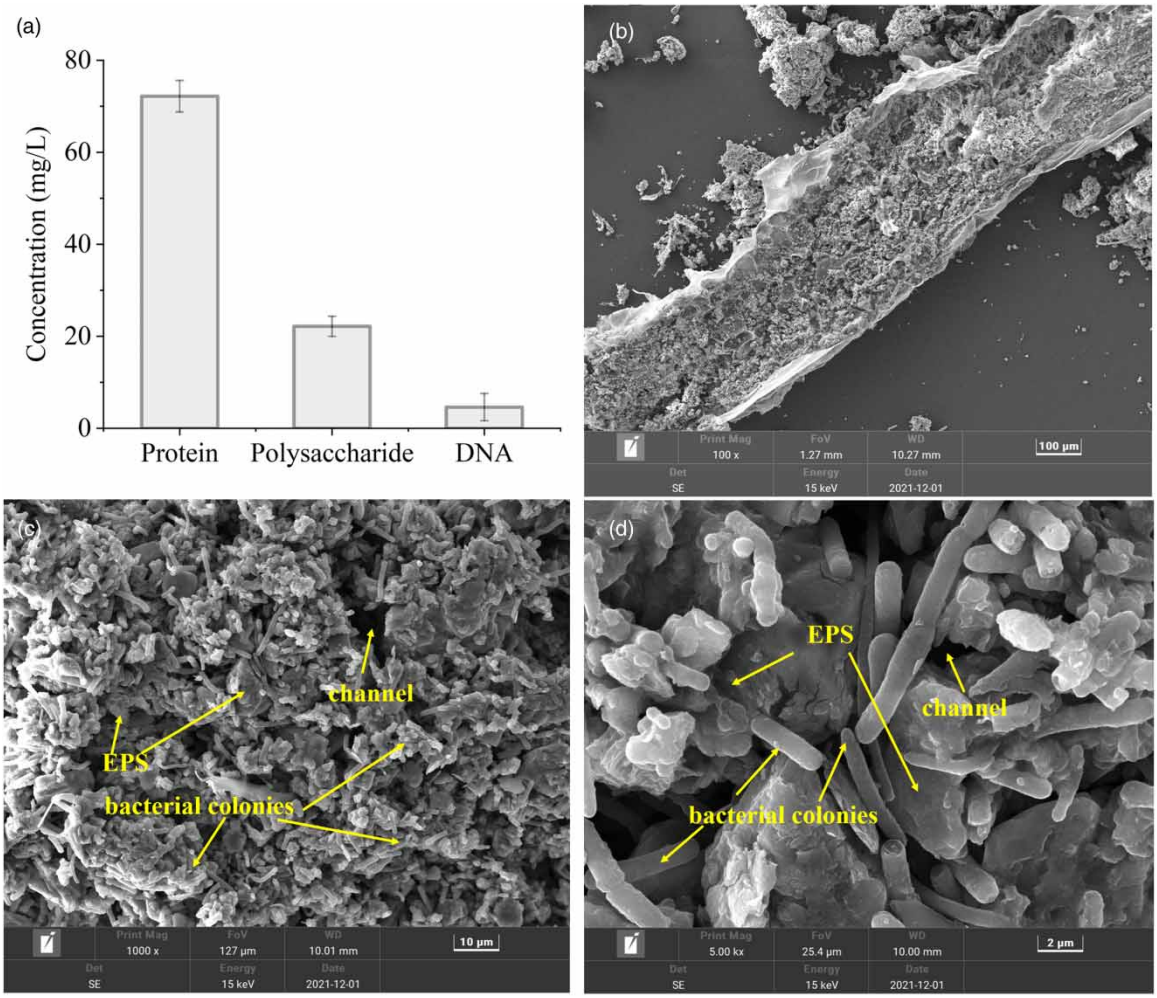


Figure 4 | The physiochemical properties of natural biofilms using (a) components analysis and SEM observation at (b) 10 μm; (c) 5 μm; and (d) 2 μm.

Functional groups of natural biofilms

The FTIR spectra (Figure 5) revealed the bonds and functional groups of the natural biofilms. Functional groups of natural biofilms were mainly located in three regions of 3,700–2,500 cm^{-1}; 1,750–1,250 cm^{-1}; and 1,100–900 cm^{-1}, respectively. Moreover, the peaks at 3,296; 3,070; 1,655; 1,530; and 1,233 cm^{-1} observed were the asymmetric N–H stretching (Amide A), aromatic C–H stretching (Aromatic amino acids), C = O stretching (Amide I), C–H stretching or N–H bending (Amide II), and C–N stretching (Amide III), respectively, which were the characteristic peaks of proteins (Lotti *et al.* 2019). The peaks at 1,233; 1,072; and 1,021 cm^{-1} found were the C–OH stretching, C–H in-plane bending, and C–O–C stretching, respectively, which were assigned to characteristic peaks of polysaccharides (Lotti *et al.* 2019). The peak at 950 cm^{-1} was the characteristic peak of nucleic acids (DNA) (Lotti *et al.* 2019). The result of FTIR indicated that natural biofilms contained carbohydrates, lipids, amines (C–H stretching, 2,930; 2,853; and 1,233 cm^{-1}), methyl, and methylene (CH_3 bending or CH_2 stretching, 1,450 cm^{-1}) in additions to proteins, polysaccharides and DNA (Lotti *et al.* 2019; Wang *et al.* 2019b).

DISCUSSION

Unique physiochemical and biological properties of natural biofilms

Most studies at present have only focused on the structural properties of EPSs extracted from activated sludge in different wastewater treatment processes (Felz *et al.* 2019; Lotti *et al.* 2019; Guo *et al.* 2020). However, few studies have addressed

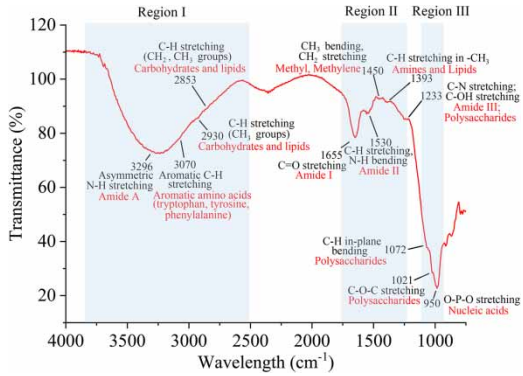


Figure 5 | Fourier transform infrared spectroscopy of natural biofilms.

the physiochemical properties of nature biofilms in heavily polluted rivers (Cheng *et al.* 2018; Ramazanpour Esfahani *et al.* 2021). Compared with previous studies, our study comprehensively reveals the unique physiochemical and biological properties of natural biofilms for the first time. As found in previous studies, the main components of natural biofilms are polysaccharides and proteins (Guo *et al.* 2020). Moreover, the FTIR spectra depicted the functional groups of natural biofilms also contained carbohydrates, lipids, amines, methyl, and methylene (Figure 5). The interaction and combination of these specific functional groups were beneficial to the flocculation, aggregation, and adhesion of microorganisms (Jiang *et al.* 2021). As parts of the active composition of biofilms, these microorganisms play an important role in the biogeochemical cycling and biodegradation of pollutants (Manirakiza *et al.* 2022). Therefore, the biological properties of natural biofilms require further analysis. Previous studies have reported the microbial community structures of naturally grown biofilms and biofilms formed in wastewater treatment processes, respectively (Supplementary material, Table S2). Compared to these biofilms dominated by bacterial phylum *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*, natural biofilms have unique bacterial communities, the most abundant populations belonged to the phyla *Campilobacterota*, *Proteobacteria*, *Bacteroidota*, *Firmicutes*, and *Desulfobacterota* (Figure 2(a)). In addition, the fungal community of natural biofilms appeared to be particularly distinctive compared to previously reported studies (Supplementary material, Table S2). The unique physiochemical and biological properties of natural biofilms might lead to its special functions.

The purification capacity of natural biofilms for different pollution indicators of heavily polluted waterbodies

Biofilm activities, including physical biosorption of EPSs and biodegradation by microorganisms, are the basis of river self-purification (Hou *et al.* 2022). However, the proportion of physical biosorption of EPSs and biodegradation by microorganisms in self-purification of natural biofilms is still unknown. Our study demonstrated through indoor experiments that the purification capacity of natural biofilms was dominated by the biodegradation function of microorganisms rather than the physical biosorption of EPSs (Figure 1). Although most current studies have focused on the biosorption of EPSs in biofilms to various exogenous toxic compounds, including heavy metals and refractory organic compounds (phenols, antibiotics, polycyclic aromatic hydrocarbons, etc.) (Tian *et al.* 2019). Extensive studies have found that the biosorption of these pollutants includes adsorption, surface chelation, complexation, ion exchange, and precipitation. (Jiang *et al.* 2021). Our study found that this biodegradation of microorganisms alleviated the pollution of various tropic indicators, such as COD, TP, NH_4^+-N, and NO_3^--N, but the purification ability of each indicator was significantly different. Moreover, functional microorganisms with different degrading functions, such as hydrocarbon degradation, aromatic compound degradation, nitrate/nitrite respiration, nitrate denitrification, etc., have always been a research hotspot in wastewater treatment (Xu *et al.* 2012; Zhou *et al.* 2018). We proposed a conceptual model of the purification capacity of natural biofilms in heavily polluted waterbodies (Figure 6). The natural biofilm acts like a filter pump to clean pollutants from water bodies. The bacteria and fungi in natural biofilm act as the cleaners of these pollutants.

The potential biological mechanisms of natural biofilms for remediation of polluted water bodies

We further analyzed the potential mechanisms of natural biofilms for the remediation of polluted waterbodies based on previously reported studies. The primary microbial communities of natural biofilms at the phylum level were *Campilobacterota*,

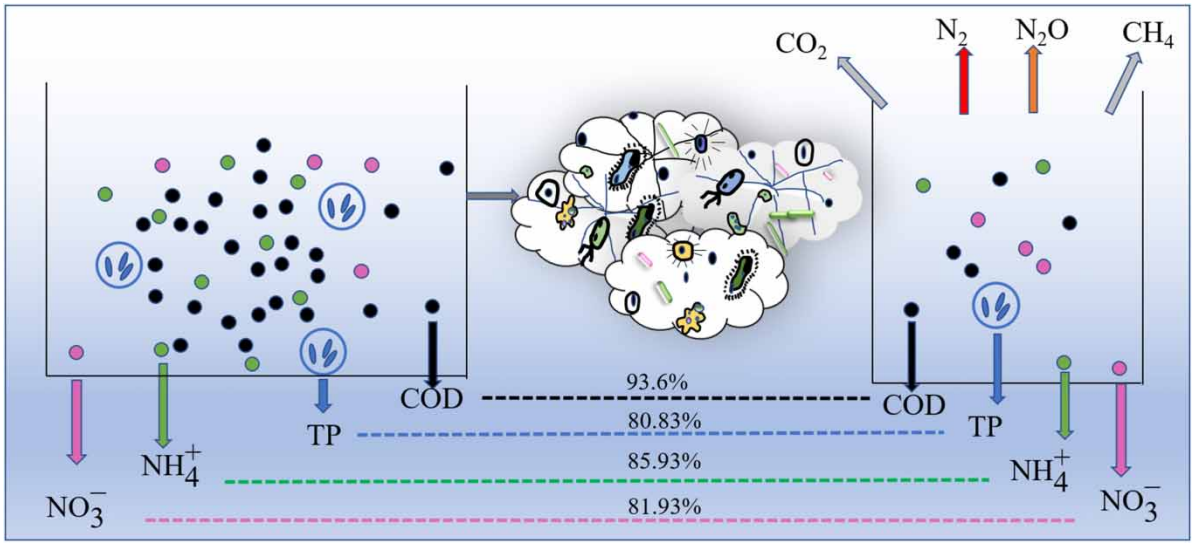


Figure 6 | The conceptual model for the purification capacity of natural biofilms for heavily polluted waterbodies.

Proteobacteria, *Bacteroidota*, *Firmicutes*, and *Desulfobacterota* (Figure 2). *Campilobacterota* were rarely found in either naturally grown biofilms or biofilms formed during wastewater treatment processes but were well known for degrading organic matter (Zhu *et al.* 2022b). *Proteobacteria* typically occupy dominant proportions in the aquatic ecosystem and various municipal wastewater treatment reactors (Yuan *et al.* 2020; Zhang *et al.* 2020). Moreover, *Proteobacteria* were directly associated with ammonia oxidization and denitrification processes (Cai *et al.* 2016; Song *et al.* 2021), which might be the reason why indoor experiments showed efficient ammonium and nitrate removal performance. *Bacteroidota* is always involved in the degradation of carbohydrates (Li *et al.* 2021a). Moreover, a previous study has found that *Proteobacteria* and *Bacteroidota* play important roles in denitrifying phosphorus removal (Qu *et al.* 2021). *Firmicutes*, which participated in refractory organic compounds decomposition and microbial nitrogen fixation, could be promoted to grow by a high concentration of organic matter (Li *et al.* 2021a). At the genus level, *Pseudarcobacter*, *unclassified_f_Arcobacteraceae*, *Sulfuricurvum*, *unclassified_f_Comamonadaceae*, *Sulfurimonas*, *Acinetobacter*, *Maikia*, *Paludibacter*, and *Flacobacterium* were the most abundant genera in natural biofilms. *Pseudarcobacter*, which are responsible for the transmitted electrons, are significantly related to nutrient elements metabolisms (Luo *et al.* 2022). Previous studies demonstrated that many members of the *Arcobacteraceae* family are nitrate-reducing bacteria (Gulliver *et al.* 2020). *Sulfuricurvum* and *Sulfurimonas* play important roles in the removal of toxic organic matter and nitrate (Li *et al.* 2019; Fida *et al.* 2021). *Acinetobacter* and *Comamonadaceae* are reported as typical phosphorus-accumulating organisms (PAOs) that are widely applied in wastewater treatment because of their abilities to remove phosphorus (Zhao *et al.* 2022). *Paludibacter* were proposed as common sulfate and refractory organics degraders (Liang *et al.* 2013; Wang *et al.* 2019a). The presence of *Flacobacterium* can promote the denitrification ability of natural biofilms at low temperatures (Qu *et al.* 2021).

At the fungal phylum level, *Chytridiomycota* have been used in the production of antibiotics, organic acids, hormones, vitamins, and in the brewing industry (Money 2016). Most species in the *Ascomycota* phylum can decompose cellulose and chitin (Czaplicki *et al.* 2018; Challacombe *et al.* 2019). At the fungal genus level, the beneficial contributions of *Kazachstania* are well-established in various food processes (Spanoghe *et al.* 2017; Urubschurov *et al.* 2018). *Plectosphaerella*, which have previously been shown to be potential biocontrol agents, are effective against potato cyst nematodes (Kusstatscher *et al.* 2019). These results confirmed that natural biofilms contain a large number of functional microorganisms that can purify polluted water bodies, and are worthy of our further exploration of patterns and strategies for their extensive application in polluted water.

CONCLUSION

In this study, the inherent purification capacity, physiochemical, and biological properties of natural biofilms collected from a heavily polluted river were synchronously investigated. Outdoor sampling and indoor experiments demonstrated the superior

purification effect of natural biofilms on heavily polluted water. Indoor experiments showed that the purification capacity of natural biofilms was dominated by the microbial biodegradation rather than physical biosorption of EPSs, and after 14.0 days of incubation, the removal rates of COD, TP, NH_4^+-N, and NO_3^--N could reach 93.6, 80.83, 85.93, and 81.03%, respectively. The SEM, FTIR spectra, and components analysis revealed that natural biofilms were mainly composed of polysaccharides and proteins, and also contained small amounts of functional groups such as carbohydrates, lipids, amines, methyl, and methylene. High-throughput Illumina MiSeq sequencing analysis illustrated that the dominant phyla in the bacterial community structure were *Campilobacterota* (52.65%), *Proteobacteria* (28.45%), *Bacteroidota* (9.51%), *Firmicutes* (4.56%), and *Desulfobacterota* (1.99%), and the major phyla in fungal community structure were *Chytridiomycota* (49.42%) and *Ascomycota* (37.69%). These microorganisms might be the main degraders of riverine pollutants. However, this study only focused on the removal of limited contaminants by natural biofilms and did not identify which microorganisms were involved in the purification processes. Further investigations are recommended to pay attention to the purification effects of natural biofilms on novel pollutants, such as heavy metals and refractory organic pollutants, and to determine the changes in microbial community structures during the purification processes.

AUTHORS CONTRIBUTIONS

W.D. studied methodology, did data analysis and data curation, wrote, reviewed and edited the article. X.Z. conceptualized the study, performed methodology, investigated the study, wrote the original draft, wrote, reviewed and edited the article. W.X. studied methodology. H.P. did project administration, acquired funds, and supervised the study. H.Y. acquired funds and supervised the study. Y.D. studied methodology.

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CONSENT FOR PUBLICATION

All authors have reviewed, approved, and consented to the publish, and they are accountable for all aspects of its accuracy and integrity.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its supplementary information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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