



Review

Extracellular polymeric substances—antibiotics interaction in activated sludge: A review



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ARTICLE INFO

Article history:

Received 16 June 2022

Received in revised form

27 October 2022

Accepted 28 October 2022

Keywords:

Extracellular polymeric substances

Antibiotics

Antibiotic resistance genes

Sorption

Biotransformation

Resistance

ABSTRACT

Antibiotics, the most frequently prescribed drugs, have been widely applied to prevent or cure human and veterinary diseases and have undoubtedly led to massive releases into sewer networks and wastewater treatment systems, a hotspot where the occurrence and transformation of antibiotic resistance take place. Extracellular polymeric substances (EPS), biopolymers secreted via microbial activity, play an important role in cell adhesion, nutrient retention, and toxicity resistance. However, the potential roles of sludge EPS related to the resistance and removal of antibiotics are still unclear. This work summarizes the composition and physicochemical characteristics of state-of-the-art microbial EPS, highlights the critical role of EPS in antibiotics removal, evaluates their defense performances under different antibiotics exposures, and analyzes the typical factors that could affect the sorption and biotransformation behavior of antibiotics. Next, interactions between microbial EPS and antibiotic resistance genes are analyzed. Future perspectives, especially the engineering application of microbial EPS for antibiotics toxicity detection and defense, are also emphatically stressed.

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1. Introduction

Antibiotics are the most frequently used pharmaceuticals for preventing and treating infectious diseases in humans and animals, with global consumption of 42 billion defined daily doses in recent years [1]. Since the antibiotics used are only partially metabolized in the human and animal digestive tracts, large amounts of antibiotics are discharged into the aquatic ecosystem from point sources (pharmaceutical industries, hospitals, and households) and nonpoint sources (agricultural and urban runoffs) [2], leading to severe pollution throughout the world. Recent works revealed that the average concentrations of typical antibiotics in different environmental matrices (such as wastewater, sewage, river sediment, and soil) ranged from $\mu\text{g L}^{-1}$ to ng L^{-1} [3,4].

Municipal wastewater treatment plants (WWTPs) are considered the core facilities for antibiotics reception and dissemination [5]. However, WWTPs are not specifically constructed for antibiotics treatment and removal [2]. Therefore, the removal of

antibiotics varies over orders of magnitude during WWTPs operation, depending on the physicochemical properties of antibiotics, sludge activities, and the operational parameters of WWTPs [2]. The existence of antibiotics and their residues not only inhibits microbial activity and subsequently affects the microbial community composition but also promotes the growth of antibiotic-resistant bacteria and the subsequent dissemination of antibiotic resistance genes (ARGs) [6].

Extracellular polymeric substances (EPS), the typical high-molecular-weight microbial polymers composed of hydrocarbons, proteins, humic organics, etc., are responsible for the adsorption of biotoxins, mediating toxic inhibition, and protecting organisms against external threats [7]. In addition to being an extracellular physical barrier, EPS could also provide many functional groups (e.g., carboxyl, hydroxyl, amino, phosphoryl, and sulfhydryl groups) for contaminant adhesion and further decrease their exposure risk and toxic stress [8]. Undoubtedly, EPS play a dominant role in antibiotics adsorption, preventing their direct contact with microbes to endow microbes with resistance to antibiotics [1].

Recently, several studies have confirmed and emphasized the role of EPS in antibiotic stress resistance during WWTPs operation [2,9,10]; however, systematic and in-depth analyses of EPS

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feedback under different antibiotics exposures, especially the complex interactions and potential mechanisms between the antibiotics and EPS are still lacking, which limits the potential application of sludge EPS for antibiotics removal. Therefore, the interactions between EPS and antibiotics were systematically elucidated and analyzed. This study summarized the basic knowledge of EPS and its importance in resisting antibiotics stress. Subsequently, the performance and potential mechanisms of EPS concerning antibiotics removal were evaluated. Several critical operating factors affecting EPS-antibiotics interactions were also analyzed in detail. Furthermore, recent advances in EPS and antibiotics resistance genes (ARGs) were also analyzed. Finally, future opportunities for functional and engineering applications of EPS were outlined. Overall, this review aims to provide insights into the complex interactions between EPS and antibiotics to achieve an optimized protection approach to effectively alleviate antibiotics inhibition in WWTPs.

2. Effects of EPS composition and functions on antibiotics exposure

2.1. EPS composition and functions

Microbial EPS are complex biopolymer mixtures of proteins, polysaccharides, nucleic acids, etc., which can be divided into tightly bound EPS (TB-EPS), loosely bound EPS (LB-EPS), and soluble EPS (S-EPS) according to their binding degree with cells [11–13]. Generally, EPS present a network-like structure with different functional groups (such as carboxyl-, phenolic-, sulfhydryl-, phosphoric-, and hydroxyl-related groups) and polar groups (aromatic-, aliphatic-, hydrophobic-regions, etc.), which play a vital role in sludge aggregation, floc formation, and pollutant biotransformation [14,15]. In addition, EPS is closely related to multiple sludge properties, including hydrophilicity/hydrophobicity ratio, bio-sorption, bioflocability, settleability, surface-charge distribution, and biodegradability [16]. In addition, certain growth conditions, such as nutrient limitation and the presence of toxins (e.g., heavy metals and antibiotics), may lead to the secretion of EPS and activate its barrier effect to resist the unfavorable environment and reduce oxidative stress.

Recently, tremendous works have clarified the role of EPS in environmental remediation and stress resistance, especially for emerging contaminants such as antibiotics and ARGs [17–19]. Sludge EPS, especially proteins and polysaccharides with abundant functional groups, such as carboxyl, amine, and hydroxyl groups, can effectively bind with antibiotics and form a stable EPS-antibiotics complex, thereby achieving the efficient removal of antibiotics (>60%) [20]. Notably, EPS structures and binding performances with antibiotics are greatly affected by the associated EPS molecules and environmental conditions (e.g., pH, temperature, substrates, and presence of toxins) [7]. For example, changes in environmental conditions such as pH and temperature might affect the stability of the EPS-antibiotics complex [21,22]. The types of substrates and the presence of toxins (e.g., heavy metals and antibiotics) might change the compositions, contents, and secondary structure of EPS, subsequently affecting the adsorption capacity and rate. In addition to adsorption, antibiotics can be degraded or transformed due to the presence of abundant exoenzymes, including hydrolases for carbohydrates, amylases for starch, and proteases for proteinaceous substrates [23,24]. The porous structure and complex matrix of EPS are helpful to capture antibiotics and retain exoenzymes, which makes EPS important sites to digest these components before they become accessible to microbial cells [7]. Additionally, multiple redox-active components (e.g., cytochromes, flavins, and phenazine) exist in the EPS matrix,

which could facilitate antibiotic transformation by acting as electron transfer media [25,26]. Considering the multiple complex effects of EPS on antibiotics defense and removal, the detailed feedback performances, potential functional features, and relations to group behaviors should be further clarified.

2.2. Importance of EPS resistance to antibiotics exposure

EPS account for as much as 50–90% of sludge flocs. They have an important role in antibiotics removal in traditional WWTP operation [27], in which the adsorption of antibiotics onto sludge EPS, as well as the potential transformation, played an important role in antibiotics removal, compared to the hydrolysis and volatilization mechanism [2]. For example, several studies have confirmed that microbial EPS are an important potential reservoir that could hinder sulfamethizole and sulfonamide antibiotics diffusion, hence alleviating direct cell-antibiotic interactions and the subsequent inhibition effects [8,10].

Diffusion of antibiotics (e.g., fluoroquinolones and β -lactams related) led to a large quantity generation of reactive oxygen species, negatively affecting the biomass functions via the deterioration of respiratory rate, inhibition of enzymatic activities, and damaging of cells and intracellular DNA [28,29]. The protection provided by EPS reduces the negative effects of antibiotics on catalase and superoxide dismutase, thereby mediating the stress of reactive oxygen species in cells [8,30]. In contrast, after the extraction of the EPS, an obvious decline in enzymatic activity was observed, accompanied by the mortality of the microbes, release of intracellular substances, and declining respiration rates under sulfamethizole exposure [8,31]. Moreover, lower community richness and diversity were observed under sulfamethizole exposure without EPS protection [8].

3. Performance and potential mechanisms of EPS resistance to antibiotics stress

3.1. Adsorption of antibiotics onto EPS

Once toxic antibiotics enter the WWTP systems and contact sludge, abundant functional groups in the dense network structure of EPS bind with antibiotics immediately and serve as a permeability barrier against antibiotics erosion. Three stages of rapid adsorption, stable adsorption, and saturation adsorption could be observed during antibiotics sorption onto sludge EPS [18,32]. Among the kinetic models, in comparison with the pseudo-first-order reaction kinetics equation, the pseudo-second-order kinetics equation exhibited a better fit for antibiotics (e.g., trimethoprim, sulfonamide, tetracycline) adsorption simulation, implying that the adsorption was driven by chemical action [17,18,33,34]. Additionally, the sorption isotherm analysis revealed that the Freundlich equation might be much more suitable for illuminating the sorption characteristics between EPS and antibiotics (e.g., quinolone, sulfonamide, tetracycline, ciprofloxacin) than the Langmuir model, indicating that multilayer adsorption or even more complex sorption might occur on the microbial EPS surface [32,35–37].

The main mechanisms related to antibiotics adsorption onto EPS mainly include (1) electrostatic interactions (e.g., cation bridging, π - π conjugation reaction, anion exchange), (2) hydrogen bonding, (3) hydrophobic interactions, and (4) surface complexation [36,38,39], and are listed in Fig. 1a. During the adsorption process, functional groups (i.e., carboxyl, hydroxyl, and amine) of proteins in EPS were bound with antibiotics, and formed a stable and compact EPS-antibiotics complex, thereby reducing the toxicity of antibiotics (Fig. 1b) [40]. The interaction order of EPS components to tetracycline exhibited a declining trend of tyrosine residues $\rightarrow$

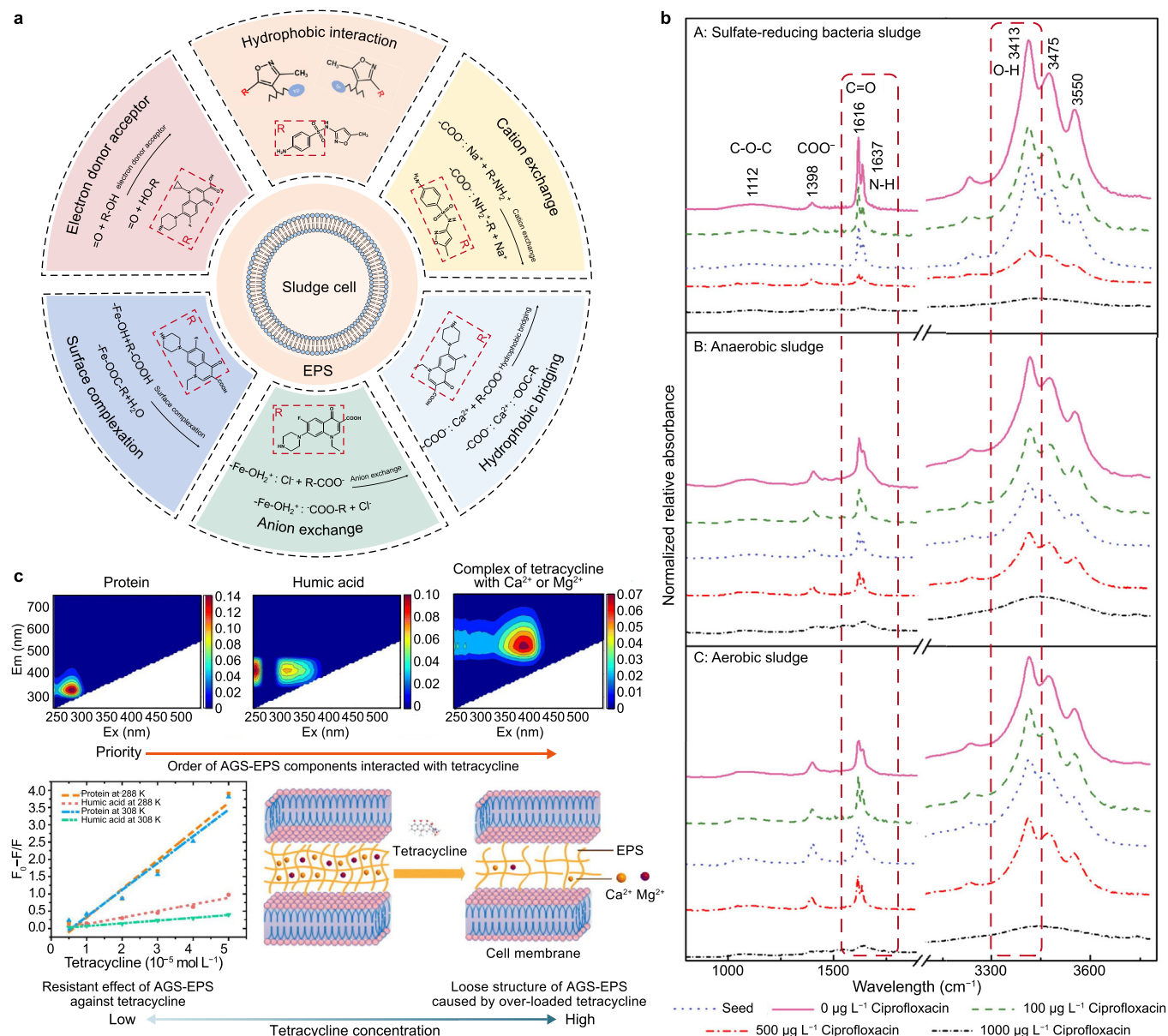


Fig. 1. a, Sorption mechanisms of different antibiotics onto sludge EPS. b FTIR spectra of EPS extracted from sulfate-reducing bacteria sludge (A), anaerobic sludge (B), and aerobic sludge (C) modified from Ref. [44], copyright (2018) ACS publications. c, The order priority of sludge EPS components interacted with tetracycline, modified from Ref. [1]; copyright (2021) Elsevier.

tryptophan → humic acid, and the larger molecular weight components in EPS exhibited a priority interaction trend with tetracycline antibiotics than the smaller molecular weight ones (Fig. 1c) [40]. [39] reported that even though sulfamethazine could be both adsorbed by EPS-related humic-like and protein organics, the binding strength of antibiotics onto protein was almost >2.0 orders of magnitude higher than that of humic-like EPS; because the percentage of proteins in EPS was approximately 3–4 times higher in comparison with the humic-like organics, the proteins undoubtedly played a predominate role in antibiotics removal, which was closely related to their higher binding ability and larger percentage distribution. Similarly, Xu et al. [39] observed that the static quenching of fluorophores in EPS proteins with 25 mg L⁻¹ sulfamethazine adsorption and the Gibbs free energy of the sulfamethazine binding onto sludge EPS was thermodynamically favorable,

which revealed a spontaneous binding process and stable EPS-antibiotics complex formation [9]. reported that the zeta potential of the EPS-sulfamethoxazole complex increased with increasing concentrations. Generally, the higher the zeta potential value is, the stronger the electrostatic repulsion [41]; thus, the adsorption of antibiotics decreased the negative charges of sludge and changed the configuration of EPS [42]. reported that the biomolecule size of the EPS declined as tetracycline concentrations increased from 0 to 100 mg L⁻¹, indicating that the continuous toxic stress of antibiotics caused the EPS structure to fold. In addition, the secondary structure of the protein in the EPS (e.g., α -helix/ β -sheet + random coil) and β -sheet value) increased with the steady increase in antibiotic concentrations [43]. Overall, these important physical structures and chemical characteristic variations would be helpful to improve the adsorption capacity of EPS and facilitate

adsorption processes, including hydrophobicity-dependent and hydrophobicity-independent adsorption, which played a key role in antibiotics adsorption onto sludge EPS and the subsequent toxic resistance [2]. Therefore, the chemical compositions and structures of the microbial cells changed insignificantly because EPS protected cells and alleviated antibiotics stress.

After binding with antibiotics, the expansion of polymers and the stretching of the peptide chains (conformation of protein) caused a more irregularly loose and porous EPS morphology, which is meaningful to mass transfer and antibiotics capture [39]. However, the variation in the sludge EPS is not irreversible, and antibiotics might be released under the effects of photolysis, oxidation, and heating [42]. The highly mobile characteristics of the EPS-antibiotic complex exist in dissolved form, and diffusion and dissemination risks of antibiotics resistance in aquatic environments may exist [42]. Therefore, the EPS-antibiotics complex still poses a potential ecological risk.

3.2. EPS synthesis regulated by quorum sensing

In addition to the adsorbing effect, the AHL-mediated quorum sensing (QS) system was activated by antibiotics pressure at the same time since a large number of EPS was synthesized and secreted after gene expression, forming a network structure outside the cells (hindering the direct contact between antibiotics and microbes) to alleviate toxic stress (Fig. 2). QS mechanism regulation has been regarded as the main reason for the regulation of EPS production, adjusting bacterial abundance and maintaining functional bacterial activity, especially under antibiotics stress [10]. After the QS system was activated, second messenger molecules such as N-acylhomoserine lactones (AHLs) were synthesized, which led to the main expression of intracellular genes of sludge [45], especially the genes related to overexpressed pressure induction, transcriptional regulation and EPS synthesis (such as *lapA* and *lapF*) [46–48].

Briefly, short-chain AHLs, including C4-HSL, C6-HSL, and C8-HSL, were secreted and enhanced EPS production under antibiotics stress, which improved the antibiotics tolerance of the biomass [50]. In addition, the production of long-chain AHLs (e.g., C10-HSL, C12-HSL, and C14-HSL) benefited antibiotics stress release

in sludge reactors [50]. Under the effect of AHLs, the content of EPS, especially the protein and polysaccharides, increased rapidly. Considering that the antibiotics diffusion coefficient in polysaccharides and glycoproteins was only 40–72% of that of water [51], the diffusion of antibiotics was effectively retarded, and the direct interaction between antibiotics and intercellular cells was alleviated by the newly generated EPS [31]. In addition, an increase in tryptophan, tyrosine, and humic substances of sludge EPS, as well as functional groups and binding sites, were observed for antibiotics defense [52].

Although most of the EPS functional groups were stable under antibiotics resistance, the compositions of the EPS observed via the FTIR spectra [53] changed slightly, indicating that the microbes secreted different types of components to resist or reduce the damage of biotoxins to bacteria. For the predominant fractions of sludge EPS, the content and percentage of protein and polysaccharide varied obviously for both TB-EPS and LB-EPS once antibiotics resistance occurred. For example [54], observed that the abundant existence of sulfamethoxazole (10 mg L^{-1}) led to the protein/polysaccharide ratio of LB-EPS increasing from 2.6 to 3.5, indicating that the stress-induced microorganisms to secrete more protein (protein was more responsible for stress release than the polysaccharide). Therefore, EPS-related proteins play an essential role in antibiotics binding via complex interactions, including hydrophobic effects, cation bridging, and polymer entanglement, to form a dense network structure to guard against adverse stress [53]. The increased protein/polysaccharide ratio also indicated that the hydrophobicity of sludge samples increased [9,44].

In addition to the changes in EPS compositions, the types of EPS also varied greatly under antibiotics stress. For example [55], reported that the content of TB-EPS sharply increased from 34 mg L^{-1} to 46 mg L^{-1} under the stress of 5 mg L^{-1} oxytetracycline antibiotics via QS feedbacks. Subsequently, the conversion of TB-EPS to LB-EPS was also observed as a protective response to oxytetracycline antibiotics stress [55]. Specifically, the oxytetracycline antibiotics bound or adsorbed onto LB-EPS promptly reacted with TB-EPS, leading to the destruction of the chemical composition of TB-EPS and an obvious simultaneous increase in LB-EPS [55]. Overall, TB-EPS served as the first protective barrier for microorganisms to defend against oxytetracycline antibiotics exposure

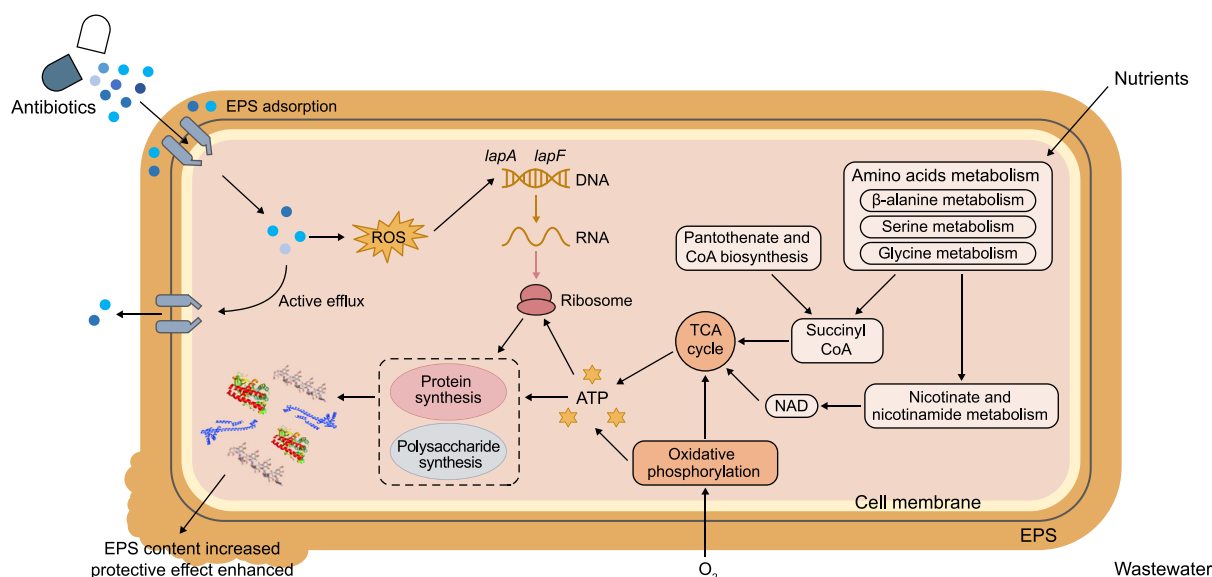


Fig. 2. EPS synthesis and regulation under different antibiotics exposure via QS, reprinted with permission from Ref. [49]; copyright (2022) Elsevier.

owing to their dense and tight structure, which may play a vital role in resisting antibiotics toxicity [56].

However, the resistance ability of EPS is still limited, and overloaded antibiotics gradually consume the active sites and finally deteriorate the EPS function [57]. For example [58], stated that a high concentration (20 mg L^{-1}) of sulfamethoxazole led to the observations of polysaccharide destruction, amino acid denaturation, and protein secondary structure destruction, as well as the conservation of tryptophan-like proteins into aromatic rings and, finally, obvious changes in the EPS composition. In addition, the fluorescence intensity of aromatic proteins and humic acid-like organics declined sharply to almost quenching [58]. This may lead to the penetration of the antibiotic into sludge cells, while the latter may develop ARGs to mitigate lethal effects under long-term exposure to antibiotics. Although the increased sulfadiazine may induce microbes to generate more EPS to release stress, the inevitable occurrence of ARG blooms would be observed under long-term stress [59]. Additionally, the molecular structure of the protein in the EPS was destroyed under 40 mg L^{-1} ciprofloxacin stress; subsequently, the humic acid-like substance became the predominant component of sludge EPS [60].

3.3. Transformation of antibiotics promoted by EPS

The adsorbed antibiotics would be dynamically complexed by EPS [10], and many types of extracellular active degradative enzymes exist in EPS, such as α -amylase, β -amylase, and protease

[7,23], which would hydrolyze those higher biomolecules into lower molecular weight organics and thus enhance the removal of antibiotics [24,61]. In addition, antibiotics can also be degraded into nontoxic substances via biological transformation [61–63]. [64] reported that a series of enzyme-catalyzed reactions, including amide hydrolysis, substituent oxidation, and sulfide oxidation, occur during the transformation of five antibiotics, including amoxicillin, ampicillin, clindamycin, daptomycin, and linezolid. They also reported a noteworthy biotransformation potential of antibiotics among the soluble extracellular enzymes extracted from activated sludge via the measurement of protease and peptidase activity [64].

In addition to enzyme-related transformation, the photochemical behavior of EPS was also beneficial for antibiotics transformation (Fig. 3). Briefly, the portions of EPS with higher C/H and O/C ratios, leading to the phototransformation of EPS generate reactive species (triplet intermediates, $\cdot\text{OH}$, and $^1\text{O}_2$), are more susceptible to antibiotics photolysis [65]. [66] reported that the transformation of tetracycline under illumination was enhanced from 6.6% to 95.7% by reactive species (triplet intermediates ($^3\text{EPS}^*$), hydroxyl radicals ($\cdot\text{OH}$), and singlet oxygen ($^1\text{O}_2$)) via a series of photochemical reactions, including demethylation, deamination, and oxidation. It should be noted that the hybrid effects of the chemical characteristics of sludge EPS and the intensity of illumination on antibiotics reduction are important and practical, especially in natural aquatic environments involving massive amounts of EPS, such as estuaries and wetlands [66]. The

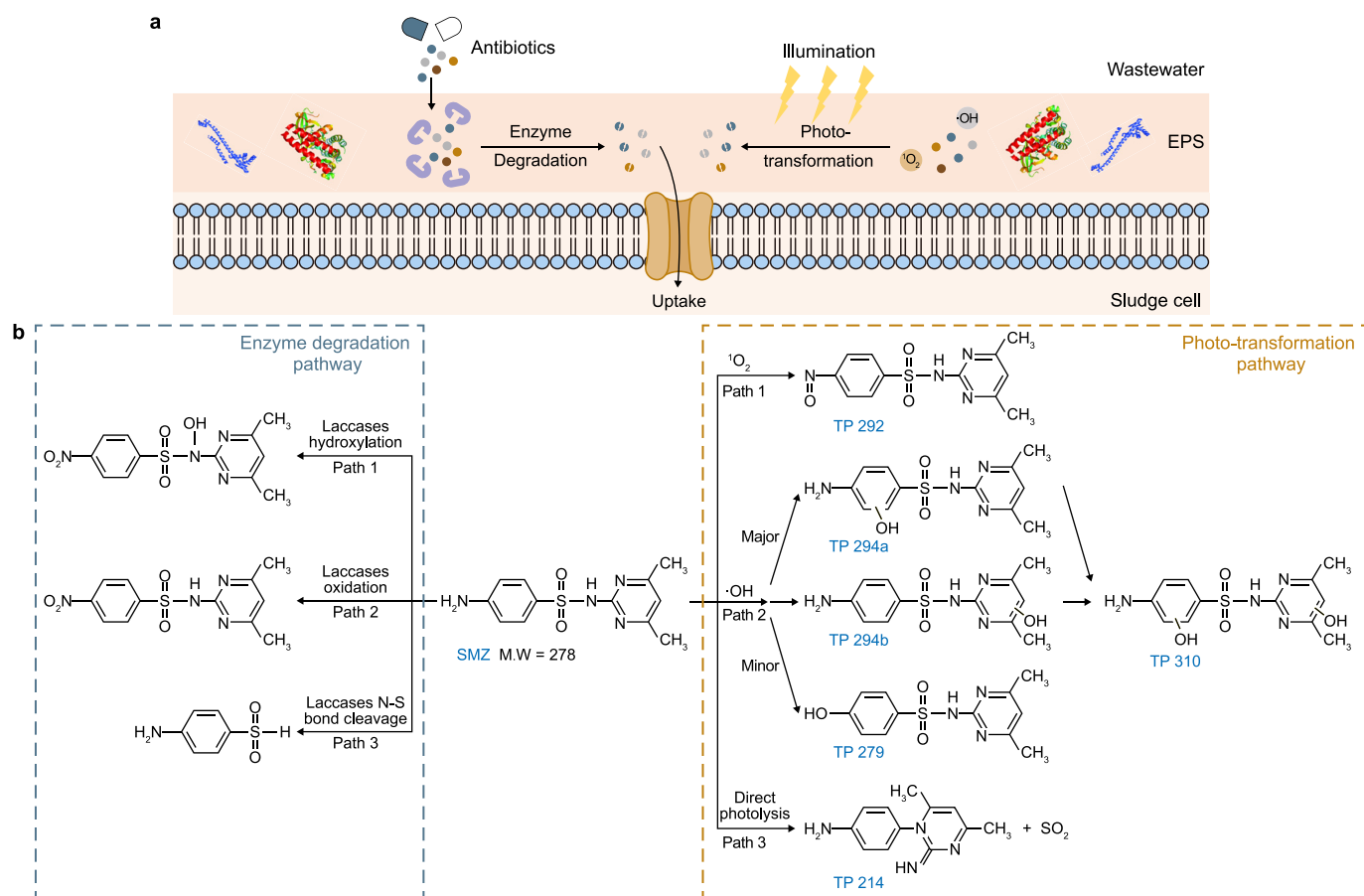


Fig. 3. a, Transformation process of antibiotics on EPS. b, Proposed biotransformation pathway for antibiotics enzyme degradation (blue box) and photo-transformation (yellow box), reprinted with permission from Ref. [67], copyright (2019) Elsevier.

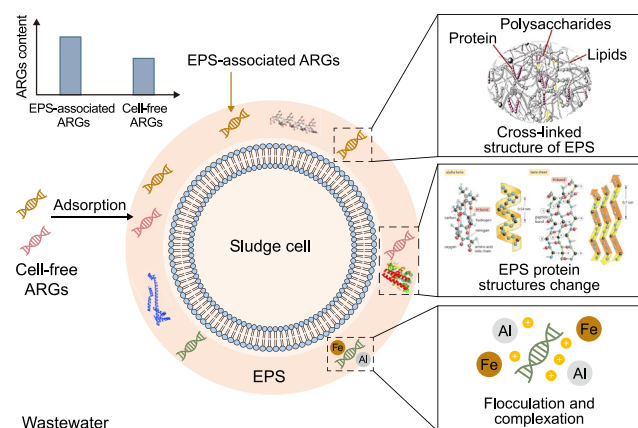


Fig. 4. Mechanisms of antibiotic resistance genes adsorption on EPS.

photochemical sensitivity characteristics of sludge EPS would strengthen the natural attenuation capability of natural waters and provide novel methods for antibiotic removal.

3.4. Mitigating ARGs proliferation by EPS

Refractory antibiotics cannot be completely biodegraded in traditional WWTPs. Microbes develop genetic mutations to mitigate lethal effects under long-term antibiotic exposure, even at the $\mu\text{g L}^{-1}$ level [68,69]. Abundances of ARGs generally exhibited an increasing trend with the increasing of antibiotic concentrations [44,70], whereas the generated antibiotic resistance might be transmitted and spread to other bacteria [71]. A bacterial host cell can acquire antibiotic resistance through the following three different routes: vertical gene transfer, de novo mutation, and horizontal gene transfer (HGT) [71]. During wastewater treatment, HGT (including transduction, conjugation, and transformation) is considered a major avenue for ARGs proliferation because ARGs can potentially be acquired by new bacteria and obtain resistance via natural transformation in downstream environments [58,72]. ARGs can exist in both extracellular and intracellular forms, and either conjugation or transduction might be the major route of transmission for intracellular ARGs in the cell [73], whereas extracellular ARGs can be captured by several nonresistant bacteria, or mobile genetic elements (MGEs), such as integrons or abiotic pellets through transformation, leading to the dissemination and proliferation of extracellular ARGs [74]. Generally, extracellular ARGs in cell-free form have received more attention due to their easy diffusion and proliferation via HGT [44].

EPS with a strong adsorption capacity might selectively capture some extracellular ARGs, retard extracellular ARGs proliferation and interaction, and weaken ARGs HGT [19,75,76]. Compared with cell-free ARGs, the abundance of EPS-associated ARGs (e.g., *sulI*, *sulII*, *bla_{TEM-1}*, *tetA*, *tetO*, *tetQ*, *tetW*) were more abundant than cell-free ARGs (0.2–4.6 orders of magnitude higher) [19,77], and corresponding mechanisms of antibiotic resistance genes adsorption on EPS are as follows: (1) the vast net-like structure of the EPS played a key role in ARGs separation and retention [78], via the ion bridging effect, hydrophobic effect and polymer entanglement [76,79]; (2) the abundant existence of colloids and flocs in wastewater enhanced the retention of ARGs [76], and facilitated ARGs removal [80]; and (3) the potential changes in the stretch degree for peptide chains and amino groups of protein in sludge EPS [39,76] led to an increase in surface area, which improved the ARGs adsorption capacity of sludge EPS [81,82] (Fig. 4).

Considering that the relative abundances of some EPS-

associated ARGs were higher than those of corresponding intracellular ARGs (e.g., *sulI*, *bla_{TEM-1}*, and *tetA*) and cell-free ARGs (e.g., *sulI* and *sulII*) [77], the transformation risks of extracellular ARGs were 3.6–4.6 logs higher than those of cell-free ARGs [19,77], which could lead to the risk of EPS-associated ARGs HGT. Additionally, the environmental risk exhibited by adsorbed extracellular ARGs was much higher than that of the free extracellular ARGs because of their special enzymatic hydrolysis protection effect, undoubtedly allowing them to persist in the aquatic environment for a long period [83].

4. Factors influencing EPS-antibiotics interactions

The chemical structure and composition of the microbial EPS during wastewater biological treatment processes greatly affect the antibiotics biosorption and removal process. It is closely related to the variation in the operational parameters, such as temperature, anion or cation types, solution pH, antibiotic concentrations and types, and the types of biological treatment processes [84]. The potential affecting factors are summarized and discussed here to clarify the interaction characteristics of antibiotics and EPS (see Table 1).

4.1. EPS chemical characteristics

The chemical characteristics of sludge EPS strongly correlate with the sludge type and environmental conditions and strongly affect antibiotics adsorption [2]. Here, the typical chemical compositions and characteristics of different sludge EPS are summarized and listed in Table 2. Specifically, a noteworthy observation of higher EPS production and a higher portion of α -helices was found for aerobic sludge EPS than for the anaerobic sludge systems [44,82,85]. Compared with the floc sludge, higher contents of proteins with larger molecular weights (such as aromatic and tryptophan proteins) detected in the granular sludge than those in flocs sludge might be the reason that the granular sludge exhibits superior resistance to antibiotics [1,86]. Additionally, higher contents were preferentially observed in granular sludge than floc sludge, which might be the main reason for its excellent resistance to the toxic stress of antibiotics [58,87]. Correspondingly, the EPS of sulfate-reducing bacterial sludge possessed more functional groups and higher affinity and binding strength for antibiotics adsorption than those of activated sludge and anaerobic sludge [44].

Substrates, as the main energy source of microorganisms, greatly affect EPS synthesis and chemical compositions [44,99]. For example, the percentage of protein in heterotrophic sludge was much higher than that of polysaccharides, whereas autotrophic sludge exhibited a converse trend [100]. Reportedly, the abundant existence of carbohydrate-related substrates would be beneficial for the synthesis of polysaccharides in sludge EPS [101,102], while protein content increased with the presence of peptone and serine-related substances (protein-like) [49]. Additionally, sludge fed with starch has a higher polysaccharide content in EPS than sludge fed with glucose [103]. Similarly, the protein content in EPS increased with increasing polymerization degree [20]. [100] observed that sludge fed with easily biodegradable substrates would generate more protein in EPS, restraining humic acid production. Undoubtedly, the obvious variation in the sludge EPS composition would affect the distribution of the functional groups and adsorption regions (hydrocarbons, proteins, polysaccharides, and nucleic acids) and finally lead to different antibiotics removal efficiencies [49].

4.2. Types of antibiotics

As shown in Table 3, the widely used antibiotics could be

Table 1
EPS response under different antibiotics exposure.

Antibiotics types	Concentration	EPS source	EPS response	References
Tetracycline	100 $\mu\text{g L}^{-1}$	Activated sludge	EPS concentration (in mg per g VSS) increased from 66 to 181	[57]
Ciprofloxacin	300 $\mu\text{g L}^{-1}$	Biofilm	TB-EPS concentration (in mg per g VSS) increased from 133 to 180	[88]
Tetracycline	500 $\mu\text{g L}^{-1}$	Aerobic granular sludge	EPS concentration (in mg per g VSS) increased from 63.8 to 94.1	[89]
Ciprofloxacin	500 $\mu\text{g L}^{-1}$	Aerobic Sludge	Tryptophan-like proteins in EPS (in mg per g VSS) increased 64.04 to 74.33	[44]
Sulfamethoxazole + Erythromycin	500 + 50 $\mu\text{g L}^{-1}$	Anammox sludge	EPS content increased to 187 mg per g VSS	[9]
Sulfamethoxazole	1 mg L^{-1}	Anammox sludge	EPS content increased and its structure became compact	[43]
Tetracycline	2 mg L^{-1}	Aerobic sludge	The relative hydrophobicity and flocculability of EPS improved	[90]
Sulfamethoxazole	2.5 mg L^{-1}	Anammox sludge	The structure of extracellular proteins became compact	[1]
Sulfamethazine	2.5 mg L^{-1}	Activated sludge	The spatial structure of EPS changed to fold	[39]
Norfloxacin + Trimethoprim + Sulfamethoxazole + Tetracycline	1 + 1 + 1 + 1 mg L^{-1}	Aerobic sludge	Bound-EPS content increased from 68 ± 12 to $120 \pm 10 \text{ mg g}^{-1}$ MLSS	[91]
Erythromycin	10 mg L^{-1}	Anammox sludge	The dense structure of EPS became loose	[92]
Erythromycin	10 mg L^{-1}	Activated sludge	Protein in EPS (in mg per g VSS) increased 18 to 48	[93]
Erythromycin + Sulfamethoxazole + Tetracycline	1 + 10 + 1 mg L^{-1}	Anammox sludge	The protein/polysaccharide ratio and the hydrophobicity of EPS increased, the aggregation of sludge improved	[94]
Tetracycline	$5 \times 10^{-5} \text{ mol L}^{-1}$	Aerobic granular sludge	The fluorescence of humic acid-like organics and proteins were nearly quenched, gel strength of EPS was decreased	[58]
Erythromycin ethylsuccinate	0.01 mol L^{-1}	Activated sludge	The fluorescence of the aromatic protein fraction and the soluble microbial by-products-like substances was quenched	[95]
Sulfamethoxazole	20 mg L^{-1}	Anammox Sludge	The random coils of EPS increased, and protein structure became loose (α -helices decreased)	[1]
Ciprofloxacin	40 mg L^{-1}	Biofilm	Protein-like substances decomposed, percentage of humic acid-like substance increased	[60]
Tetracycline	50 mg L^{-1}	Anammox granular sludge	EPS binding sites were occupied completely, viscoelasticity decreased, the sturcture stability was disrupted	[52]
Erythromycin	50 mg L^{-1}	Anammox sludge	EPS decomposed and reduced, while the concentration of SMP was suddenly increased	[92]

Table 2
Chemical characteristics and compositions of different sludge EPS cultured with different substrates.

EPS source	Carbon source	Extraction method	EPS content (mg per g VSS)	Protein (mg per g VSS)	Polysaccharide (mg per g VSS)	Humic acid (mg per g VSS)	Protein/Polysaccharide	Zeta potential (mV)	α -helix (%)	References
Aerobic ammonium-oxidizing bacteria	Inorganic wastewater	Heat extraction	49.41	17.77	31.74	—	0.56	—	21.93	[82]
Anaerobic ammonium-oxidizing bacteria	Inorganic wastewater	Heat extraction	—	53.12	30.12	—	2.64	—	23.13	[82]
Activated sludge	Municipal wastewater	Heat extraction	31.84	21.09	10.76	—	1.96	—	16.70	[82]
Activated sludge	Municipal wastewater	Cation exchange resin method	22.95	11.10	5.70	6.14	1.95	−14.70	—	[96]
Nitrifying activated sludge	Without any organic carbon	Cation exchange resin method	86.69	20.30	47.20	19.19	0.43	−16.97	—	[96]
Anammox sludge	Synthetic nitrogen wastewater	Cation exchange resin method	57.13	33.00	8.95	19.97	3.69	−13.70	—	[96]
Anammox granules	Municipal wastewater	Acid-alkaline extraction method	210	134.4	14.7	—	9.14	—	30.5	[97]
Anammox granules	Synthetic wastewater	Heat method	74.0	62.16	11.84	—	5.25	—	37.7	[98]
Anammox sludge	Synthetic wastewater	Heat method	324.3	204.3	28.8	—	7.09	12.3	16.79	[43]

Table 3
Interactions between EPS and antibiotics.

Target antibiotics	Antibiotics structure	EPS source	Adsorption Isotherms	Biosorption model	Action sites	Driving force	log Kow	Acid dissociation constants (pK _a)	References
Sulfamethazine (SMZ) C ₁₂ H ₁₄ N ₄ O ₂ S M.W = 278.3 g mol ⁻¹		Activated sludge	—	—	Tryptophan-like protein, humic-like organics	Hydrophobic interaction	0.19	2.65, 7.65	[39]
Sulfamethoxazole (SMX) C ₁₀ H ₁₁ N ₃ O ₃ S M.W = 253.3 g mol ⁻¹		Klebsiella sp. J1	—	Pseudo-second-order model	Tyrosine, Tryptophan	Hydrophobic interaction	0.89	1.85, 5.70	[18]
Sulfamethizole C ₉ H ₁₀ N ₄ O ₂ S M.W = 270.3 g mol ⁻¹		Biofilm	Freundlich model	—	Protein-like and humic-like organics	—	—	—	[8]
Sulfadiazine (SDZ) C ₁₀ H ₁₀ N ₄ O ₂ S M.W = 250.3 g mol ⁻¹		Activated sludge	—	—	tyrosine-like substances	—	-0.09	2.10, 6.18	[107]
Ciprofloxacin (CIP) C ₁₇ H ₁₈ FN ₃ O ₃ M.W = 331.3 g mol ⁻¹		Excess sludge	Freundlich model	—	Protein-like substances	—	0.28	6.0, 8.8	[36]
Sarafloxacin (SAR) C ₂₀ H ₁₇ F ₂ N ₃ O M.W = 385.4 g mol ⁻¹		Excess sludge	Freundlich model	—	—	—	1.07	5.6, 8.2	[36]
Moxifloxacin (MOX) C ₂₁ H ₂₄ FN ₃ O ₄ M.W = 401.4 g mol ⁻¹		Excess sludge	Freundlich model	—	—	—	0.95	6.4, 9.5	[36]
Tetracycline (TET) C ₂₂ H ₂₄ N ₂ O ₈ M.W = 444.4 g mol ⁻¹		Anammox granular sludge	—	—	Tryptophan, tryptophan type-proteins and humic acids	Hydrophobic force	-1.30	3.3, 7.7, 9.7	[52]
Tetracycline (TET) C ₂₂ H ₂₄ N ₂ O ₈ M.W = 444.4 g mol ⁻¹		Pseudomonas sp. TC952.	Langmuir model	First-order kinetic model	Tyrosine and tryptophan	Hydrophobic interaction	-1.30	3.3, 7.7, 9.7	[49]
Oxytetracycline C ₂₂ H ₂₄ N ₂ O ₉ M.W = 460.4 g mol ⁻¹		Sludge	Langmuir model	Pseudo-second-order model	—	—	-1.12	3.2, 7.5, 8.9	[108]
Chlortetracycline C ₂₂ H ₂₃ ClN ₂ O ₈ M.W = 478.9 g mol ⁻¹		Sludge	Langmuir model	Pseudo-second-order model	—	—	-0.62	3.3, 7.6, 9.3	[108]
Trimethoprim (TMP) C ₁₄ H ₁₈ N ₄ O ₃ M.W = 290.3 g mol ⁻¹		Activated sludge	—	Pseudo-second-order kinetic model.	Tyrosine and tryptophan	—	0.91	3.23, 6.76	[96]
Ciprofloxacin (CIP) C ₁₇ H ₁₈ FN ₃ O ₃ M.W = 331.3 g mol ⁻¹		Secondary-treated sludge	Langmuir model	Pseudo-first-order kinetic equation	—	—	0.28	6.16, 8.63	[109]

classified into β -lactams, quinolones, tetracyclines, macrolides, sulfonamides, etc., according to their physicochemical structure and molecular properties. The overall biosorption performance of antibiotics by EPS was primarily related to their physical-chemical properties, including their molecular structure, solubility, acid dissociation constants (pKa), partition coefficient, speciation, hydrophobicity, and octanol-water coefficient (log Kow). For example, the complexity of molecular structures could affect their adsorption by EPS. For example, increasing the bulky isobutoxy group at position N1 would lead to a decline in the sorption ability of ofloxacin, whereas the opposite trend was observed for cyclopropyl and ethyl groups in norfloxacin and ciprofloxacin [104]. The torsion of the ring, as well as the changes in the planar structure of the molecule caused by the bulky group, promoted the steric hindrance of possible H-bonds and benefited the subsequent sorption [104]. In addition, antibiotics are amphoteric molecules relative to their pKa values, which can be cationic, neutral, or anionic with the variation of the aqueous pH [2]. Additionally, the hydrophobicity of antibiotics is tightly related to their adsorption ability. For example, antibiotics with log Dow <1 or log Kow <2.5 tend to exhibit a low adsorption potential, while antibiotics with log Dow >3 or log Kow >4 have high adsorption potential [22,105]. For example, the adsorption of clarithromycin (log Kow = 3.16) onto sludge was better than that of sulfamethoxazole (log Kow = 0.89) owing to its strong adsorption characteristics [106].

In addition to the chemical molecular structure, the biodegradability of the antibiotics is affected by the stereochemical groups, which are closely related to the stability and complexity of the antibiotics. Usually, low molecular-weight antibiotics with unsaturated aliphatic groups would be more preferentially degraded than long-chain antibiotics with branched structures and aromatic groups [110]. For example, fluoroquinolone antibiotics are more recalcitrant and resistant than the other antibiotics due to the special fluorine atom in their structure [111,112], which finally leads to the long half-life time of antibiotic transformation in activated sludge [111,113,114]. In addition, the real wastewater environment always contains multiple antibiotics, and the corresponding competitive adsorption might mutually affect their adsorption and biotransformation behaviors.

4.3. Wastewater chemical properties

Wastewater chemical properties (e.g., operational pH and ionic strength) also affected antibiotics adsorption onto the microbial cell surfaces. For example, the adsorption process and mechanism of antibiotics on activated sludge were highly pH dependent, and their reduction efficiency was restricted to a specific pH condition [115]. The main reason might be that aquatic pH affected the patterns of antibiotics and EPS. Specifically, ionic antibiotics may exist as cations, zwitterions, or anions formed under different solution pH values [116]. Usually, the maximum adsorption capacity of antibiotics onto sludge EPS is observed under near-neutral pH conditions [2,36], which could be demonstrated by the observation of the solid-liquid distribution coefficient (K_d values) or adsorption capacity of antibiotics reaching its maximum value under neutral conditions.

Generally, those antibiotics predominantly presented as the cationic phase under acidic conditions because of the binding of $-\text{NH}$ or $-\text{N}$ onto H^+ , as well as the abundant existence of electro-positive microbial cell floc in biosolids [117]. Therefore, the K_d value (or adsorption capacity) declined obviously owing to the electrostatic repulsion between antibiotics and EPS [117]. For comparison, the loss of H^+ from the $-\text{COOH}$ groups of the antibiotics, accompanied by increasing pH, might be the main reason for the cationic bridging between the metals and $-\text{COOH}$ groups within the

antibiotics in the sludge EPS [117]. In addition, the antibiotics could combine with sludge via cationic exchange and electrostatic attraction. Specifically, the cation fractions of the antibiotics continuously declined under basic conditions once they adsorbed onto the sludge EPS, similar to the K_d values, indicating a strong electrostatic interaction between the negatively charged sludge flocs and those cationic antibiotics [36,117]. In addition to the antibiotics type, the secondary structure of the EPS also changed widely with variations in pH [36]. The main reasons could be summarized as follows: (1) the transformation of proteins from helical to unordered random conformation; (2) the smaller and looser EPS structure leading to higher binding sites of the sludge EPS, better adsorption of antibiotics, and further release of extracellular proteins [118].

The existence of multiple metal species within the real water environment may also mutually affect the adsorption of the antibiotics. For low concentrations of heavy metals, the bridging and complexation of metal cations promoted the antibiotics sorption onto the microbial surfaces [36,119,120]. In contrast, a high concentration of metal ions competed with antibiotics for binding sites, which shielded adsorption sites and finally decreased adsorption efficiency [121,122]. For example [116], observed that the abundant existence of Ca^{2+} (121 mg L^{-1}) and Mg^{2+} (269 mg L^{-1}) (divalent cations) in saline sludge efficiently decreased the sorption performance of norfloxacin antibiotics onto the EPS surface from 91.6% to 60.5%. Moreover, ion valence would also affect the sorption performance of antibiotics onto the EPS surface. Previous research also reported that bivalent cations have a stronger competitive adsorption capacity than monovalent cations, which might be related to the electronegativity of bivalent ions (Ca^{2+} and Mg^{2+}) is higher than that of monovalent cations (Na^+ and K^+) [123,124]. The stronger the ability of metal ions to attract electrons, the more significant complexation between EPS and metals [124].

In addition to heavy metals and inorganic ions, coexisting substances (such as various dissolved organics and surfactants) in wastewater may damage the hydrophobic interaction between the EPS-antibiotics complex, and subsequently cause antibiotics to be desorbed from EPS [39], finally causing the release of antibiotics into the environment.

4.4. Operating conditions

Considering that the origins and chemical components of EPS are quite complex, the production and defense process of EPS may be influenced by many operational parameters, such as temperature and solid retention time.

Temperature is a vital factor influencing EPS production and antibiotics adsorption. Usually, the productivity of EPS increases gradually with increasing temperature and reaches its maximum production under the optimum growth temperature, which mainly depends upon the bacterial strain. Recent work revealed that the optimal temperature for EPS generation was approximately $25\text{--}30^\circ\text{C}$ [14]. Correspondingly, the removal of antibiotics also improved due to the increase in EPS content and the promotion of microbial activities, while an excessive temperature restricted the growth of microbes, which led to the transformation of EPS and a low antibiotics removal rate [21,22]. Interestingly, a decrease in polysaccharide content was observed with decreasing temperature, while the protein content increased as the temperature dropped [125].

Mild temperatures benefited the EPS-antibiotics binding processes due to the decline in the migration speed of antibiotics at low temperatures. Thermodynamic adsorption analysis revealed that the maximum unit adsorption capacity of antibiotics

decreased and the adsorption equilibrium constant increased with increasing temperature, indicating a stronger interaction between EPS and antibiotics at higher temperatures [18,126,127]. This phenomenon might be closely related to the increasing disorder of the reaction. Additionally, the above results also indicated that the antibiotics sorption was exothermic under natural conditions (the adsorption capacity declined with increasing temperature) [128]. The isothermal titration microthermal result demonstrated that the EPS-antibiotics complex was quite stable, as evidenced by the negative Gibbs free energy observation after the antibiotics bind to the sludge EPS [16]. When hydrophobic interactions are damaged under high temperatures, the antibiotics may desorb from EPS, which causes a risk of antibiotics release into the environment.

In addition to the temperature, the solid retention time is another key operational parameter for ESP production, affecting EPS-antibiotics interactions. The sorption ability of EPS decreased sharply after reducing the solid retention time due to the decreased biomass concentration in the reactors [129]. Conversely, a higher biomass concentration was observed with increasing solid retention time, which enhanced the opportunity for the interaction between antibiotics and the sludge EPS [129]. However, a higher solid retention time enriched those slow-growing rate microorganisms, increased the diversity of microbial communities, and promoted the secretion of degradative enzymes in EPS, therefore improving the antibiotic removal efficiency [105].

5. Future perspectives

5.1. Developing an early warning system for biotoxin inhibition based on EPS variations

The operational efficiency of WWTPs is susceptible to interference by influent antibiotics. Long-term exposure to antibiotics stress would cause a series of changes in the metabolic pathway of microbial cells and even possibly lead to death, which would prolong the start-up time and increase the process risk [53,70,130]. As a result, monitoring and analyzing the microbial response performance to antibiotic variations is crucial for the stable operation and optimization of biological wastewater treatment systems [131]. However, traditional antibiotics inhibition detection methods, such as enzyme activity detection and biological acute toxicity tests [131,132], are time-consuming and expensive.

Additionally, EPS exists on the surface of microbial cells or fills the internal voids of their aggregates. Antibiotics entering the wastewater biological treatment system adhere to the sludge floc and cause different variations in some physical parameters (e.g., zeta potential and contact angle) and chemical composition (e.g., α -helix/(β -sheet + random coil) and β -sheet value) of EPS [43,133]. For example, the zeta potential of EPS was obviously increased with exposure to antibiotics (e.g., sulfamethoxazole and erythromycin) [9], whereas it gradually declined after binding with heavy metals (e.g., As(III), Cd(II), Pb(II), and Cr(VI)) [43,134]. In addition to zeta potential, the secondary protein structure of EPS showed a different trend under the stress of As(III) and sulfamethoxazole (SMX). The α -helix/(β -sheet + random coil) of protein in EPS increased under SMX stress, whereas the value decreased under As(III) stress [43]. The β -sheet value decreased with increasing As(III) dosage but increased with the increasing sulfadiazine, ciprofloxacin, and sulfadiazine-ciprofloxacin [43,135].

Therefore, based on the characteristic response of EPS physicochemical parameters to antibiotics' toxic effects, it is vital and feasible to develop an innovative in-situ and real-time method for analyzing EPS "fingerprint signals", which can identify the degree of biotoxin inhibition in wastewater biological treatment systems. The linkage response mechanisms of the physicochemical signals of sludge EPS

and the microbial community distribution of the abovementioned wastewater treatment systems should be evaluated to identify the key signaling molecules. Combined with cluster analysis and dimensionality reduction optimization, a biotoxicity identification method based on EPS signaling molecules should be constructed.

5.2. Strengthen the antibiotics biotransformation abilities of EPS

When a large amount of antibiotic-containing wastewater enters a WWTP in a short time, in addition to early warning detection, it is more important to reduce its toxicity and inhibition to activated sludge. Therefore, the antibiotics biotransformation ability of activated sludge in WWTPs is essential to relieve the toxicity inhibition of antibiotics [64]. Previous studies reported the presence of bioactive enzymes in sludge EPS, such as laccases which can promote the biotransformation of antibiotics and improve the removal efficiencies for persistent antibiotics [64,136]. However, the production of bioactive enzymes is controlled at the cellular level by various genes and is low due to the limited expression level of synthase in vivo.

Synthetic biology has been successfully used to build engineered microbial strains or consortia with excellent toxin resistance ability and efficient pollutant degradation capacities [7,137–139]. For example [137], reported that the *Rhodococcus* sp. strain p52 harboring two mega plasmids, i.e., pDF01 and pDF02, decreased the dibenzofuran content (120 mg L^{-1}) in the synthetic wastewater by 32.6–100% compared to that in the reactor without the genetic bioaugmentation strain. It is also suitable for modifying and producing EPS [7]. Therefore, exploring microbes modified by functional enzyme genes such as laccase enzyme, and improving the efficient transformation capacity of EPS on antibiotic, may be an effective choice to reduce the inhibition effects of antibiotics. In addition, considering the unforeseeable effects of genetic engineering technology, it is necessary to pay more attention to the long-term effects and risk assessment of engineered microbes in actual wastewater treatment applications.

5.3. Reducing the ARGs release risks from sludge EPS

Generally, traditional biological wastewater treatment, such as activated sludge treatment, often fails to completely remove wastewater antibiotics because of its limited adsorption and biotransformation ability [64]. Residual antibiotics and their metabolites exert continuous selective pressure, which promotes the development of ARGs [140]. Previous studies reported that ARGs could be adsorbed onto sludge EPS and form a stable EPS-ARGs complex [76], and the absolute abundance of EPS-associated ARGs reached 1.49×10^7 – 4.45×10^9 copies per g VSS in sludge [77]. However, the ARGs adsorbed on sludge EPS would be partially released due to the variation of the environmental conditions [49]. Considering that most EPS-associated ARGs are contained in sewage sludge after dewatered treatment, the destruction of the EPS-ARGs complex may cause a secondary release of ARGs into the environment from sewage sludge.

Currently, the estimated sewage sludge productivity in China is approximately 39.2 million tons (with 80% water content) every year [141]; as many as 10^{19} – 10^{22} copies of extracellular ARGs could be enriched in sludge EPS [77]. Usually, most EPS-associated ARGs are concentrated in excess sludge after dewatering treatment and entered into the environment via fertilizer and land applications. Those remaining antibiotics are partially entered into crops and subsequently transferred to the food chain, and the HGT of ARGs were inevitably [77]. Therefore, additional treatment processes are urgently needed to further remove these antibiotics and their ARGs before sludge agricultural utilization, especially the transformation

of EPS-associated ARGs, which would be helpful to control antibiotic resistance dissemination risk.

6. Conclusions

This review summarizes the performances, mechanisms, and influencing factors of EPS resistance to antibiotics stress in the wastewater treatment process. Regarding the adsorption of antibiotics, chemical adsorption, especially hydrophobic effects, hydrogen bonding, ionic interactions, and cation bonding, are the main mechanisms that occur during the adsorption of antibiotics onto EPS. Moreover, new EPS were synthesized under the regulation of the AHL-mediated quorum sensing system to resist the stress of antibiotics. Those adsorbed antibiotics were subsequently transformed into nontoxic substances under the joint effect of extracellular active degradative enzymes and EPS phototransformation. Furthermore, EPS can retain extracellular ARGs, prevent ARGs leakage risks and weaken ARGs transformation. EPS-antibiotics interactions are affected by several factors, including the sludge type, substrate source, antibiotics types and concentrations, solution properties, and temperature. Although some progress has been achieved in understanding the effect of EPS on antibiotics and ARGs transformation during WWTP operation, information on the mechanisms and actual engineering of EPS and antibiotics/ARGs is still very limited and warrants further investigation.

Author contributions

Weixin Zhao: Conceptualization, Writing-Original Draft, Writing-review & editing. Jia You: Investigation, Visualization, Writing-original draft. Shilei Yin: Investigation, Writing-original draft, Writing-review & editing. Haizhou Yang: Visualization, Writing-review & editing. Shufei He: Investigation, Writing-review & editing. Likui Feng: Conceptualization, Writing-review & editing. Jianju Li: Conceptualization, Supervision. Qingliang Zhao: Conceptualization, Writing-Original Draft, Writing-review & editing. Liangliang Wei: Conceptualization, Writing-Original Draft, Writing-review & editing, Project administration, Funding acquisition.

Declaration of competing interest

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

Acknowledgements

This work was supported by the funding from the National Key Research and Development Program of China (2019YFC1803802), the National Natural Science Foundation of China (No. 51878213), the State Key Laboratory of Urban Water Resource and Environment (No. 2020TS01), and the Heilongjiang Nature Science Foundation (YQ2020E022).

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