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Review

Harnessing the Potential of Extracellular Polymeric Substances in Enhancing ANAMMOX Processes: Mechanisms, Strategies, and Perspectives

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Abstract: Anaerobic ammonium oxidation (ANAMMOX) has emerged as a promising sustainable nitrogen removal technology that offers significant advantages over conventional nitrification–denitrification processes, such as reduced energy consumption, a 60% reduction in oxygen demand, and a 90% reduction in sludge production. However, the practical application of ANAMMOX is hindered by several challenges, including the slow growth of ANAMMOX bacteria, long start-up periods, and high sensitivity to environmental disturbances. Recent studies have highlighted the crucial role of extracellular polymeric substances (EPSs) in the formation, activity, and stability of ANAMMOX biofilms and granules. An EPS is a complex mixture of high-molecular-weight polymers secreted by microorganisms, mainly composed of polysaccharides, proteins, nucleic acids, and lipids. The diverse physicochemical properties and functional groups of EPSs enable them to serve as a structural scaffold, protective barrier, sorption site, electron shuttle, and nutrient source for ANAMMOX bacteria. This review aims to provide an overview of the latest research progress on harnessing the potential of EPSs to enhance the ANAMMOX process. The characteristics, compositions, and extraction methods of ANAMMOX-derived EPSs are summarized. The mechanisms of how EPSs facilitate the enrichment, immobilization, aggregation, and adaptation of ANAMMOX bacteria are elucidated. The strategies and effects of EPS supplementation on improving the performance and robustness of ANAMMOX reactors under various stresses are critically reviewed. The challenges and future perspectives of the EPS-mediated optimization of the ANAMMOX process are also discussed. This review sheds new light on exploiting EPSs as a renewable bioresource to develop more efficient and stable ANAMMOX applications for sustainable wastewater treatment.

Keywords: ANAMMOX; extracellular polymeric substances; biofilm; granulation; nitrogen removal

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1. Introduction

With the growing concerns regarding the energy crisis, climate change, and water pollution, developing sustainable and cost-effective technologies for wastewater treatment has become an urgent need. Anaerobic ammonium oxidation (ANAMMOX), a novel autotrophic nitrogen removal process, has attracted increasing attention as an eco-friendly alternative to conventional nitrification–denitrification. ANAMMOX utilizes nitrite (NO_2^-) as the electron acceptor to oxidize ammonium (NH_4^+) directly into nitrogen gas (N_2) under anoxic conditions [1], with a small amount of nitrate (NO_3^-) as the byproduct [2].

Compared to traditional nitrification–denitrification, ANAMMOX offers significant benefits, such as a 60% reduction in oxygen demand, a 90% reduction in sludge production, and 60% lower operating costs [3,4]. Moreover, ANAMMOX emits much less nitrous oxide (N_2O), a potent greenhouse gas, than denitrification [5]. Due to these advantages, ANAMMOX has been successfully applied for treating many kinds of wastewater, such as sludge digester liquor, landfill leachate, and livestock manure [6].

At present, there are limited applications of ANAMMOX in mainstream urban domestic sewage and industrial wastewater. One of the reasons for this is the low concentration of ammonia nitrogen, the unstable nitrogen load, and the seasonal temperature changes in sewage. These problems make the stable operation of the ANAMMOX process difficult. However, some studies are attempting to solve these problems. Gao et al. adopted the step-feed anoxic–oxic (A/O) process coupled with partial denitrification–anammox (PD/A) in order to achieve stable operation from 26.8 °C and 13.1 °C for 200 days [7]. The ANAMMOX activity (SAA) was 5.60 mg NH_4^+-N/g MLSS/d, and the abundance of ANAMMOX bacteria increased, even at 14 °C. In a cold region (Helsinki, Finland), a full-scale ANITA^{TM} Mox bioreactor is used, with a maximum influent nitrogen load of 1000 mg/L and an effluent with the nitrogen concentration reduced to 180–200 mg/L [8]. The COD 1500–900 mg/L both in and out of the water achieves stable operation within 160 days, and shortens the start-up time. Because of the complex content of industrial wastewater, different industrial wastewater has different characteristics; industrial wastewater requires the shortest possible start-up time, and there is no uniform condition for current applications. The wastewater from the ammonia plant not only contains a high concentration of ammonium, but also a high concentration of methanol, which is more toxic to ANAMMOX. The nitrifying ANAMMOX unit using gel embedding technology was successfully operated, and the ANAMMOX partial start-up time was two months [9]. Some industrial wastewater contains high ammonia nitrogen and a lot of salt, such as seafood processing wastewater, textile printing and dyeing wastewater, tannery wastewater, and so on [10]. Xu et al. developed a salt-tolerant PD/A process, in which the ANAMMOX sludge maintained the high activity at a salinity of 10 g/L, and the nitrogen removal efficiency reached more than 90% [11]. When Zhang et al. used a PD/A SBBR reactor to treat pharmaceutical wastewater, the total nitrogen removal rate (NRR) could reach 80.8%, and the contribution rate of anaerobic ammonia oxidation was 3.6% [12]. In the treatment of printed circuit board (PCB) wastewater, the ANAMMOX process shows good nitrogen removal performance and the enrichment of dominant bacteria (*Candidatus* Kuenenia) [13].

Despite great promise, the engineering application of ANAMMOX still faces several challenges. A major bottleneck is the extremely slow growth rate of ANAMMOX bacteria, with a doubling time of 4–15 days [14]. This leads to a long start-up period (usually 2–4 months) for ANAMMOX bioreactors and low biomass retention [15]. In addition, ANAMMOX bacteria are highly sensitive to various environmental factors, such as temperature, pH, dissolved oxygen (DO), highly concentrated organic matter, substrate concentrations, toxic compounds, etc. [16]. Fluctuations in these parameters can severely inhibit SAA and cause process instability or even failure. Therefore, developing effective strategies to accelerate ANAMMOX enrichment, immobilize ANAMMOX biomass, and enhance the resistance of ANAMMOX bacteria to stress is of vital importance for the scale-up and optimization of this technology. In recent years, harnessing the potential of extracellular polymeric substances (EPSs) to improve ANAMMOX performance has gained growing interest. An EPS is a complex mixture of high-molecular-weight secretions produced by microorganisms, mainly composed of polysaccharides, proteins, nucleic acids, and lipids [17]. EPSs play essential roles in the formation and function of biofilms and granular sludge, two common forms of microbial aggregates used in wastewater treatment [18]. ANAMMOX bacteria can naturally excrete large amounts of EPSs during cultivation, which contributes to their aggregation, adhesion, and stress resistance [19]. Studies have shown that supplementing ANAMMOX reactors with extracted EPSs or EPS-producing bacteria can significantly promote the growth, activity, and stability of ANAMMOX microorganisms

under various conditions [17,19–21]. The mechanisms involve the provision of structural support, a protective barrier, sorption sites, electron shuttles (ESs), signaling molecules, and nutrients from EPSs to ANAMMOX bacteria and their symbiotic partners [22–24].

Therefore, this review aims to provide a comprehensive overview of the latest research progress on harnessing the potential of EPSs to enhance the ANAMMOX process. By systematically clarifying the relationship between EPSs and ANAMMOX, this review elucidates the key roles and mechanisms of EPSs in ANAMMOX biofilm/granule formation, activity, and stability. The article also summarizes the strategies and effects of EPS supplementation on improving ANAMMOX performance under various stresses, and discusses the challenges and future research directions of the EPS-mediated optimization of the ANAMMOX process. This work attempts to provide a comprehensive evaluation of the characteristics, functions, and regulatory roles of EPSs in the context of ANAMMOX, exploring potential EPS-based strategies for ANAMMOX enhancement. The insights provided herein may contribute to a deeper understanding of the ANAMMOX microbial ecology process and the development of EPS-oriented engineering techniques for ANAMMOX optimization, potentially supporting the development of more efficient and stable ANAMMOX applications for sustainable wastewater treatment.

2. Characteristics, Compositions, and Extraction of ANAMMOX-Derived Extracellular Polymeric Substances

2.1. Characteristics of ANAMMOX-Derived Extracellular Polymeric Substances

An EPS is a common component of ANAMMOX biofilms and granules. The content and composition of EPSs excreted by ANAMMOX bacteria vary with the species, growth phase, and environmental conditions [25]. In general, ANAMMOX biomass has a higher EPS content (up to 40–60% of dry mass) when compared to other types of sludge [26,27]. This may result in a more anaerobic environment inside EPSs, and may also help sensitive ANAMMOX bacteria cope with harsh conditions and improve cell aggregation. The EPS concentrations in ANAMMOX reactors were found to increase with the influent nitrogen loading rate [28], hydraulic retention time [29], and salinity [30], suggesting an active EPS production by ANAMMOX bacteria under stress. ANAMMOX-derived EPSs exhibit heterogeneous distributions in biofilms and granules (Figure 1) [31]. Confocal laser scanning microscopy (CLSM) images showed a gel-like EPS matrix surrounding the ANAMMOX cells, with decreasing density from the center to the surface of the granules [32,33]. Transmission electron microscopy (TEM) further revealed that ANAMMOX EPSs contain abundant pores and channels that can facilitate the transport of substrates and products while protecting the cells from external disturbances [34,35]. The viscoelasticity and hydrophobicity of ANAMMOX EPSs also change dynamically during reactor operations, which influence the cohesion and settling properties of ANAMMOX aggregates [36].

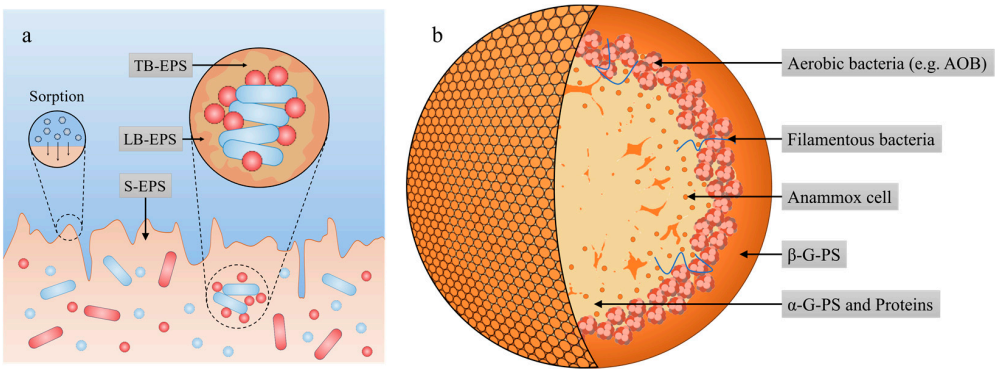

Figure 1. Schematic diagram of EPSs in anaerobic ammonia oxidation biofilm (**a**) and granular sludge (**b**). TB-EPS: soluble EPS; LB-EPS: loosely bound EPS; S-EPS: tightly bound EPS; β–G-PS: β–glucopyranose polysaccharides; α–G-PS: α-D-glucopyranose polysaccharides. Sorption: nutrients and other molecules can be trapped by sorption to EPSs.

2.2. Compositions of ANAMMOX-Derived Extracellular Polymeric Substances

The major components of ANAMMOX EPSs are polysaccharides and proteins, with minor amounts of nucleic acids, lipids, and humic-like substances [37]. The polysaccharide/protein (PS/PN) ratio of ANAMMOX EPSs varies in the range of 0.1–0.8, which is lower than that of other sludge types [38]. This suggests that proteins play a more important role in the structural stability and functional activity of ANAMMOX biofilms/granules when compared to polysaccharides. In addition, the proteins in ANAMMOX sludge have a looser secondary structure and also have more hydrophobic functional groups, which were thought to be the key to the aggregation of ANAMMOX sludge [17]. The monosaccharide composition of ANAMMOX EPS polysaccharides usually includes glucose, galactose, mannose, glucosamine, and glucuronic acids [39]. Some specific glycoconjugates, such as acidic glycoproteins and sulfated proteoglycans, were identified in ANAMMOX granular sludge, and were proposed as key molecules in mediating cell–cell adhesion and EPS gelation [40]. More importantly, EPSs also exist as skeletons of microbial aggregates. The polysaccharide branch structure and glycoprotein play a vital role in maintaining the structure of ANAMMOX granules [17]. Recently, integrated genomic and glycomic analyses revealed that ANAMMOX genera contain unique gene clusters for synthesizing novel polysaccharides and glycoproteins that are absent in other bacteria [41]. These insights shed new light on the structural basis and functional significance of ANAMMOX EPS polysaccharides. The proteins in ANAMMOX EPSs contain a high proportion of negatively charged amino acids (e.g., aspartic acid and glutamic acid) and hydrophobic amino acids (e.g., alanine, valine, and leucine) [42]. This specific amino acid profile endows ANAMMOX EPSs with amphoteric and amphiphilic properties that are beneficial for cell aggregation [43]. Metaproteomic studies identified various extracellular enzymes (e.g., hydrolases, lyases, transferases) and structural proteins (e.g., S-layer proteins, adhesins, pili) in ANAMMOX biofilm EPSs, which participate in EPS metabolism and assembly [44]. In addition, some c-type cytochromes (c-Cyts) proteins and iron-sulfur proteins were found in ANAMMOX EPSs, suggesting a potential extracellular electron transfer pathway in ANAMMOX consortia [45].

2.3. Extraction Methods of ANAMMOX-Derived Extracellular Polymeric Substances

Extracting EPSs from ANAMMOX sludge is the first step in studying their composition and function. However, there is no universal or standardized protocol for EPS extraction due to the complexity and heterogeneity of EPSs [23,46]. The most common EPS extraction methods include physical methods (e.g., centrifugation, heating, sonication) and chemical methods (e.g., using ethylenediaminetetraacetic acid (EDTA), formaldehyde, or NaOH) [47,48]. Each method has its own advantages and limitations in terms of extraction efficiency, EPS integrity, and downstream compatibility [49]. For ANAMMOX granular sludge, heating (80 °C for 30 min) coupled with centrifugation (10,000 g for 20 min) was found to be an effective and reproducible method for EPS extraction [50]. This protocol could achieve high EPS yields with minimal cell lysis and avoid chemical contaminations. A sonication-based EPS extraction protocol was also developed for ANAMMOX granules pretreated with grinding, which increased the EPS recovery by disrupting the compact granular structure [51]. For the EPS in ANAMMOX suspended sludge and biofilms, a cation exchange resin (CER) method was shown to extract higher amounts of EPSs with lower cell damage than heating and chemical methods [52]. After extraction, the crude EPS is usually purified through dialysis, filtration, precipitation, or chromatography in order to remove residual cells, salts, and impurities before chemical analysis and application [25]. The total EPS content is quantified by measuring the organic carbon or dry mass. The polysaccharide, protein, and DNA levels in EPSs are determined with colorimetric assays, such as the phenol-sulfuric acid method, Lowry method, and diphenylamine method, respectively [26]. More detailed characterizations of EPS compositions and structures can be conducted with advanced analytical tools, such as Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), X-ray photoelectron spectroscopy (XPS),

high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and size exclusion chromatography-multi-angle light scattering (SEC-MALS) [53].

3. Roles and Mechanisms of Extracellular Polymeric Substances in ANAMMOX Processes

3.1. Extracellular Polymeric Substances Facilitate ANAMMOX Biofilm and Granule Formation

An EPS is essential for the aggregation and immobilization of ANAMMOX bacteria in biofilms and granular sludge [36]. The formation of ANAMMOX biofilms and granules generally undergoes four steps as follows: (1) bacterial attachment to solid surfaces or self-aggregation; (2) production and accumulation of EPSs; (3) maturation of three-dimensional structures; and (4) detachment and recolonization (Figure 2) [54]. EPSs participate in multiple stages of this process by providing cohesion, adhesion, and mechanical strength. In the initial attachment phase, the amphiphilic and anionic properties of EPSs can help to overcome the electrostatic repulsion between negatively charged bacterial surfaces and can enable cell–surface and cell–cell interactions through hydrophobic and polymer bridging forces [55]. The polysaccharides in ANAMMOX EPSs, such as poly-β 1,6-N-acetylglucosamine, were found to mediate the adhesion and aggregation of ANAMMOX bacteria via lectin-like binding and alginate complexation [56]. The proteins in ANAMMOX EPSs, such as pilin, extracellular serine protease, and S-layer proteins, also exhibit surface adhesion and self-assembly abilities that contribute to the aggregation of ANAMMOX cells [57].

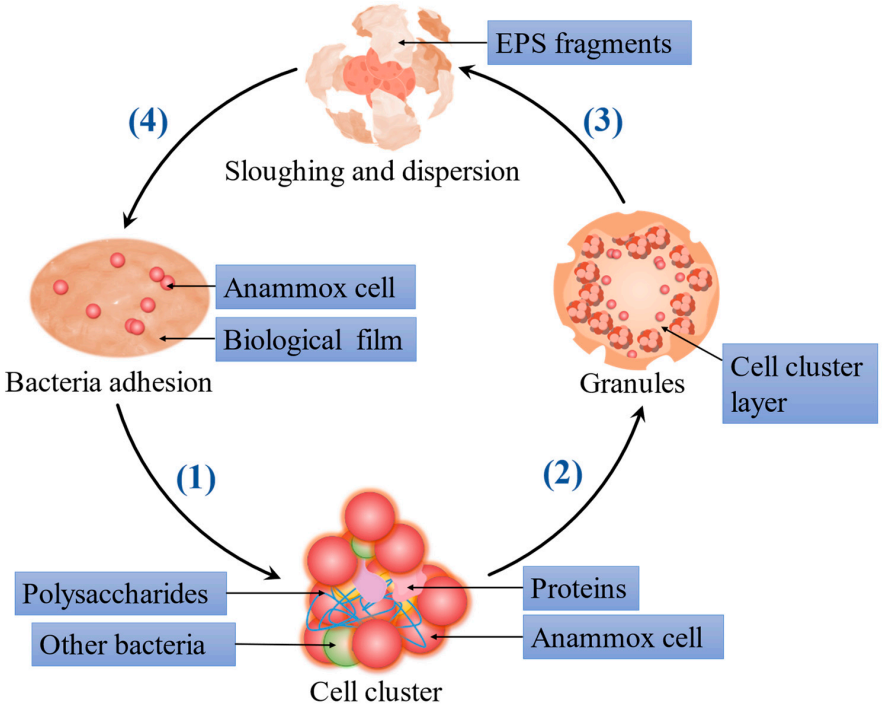

Figure 2. Granulation cycle mechanism. Mechanism based on EPS adhesion. (1) bacterial attachment to solid surfaces or self-aggregation; (2) production and ac-cumulation of EPS; (3) maturation of three-dimensional structures; and (4) detachment and recolonization.

During the EPS accumulation and maturation phases, ANAMMOX bacteria continue to secrete EPSs, which form a gel-like matrix surrounding the cells, providing a scaffold for the development of biofilms and granules. The EPS matrix can retain moisture, trap nutrients, buffer pH fluctuations, and protect cells from harmful substances, thus creating a stable microenvironment for ANAMMOX growth [22,58]. The viscoelastic and porous properties of ANAMMOX EPSs also facilitate the diffusion of substrates and products while maintaining the structural integrity of aggregates [59]. As the biofilm/granules mature, the EPS content and density increase, enhancing the settling ability, mechanical strength,

and erosion resistance of ANAMMOX aggregates [60]. In the final detachment phase, shear forces can cause the sloughing and dispersion of ANAMMOX biofilms/granules, releasing cells and EPS fragments into the bulk solution. These detached components can serve as new nuclei for ANAMMOX reattachment and regrowth, promoting the spread and resilience of ANAMMOX populations [61]. The EPS content and composition also influence the hydrophobicity, surface charge, and dispersibility of ANAMMOX flocs, regulating their detachment potential [62].

Therefore, EPSs play a critical role in the formation, structure, and stability of ANAMMOX biofilms and granules [63]. Manipulating the EPS properties and dynamics can be an effective approach to accelerate ANAMMOX granulation and optimize the ANAMMOX reactor performance. For example, dosing exogenous EPSs or EPS-producing bacteria into ANAMMOX reactors was shown to promote cell aggregation, reduce start-up time, and improve biomass retention [42,64]. Controlling the EPS content and components (e.g., PS/PN ratio) in ANAMMOX granular sludge through selective enrichment or chemical modification could also enhance the settleability, permeability, and mechanical strength of ANAMMOX granules [65].

3.2. Extracellular Polymeric Substances Enhance ANAMMOX Activity and Stability

Apart from their structural functions, EPSs also have important physiological and ecological roles in the ANAMMOX process via regulating cell activity, interspecies interactions, and stress resistance. Firstly, EPSs can serve as nutrient and energy sources for ANAMMOX bacteria and their syntrophic partners (Figure 3a). Although ANAMMOX bacteria are autotrophs that mainly rely on CO_2 fixation for growth, they can also utilize organic compounds (e.g., acetate, propionate, glucose) as a supplementary carbon source under certain conditions [66]. The EPS matrix contains various biodegradable polymers (e.g., polysaccharides, proteins, DNA) that can be hydrolyzed and fermented by heterotrophic bacteria into volatile fatty acids (VFAs) and other metabolites, which can then be assimilated by ANAMMOX bacteria [67]. This EPS-mediated metabolic interaction between ANAMMOX bacteria and heterotrophs can provide a sustainable and in situ organic carbon supply for ANAMMOX growth, especially under substrate-limited conditions [68]. A recent ^{13}C-stable isotope probing study confirmed that ANAMMOX bacteria could incorporate EPS-derived carbon into their biomass and enhance their activity in anoxic environments [69]. In addition to carbon metabolites, EPSs can also act as a source of nitrogen and phosphorus for ANAMMOX bacteria. Some studies reported that ANAMMOX bacteria could utilize NH_4^+ released from EPS biodegradation as a substrate for growth [70]. The nucleic acids (DNA and RNA) in EPSs may also serve as a potential phosphorus source for ANAMMOX bacteria, which have a high demand for phosphorus to synthesize the ladderane lipids in their cell membranes [71]. The recycling of EPS-bound nutrients within ANAMMOX aggregates can enhance the self-sufficiency and stability of the ANAMMOX process under nutrient-deficient conditions.

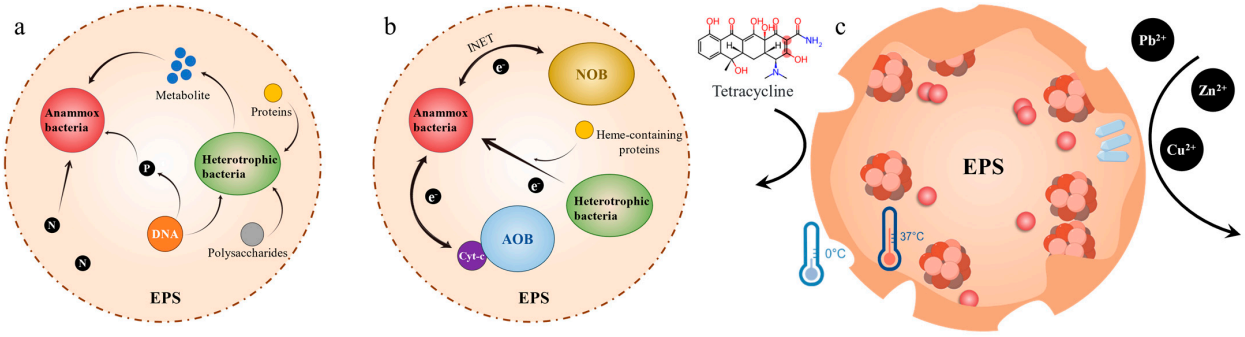

Figure 3. EPSs play an important physiological and ecological role in the ANAMMOX process. INET: interspecies electron transfer. (**a**) EPS as a source of nutrition and energy; (**b**) EPS mediates EET and INET; (**c**) EPS as a protective barrier against adverse environments.

Secondly, EPSs can mediate the extracellular electron transfer (EET) of ANAMMOX and interspecific electron transfer with symbiotic partners such as ammonia-oxidizing bacteria, nitrite-oxidizing bacteria (NOB), and heterotrophic bacteria (Figure 3b) [72]. ANAMMOX sludge contains complex and diverse microorganisms, and anammox activity and nitrogen removal efficiency are closely related to microbial metabolism and interspecific electron transfer. These electron transfers can be achieved by outer membrane cytochromes, ESs, nanoconductors, conductive pili, etc. The EPS in the denitrifying anaerobic methane oxidation–anaerobic ammonia oxidation (DAMO-ANAMMOX) system is rich in c-Cyts, which is conducive to extracellular electron transfer, indicating that direct interspecies electron transfer may exist in this system [73]. In the presence of biochar, the composition of EPSs increase, with most of the HS compounds and a few PN (proteins such as c-Cyts) having redox activity and electron shuttle capability. EPSs may mediate electron transfer to extracellular electron acceptors as a transient mediator [74]. The increase in EPSs increased the content of flavin and c-Cyts, which will construct ESs channels and indirect/direct EET channels in the EPS layer of ANAMMOX cells in order to transfer electrons to extracellular receptors. As a result, the electron transfer rate is greatly enhanced and the metabolism of ANAMMOX is improved [24].

Thirdly, EPSs can protect ANAMMOX bacteria from various environmental stresses, such as oxygen, temperature, pH, salinity, heavy metals, and antibiotics (Figure 3c). The diffusion-limiting and metal-binding capacities of EPSs can help maintain a stable microenvironment for ANAMMOX bacteria and mitigate the effects of inhibitory substances. For example, the polysaccharides and proteins in EPSs can form a viscous and hydrated gel layer on cell surfaces that can limit the penetration of O_2 and protect ANAMMOX bacteria from oxidative damage [75]. The ionizable functional groups (e.g., carboxyl, phosphoryl, and amino groups) in EPSs can bind and immobilize heavy metal ions (e.g., Cu^{2+}, Zn^{2+}, Pb^{2+}), thus reducing their bioavailability and toxicity to ANAMMOX bacteria [18,76–78]. The EPS matrix can also act as a physical barrier and adsorbent for antibiotics, alleviating their inhibitory effects on SAA [79]. In addition, some studies suggested that EPSs could cryoprotect ANAMMOX bacteria by reducing ice crystal formation and maintaining cell membrane integrity during freeze–thaw cycles [80]. In addition, denitrifying sludge (D-EPS) can also act as a preservative for ANAMMOX at low temperatures, with a higher SAA than ANAMMOX sludge (A-EPS) [81]. The EPS-mediated stress resistance mechanisms of ANAMMOX bacteria are crucial for their survival and activity in harsh environments and engineered systems.

However, excessive EPS accumulation may also exhibit negative effects on the ANAMMOX process. Thick and dense EPS layers can hinder the mass transfer of substrates and products in ANAMMOX biofilms/granules, causing diffusion limitation and creating anoxic/anaerobic microenvironments that favor the growth of competing microorganisms (e.g., NOB, denitrifiers) [82]. The nonproductive adsorption and complexation of NH_4^+ and NO_2^- by EPSs can also reduce the substrate availability for ANAMMOX bacteria and lower the nitrogen removal efficiency [31]. The overproduction of EPSs may lead to the formation of large and loose sludge flocs with poor settleability, resulting in ANAMMOX biomass washout [83]. Therefore, maintaining an appropriate EPS content and composition is important for the optimal functioning of the ANAMMOX process.

4. Extracellular Polymeric Substances Supplementation Strategies to Improve ANAMMOX Performance

4.1. Extracellular Polymeric Substances Supplementation during the Start-Up of ANAMMOX Reactors

Based on the above-mentioned roles of EPSs in the ANAMMOX process, several EPS-based strategies have been developed to enhance the performance and stability of ANAMMOX reactors under various conditions (Figure 4). It includes the rapid start-up of the ANAMMOX reactor, the relief of the substrate inhibition effect by supplementing EPSs, the enhancement of the salt tolerance of ANAMMOX by adding EPSs, and the resistance of EPSs to antibiotic stress.

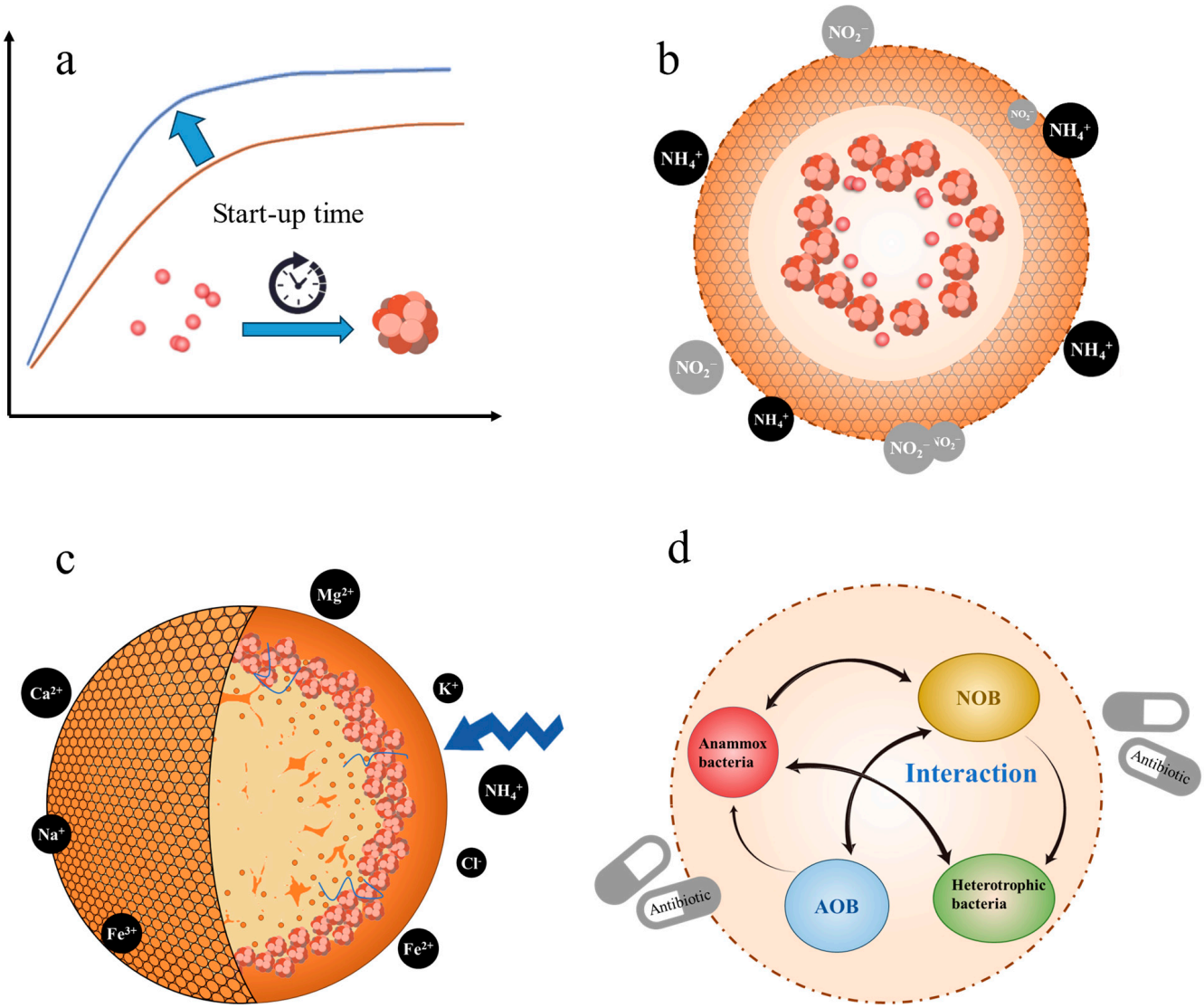

Figure 4. Examples of EPSs improving ANAMMOX performance. (**a**) fast start-up by accelerating cell aggregation; (**b**) wrap cells to reduce substrate penetration; (**c**) reduce direct contact between cells and salt ions; (**d**) microbial community diversity promotes recovery.

The long start-up time of ANAMMOX reactors is a major bottleneck for their practical application, which is largely due to the slow growth and low biomass retention of ANAMMOX bacteria. To accelerate the ANAMMOX start-up, inoculating the reactors with EPS-rich ANAMMOX sludge or EPS extracts has been proposed as a promising approach. For example, Yang et al. [84] demonstrated that dosing 60–100 mg/L of EPSs extracted from mature ANAMMOX granules into the feed of up-flow anaerobic sludge blanket (UASB) reactors could shorten the start-up time from 131 to 67 days, and could increase the NRR by 131%. The rapid granulation and enhanced SAA were attributed to the bridging and coating effects of the EPSs that promoted cell–cell adhesion and sludge aggregation. Jia et al. [34] reported that adding gel beads containing ANAMMOX sludge and calcium alginate (a model EPS) into sequencing batch reactors (SBRs) could significantly increase the biomass retention and accelerate the ANAMMOX start-up process, thus achieving a high NRR of 0.74 kg $N/m^3/d$ within 50 days.

Yang et al. [85] use response surface analysis to optimize the specific SAA with the addition of EPSs. They found that EPS-alginate beads significantly speed up the startup of the ANAMMOX process and enable the start time to be shortened from 31 to 19 days.

Presumably, this is a result of the higher mixed liquor volatile suspended solid (MLVSS) content, higher zeta potential, and lower SVI_{30}; ANAMMOX granules exhibited a stronger capacity to aggregate. This method improves the ANAMMOX reaction rate, shortens the start-up time, and enhances the ability to resist the impact load.

4.2. Extracellular Polymeric Substances Supplementation under Substrate Inhibition Stress

High concentrations of NH_4^+ (>200 mg/L) and NO_2^- (>100 mg/L) are commonly found in anaerobic digester effluents and can inhibit SAA due to the toxicity of free ammonia (FA) and free nitrous acid (FNA) [16]. To alleviate the substrate inhibition effects, several studies have explored the use of EPSs to protect ANAMMOX bacteria and improve their resilience. Wang et al. [86] found that adding 10–50 mg/L of alginate oligosaccharides (AOS, a model EPS component) into ANAMMOX SBR fed with high-strength ammonium wastewater (500 mg NH_4^+-N/L) could mitigate the FA inhibition and maintain a stable NRR of 0.33 kg $N/m^3/d$. The protective mechanisms of AOS included forming a hydrated gel layer on the cell surfaces to reduce FA penetration, providing organic substrates for ANAMMOX mixotrophy, and stimulating the antioxidant enzyme activities of ANAMMOX bacteria. Another study by Zhang et al. [87] demonstrated that dosing 50–200 mg/L of EPSs extracted from denitrifying granules into ANAMMOX reactors could enhance the ANAMMOX resistance to NO_2^- inhibition (up to 400 mg NO_2^--N/L) and increase the NRR by 92% when compared to the EPS-free control. The EPS could reduce the FNA stress by binding NO_2^- and buffering the pH, as well as improve the ANAMMOX cell aggregation and metabolic activity.

The combination of EPS supplementation with other optimization strategies, such as immobilization and adaptation, has also been reported to further improve the ANAMMOX performance under high-strength conditions. For instance, Zhu et al. [88] developed a novel EPS-based cryogel carrier by embedding ANAMMOX sludge and polyvinylpyrrolidone (PVP, a synthetic EPS) into a poly (vinyl alcohol)/sodium alginate matrix, followed by a freeze–thaw treatment. The immobilized ANAMMOX cryogel exhibited excellent mechanical strength, high porosity, and resistance to FA inhibition, maintaining an NRR of 0.81 kg $N/m^3/d$ at 400 mg NH_4^+-N/L. The adapted ANAMMOX consortia showed elevated EPS contents and hydrophobicity when compared to the non-adapted ones, suggesting a key role of EPSs in substrate inhibition resistance.

4.3. Extracellular Polymeric Substances Supplementation under Salinity Stress

Saline wastewater generated from chemical, food, and leather industries often contains high levels of NH_4^+ that require treatment before discharge. However, the high salt concentrations (>1% NaCl) can severely inhibit the SAA and cause biomass washout due to osmotic stress and ion toxicity [89,90]. To address this issue, the application of EPSs to enhance the salt tolerance of ANAMMOX bacteria has been investigated. Zhang et al. [91] isolated a salt-tolerant ANAMMOX consortium from marine sediments and characterized its EPS components under different salinity levels (0–4% NaCl). They found that the EPS content, hydrophobicity, and PS/PN ratio of the ANAMMOX consortium increased with the increasing salinity, suggesting an adaptive response to salt stress. The EPS could protect ANAMMOX cells from osmotic shock by forming a hydrated polymer network and reducing direct contact with salt ions. Bivalent cations are beneficial to the resistance of ANAMMOX to salinity stress, and the reason may be that bivalent cations increase the yield of EPSs. The amount of Na^+ sequestered in the soluble EPS was increased by the augmentation of divalent cations, which seems to contribute to the alleviation of salinity stress [92].

Based on this finding, several studies have applied the salt-tolerant ANAMMOX consortia and their EPS extracts to inoculate ANAMMOX reactors treating saline wastewater. Liu et al. [93] developed salt-resistant ANAMMOX granules through the self-assembly of salt-tolerant ANAMMOX consortia and alginate gel under saline conditions (3% NaCl). The granules exhibited compact structure, high EPS content, and stable SAA at high salt

levels, with a maximum NRR of 0.42 kg $N/m^3/d$ at 30 g NaCl/L. Studies have shown that ANAMMOX processes in low-salinity waters can adapt to high-salinity environments. Through experiments, it was found that the SAA could reach 92.2% of the original low salinity condition when the salt concentration was 20 g/L, and the adaptation of ANAMMOX bacteria to salinity was mainly achieved via increasing the content of EPSs [94].

4.4. Extracellular Polymeric Substances Supplementation under Antibiotic Stress

The presence of antibiotics in wastewater effluents from pharmaceutical, hospital, and animal farming industries poses a serious threat to the biological nitrogen removal processes, including ANAMMOX. Many antibiotics, such as tetracyclines, sulfonamides, and macrolides, have been shown to inhibit SAA by disrupting the key enzymes and cell membranes of ANAMMOX bacteria [95]. The use of EPSs to mitigate the antibiotic inhibition on ANAMMOX has emerged as a promising strategy. It reported that dosing 50 mg/L of soluble EPSs (extracted from anaerobic sludge) into ANAMMOX reactors could significantly alleviate the toxicity of tetracycline (10 mg/L) and oxytetracycline (10 mg/L) to the ANAMMOX process, with an NRR recovery rate of 85% and 78%, respectively [96,97]. The soluble EPS could act as a natural adsorbent and complexing agent for antibiotics, reducing their bioavailability and protecting ANAMMOX bacteria.

In another study, Fan et al. [98] found that adding 200 mg/L of EPSs (extracted from denitrifying sludge) into ANAMMOX reactors could enhance the resistance of the ANAMMOX process to sulfamethazine (SMZ) stress (50 mg/L), maintaining 91% of the initial NRR after 7 days of exposure. The EPS promoted the formation of compact and stable ANAMMOX granules that could reduce the diffusion and accumulation of SMZ within the sludge. The EPS also stimulated the expression of stress-responsive genes and extracellular polymeric substance synthesis genes in ANAMMOX bacteria, as revealed through metatranscriptomic analysis. Similarly, Zhang et al. [99] demonstrated that supplementing 100 mg/L of EPSs (extracted from anaerobic granular sludge) into ANAMMOX reactors could improve the ANAMMOX resilience to erythromycin inhibition (10 mg/L), with the NRR being maintained at 76% of the control level. The EPS enhanced the ANAMMOX microbial community diversity and functional redundancy, which contributed to the process stability under antibiotic disturbance.

5. Challenges and Future Perspectives

Despite the significant advancements in applying EPSs to enhance the ANAMMOX process, there are still some challenges and knowledge gaps that need to be addressed for further development and optimization (Figure 5). EPSs play a crucial role in microbial growth processes, and we have gained a certain understanding of their composition, structure, and extraction methods. Firstly, the complex composition and dynamic nature of EPSs pose challenges for their characterization and standardization. The current extraction and analytical methods for EPSs are mostly developed for aerobic activated sludge, which may not be directly applicable to ANAMMOX sludge due to the unique cell structure and metabolic pathways of ANAMMOX bacteria [42]. The recovery of EPSs is also a research direction, but there are few results at present. EPSs extracted via different methods can vary greatly in their yield, purity, and functionality, making it difficult to compare the results across studies [34]. Therefore, it is necessary to develop and validate more specific and reliable methods for characterizing the composition, structure, and properties of ANAMMOX-derived EPSs, such as using advanced spectroscopic and microscopic techniques coupled with multivariate analysis [38]. Establishing a standardized protocol for EPS extraction and characterization from ANAMMOX sludge is also crucial for ensuring the reproducibility and comparability of research findings.

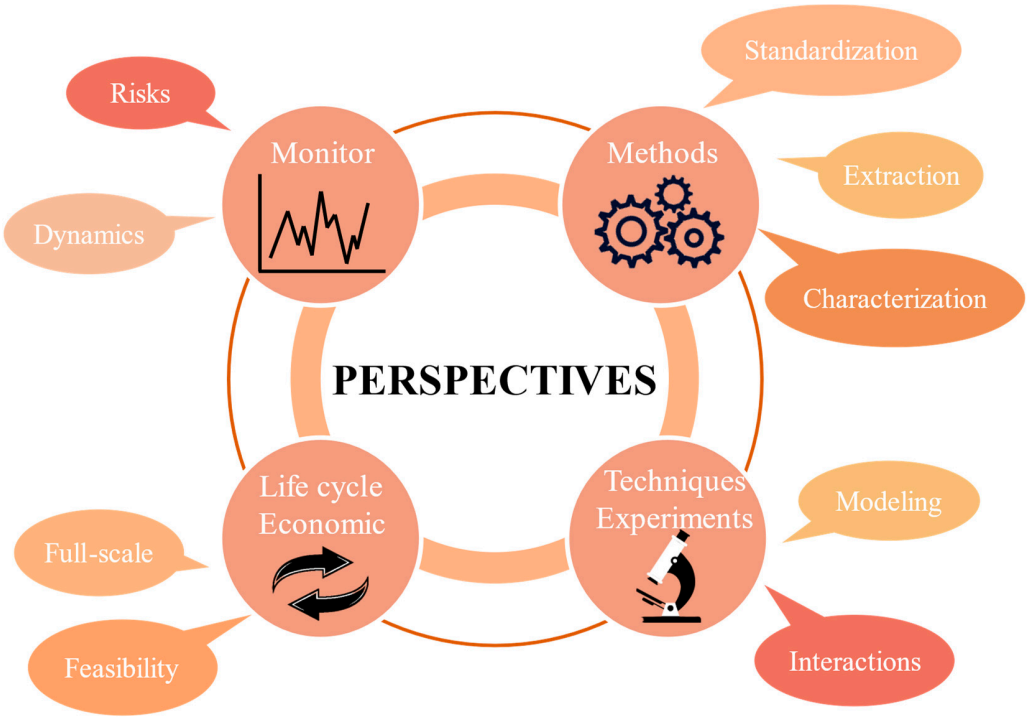

Figure 5. Possible directions in the future.

Secondly, the mechanisms underlying the interactions between EPSs and ANAMMOX microorganisms are not yet fully elucidated. While several studies have reported the beneficial effects of EPSs on the SAA and stability, the specific pathways and factors involved in EPS-mediated enhancement are still largely unknown. For instance, how do the different components of EPSs (e.g., proteins, polysaccharides, eDNA) interact with ANAMMOX cells and influence their physiology and gene expression? What are the key functional groups or structural features of EPSs that contribute to their protective and stimulatory effects on ANAMMOX? How do the EPS composition and properties change in response to different environmental stresses, and how do these changes affect the ANAMMOX community structure and function? What is the material exchange and information exchange between ANAMMOX bacteria and cooperative bacteria in EPSs? Are EPSs just the midpoint in the ANAMMOX system? Answering these questions requires an integrated approach, combining advanced analytical techniques (e.g., omics, imaging, and spectroscopy) with well-designed experiments and modeling [36]. Unraveling the molecular mechanisms of EPS-ANAMMOX interactions can provide valuable insights for optimizing the EPS-based strategies to enhance ANAMMOX performance.

Thirdly, the feasibility and cost-effectiveness of applying EPS-based strategies for full-scale ANAMMOX wastewater treatment need to be carefully evaluated. Most of the current studies on EPS supplementation for ANAMMOX enhancement were conducted in lab-scale reactors fed with synthetic wastewater, which may not reflect the complexity and variability of real wastewater compositions. The efficiency and stability of EPS-amended ANAMMOX processes in treating actual industrial and municipal wastewater with various inhibitors and fluctuating loading rates remain to be demonstrated. Moreover, the production, purification, and formulation of EPS additives for ANAMMOX enhancement may incur extra costs and environmental impacts that should be taken into account when assessing the overall sustainability of the technology. The techno-economic analysis and life cycle assessment of EPS-based ANAMMOX processes at different scales and scenarios are needed to support their practical applications and optimization [5].

Lastly, the long-term effects and potential risks of EPS amendments on ANAMMOX ecosystems and effluent quality also warrant further investigation. While EPS supplementation can promote the growth and activity of ANAMMOX bacteria, it may also stimulate

the proliferation of other microbial groups, such as heterotrophic denitrifiers and nitrite oxidizers that compete with ANAMMOX for nitrite substrates [100,101]. The excessive accumulation of EPSs in ANAMMOX reactors may lead to operational issues such as foaming, clogging, and the deterioration of sludge settleability and dewaterability [83]. The presence of high concentrations of soluble EPSs in the effluent may also affect the downstream treatment processes or receiving water bodies. Therefore, monitoring the dynamics and balancing the trade-offs of EPSs in ANAMMOX systems, based on a holistic understanding of their ecological and engineering implications, are essential for achieving sustainable and robust ANAMMOX-based wastewater treatment [41].

6. Conclusions

This review highlights the pivotal roles and emerging applications of EPSs in enhancing the ANAMMOX process for sustainable wastewater treatment. As a ubiquitous and multifunctional component of ANAMMOX biofilms and granules, EPSs not only serve as a structural scaffold and protective barrier for ANAMMOX cells, but also mediate the microbial interactions and mass transfer within the ANAMMOX consortia. The diverse composition and properties of EPSs can be harnessed to improve the start-up, activity, and resilience of ANAMMOX under various environmental stresses, such as substrate inhibition, salinity, and antibiotics. EPS-based strategies, such as EPS supplementation, EPS-producing co-culture, and EPS-based immobilization, have shown promising results in accelerating ANAMMOX granulation, shortening start-up time, and enhancing ANAMMOX stability in lab-scale studies. However, the complex nature of EPSs and the intertwined metabolic and ecological networks within ANAMMOX microbiomes pose great challenges for elucidating the mechanisms and optimizing the applications of EPS-enhanced ANAMMOX processes. Further research integrating advanced analytical techniques, reactor engineering, and modeling is needed to unravel the molecular interactions between EPSs and ANAMMOX microbes, assess the feasibility and sustainability of EPS-based ANAMMOX processes, and design robust and efficient EPS-amended ANAMMOX systems for wastewater treatment. Solving these challenges will not only deepen our understanding of the versatile functions of EPSs in environmental bioprocesses, but also open up new avenues for harnessing the potential of EPSs in developing resilient and sustainable ANAMMOX-based technologies for waste-to-resource recovery.

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