# Extracellular biopolymers recovered as raw biomaterials from waste granular sludge and potential applications: a critical review

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## Abstract

Granular sludge (GS) is a special self-aggregation biofilm. Extracellular polymeric substances (EPS) are mainly associated with the architectural structure, rheological behaviour and functional stability of fine granules, given that their significance to the physicochemical features of the biomass catalysing the biological purification process. This review targets the EPS excretion from GS and introduces newly identified EPS components, EPS distribution in different granules, how to effectively extract and recover EPS from granules, key parameters affecting EPS production, and the potential applications of EPS-based biomaterials. GS-based EPS components are highly diverse and a series of new contents are highlighted. Due to high diversity, emerging extraction standards are proposed and recovery process is capturing particular attention. The major components of EPS are found to be polysaccharides and proteins, which manifest a larger diversity of relative abundance, structures, physical and chemical characteristics, leading to the possibility to sustainably recover raw materials. EPS-based biomaterials not only act as alternatives to synthetic polymers in several applications but also figure in innovative industrial/environmental applications, including gel-forming materials for paper industry, biosorbents, cement curing materials, and flame retardant materials. In the upcoming years, it is foreseen that productions of EPS-based biomaterials from renewable origins would make a significant contribution to the advancement of the circular economy.

**Keywords**: Extracellular polymeric substances; waste granular sludge management; extraction and recovery; biomaterials; circular economy

## 1. Introduction

Over 300 million tonnes of polymers are worldwide manufactured per year, where consuming merely approximately 6% of fossil produced, yet expecting to increase to 20% in the coming three decades (Payne et al., 2019). Whilst those of petrochemical origin dominate the polymers industry (about 99% in 2015). Together with environmental concerns, biopolymers produced from renewable resources become attractive and are currently the focus of intensive research efforts (Kreyenschulte et al., 2014). Due to the cost-effective production process, the application of biopolymers has become economically viable. Microbial polymers include intracellular and extracellular polymers. Compared with limited intracellular polymers (e.g. polyphosphate, glycogen) (Feng et al., 2020), extracellular polymeric substances (EPS) have drawn emerging commercial interest. EPS are the “sticky” materials secreted by bacterial consortia during cell metabolism and form a complex and diverse biopolymeric matrix consisting of proteins (PN), exopolysaccharides (PS), DNA, lipids, glycoprotein, S-layer and humic-like substances, etc. (Seviour et al., 2018). Unlike other natural sources, EPS can be extracted from a large range of biomass, holding specific superiorities compared with other polymers, because they are prevalent, bio-based, biodegradable, highly-productive rate and easier extraction procedure than oil-based synthesizing polymers.

Currently, more studies on wastewater treatment plants (WWTPs) gradually shift from the aquatic environment protection (i.e., pollutant removal and water quality control) towards energy/resource recovery (Hao et al., 2019; Kehrein et al., 2020). The main waste product in WWTP is excess sludge and its processing cost accounts for nearly half of the total operational capital (de Valk et al., 2019). As reported, EPS take a large fraction of sludge dry weight, making EPS applicable in various fields including directly used as biomaterials or as rheological-modification additives, industrial sizing chemicals, and medical reagents (Feng et al., 2019; Lin et al., 2015). With this regard, EPS recovered from waste sludge holding the promises of achieving resource recovery and contributes to a circular economy (Seviour et al., 2018; van Leeuwen et al., 2018).

Granular sludge (GS) technologies gain more attention due to the unique advantages of efficient biomass retention and more compact reactors than traditional activated sludge systems. Biogranulation treatment units have many advantages, including 1) an excellent settleability; 2) an enhanced biomass withholding; 3) capacity of treating high level of carbon/nutrient load pollutants; 4) increased toxicity tolerance (McSwain et al., 2005; Pronk et al., 2015). Characterisations correlated to different biological granule types originating from wastewater treatment units as well as their EPS contents are summarised in **Table 1**. Microbial granules exhibit a large size (> 0.2 mm), compact and spheroidal structure, and high settling velocities. Highly-diverse microbial communities inhabit in distinct granules (Nancharaiah and Reddy, 2018). The granule formation is governed by the microbial EPS, which are much higher than in any other types of biofilm (Ding et al., 2015). Some important functions concerning EPS have been described, such as a protective barrier against detrimental environment, maintenance of a stable structure, nutrient source, and organic substance sorption (Seviour et al., 2018). A significant yield of extracellular biopolymers has been extracted from different kinds of granules, e.g. about 25% of organic matter (volatile suspended solids, VSS) in aerobic granules (Felz et al., 2016); 40%-59% of VSS in anammox GS (Feng et al., 2019; Lotti et al., 2019a; Ni et al., 2010a). Currently, aerobic granules hold the promising for resource recovery (i.e., phosphate, bioplastic, EPS) from wastewater, and perhaps can be integrated with the conventional water treatment process (Van der Hoek et al., 2016). Meanwhile, it should be noticed that large amounts of EPS are expected from anaerobic granules, given that there are over 1000 full-scale anaerobic reactors such as Upflow Anaerobic Sludge Blanket (UASB) reactors worldwide (Lim and Kim, 2014). The demonstration of EPS extraction from anaerobic granules would expand the great potential towards resource recovery. Interestingly, in many studies, the autotrophic anammox GS have been discovered to contain a large number of EPS contents (~594 mg/g VSS) compared with heterotrophic granules (Feng et al., 2019; Lotti et al., 2019a; Ni et al., 2010a). Like the conventional activated sludge technologies, waste granular sludge (WGS) from both full-scale and lab-scale GS reactors (Guo et al., 2020; Lin et al., 2015; Lotti et al., 2019b) is a concern due to the high processing cost. Given the rapidly increasing number and size of AGS and anammox-GS installations, together with the large number of current AnGS reactors, strategies for the efficient WGS management are demanded. The aforementioned information suggests granulation technology holds great potential for EPS recovery as the raw biomaterials. Further study is necessarily dedicated to better designing and managing future biorefinery large-scale implementation, particularly in enhancement of quantity and quality of the biomaterials.

This present review mainly focuses on the current status of EPS secreted from granular biomass, including anaerobic, aerobic, anaerobic ammonium oxidation (anammox) granules, hereafter referred to as AnGS, AGS, and anammox GS, respectively. To our best knowledge, this is the first review concerning on GS-based EPS. This present review aims at providing recent advances towards the conversion of EPS from the original sources - waste granular biomass to value-added products (i.e. biomaterials) (Fig. 1). The following aspects are highlighted: 1) new identified EPS components and EPS distribution in different granules; 2) EPS extraction and recovery; 3) key parameters affecting EPS production; 4) potential applications of EPS-based biomaterials. Fundamental protocols and technologies involved in these four essential aspects are introduced in different sections. As the prerequisite for the application of EPS-based biomaterials (Section 6), the guarantee for GS-based EPS production relies on the performance stability of GS installations. The granule stability and granulation process are keenly associated with the EPS components and distribution among the granules (Section 2, 3), resulting in distinct physicochemical properties. Prior to the application, how to obtain the maximum EPS extraction efficiency can be attributed to two aspects. One is to assess different extraction and recovery methods (Section 4). Another is to find out the key parameters inducing massive GS-based EPS production, but without invalidating reactor performance (Section 5). Challenges and prospects are presented as well.

## 2. High diversity of GS-derived EPS contents: emerging classification criteria and new components appreciated

EPS are secreted by the bacterial consortium during metabolism and their accumulation helps to bridge bacterial cells and other particles into aggregates. Several definitions of EPS forms are illustrated in **Table 2**. The most common one is according to the distribution: bound (B-EPS) and soluble EPS (S-EPS) (Raszka et al., 2006; Su et al., 2013). B-EPS are closely bound with cells that are further subdivided into loosely-bound (LB-EPS) and tightly-bound EPS (TB-EPS). The TB-EPS contents in biomass are practically higher, which may contribute to different properties of sludge/biofilm (Sheng and Yu, 2006). According to their nature, EPS can be also classified as slime and capsular (More et al., 2012; Raszka et al., 2006). Recently, emerging criteria are proposed to denominate EPS according to their specific physical, chemical or structural characteristics. For instance, given the electrical charge characterisation, EPS can be divided into anionic/cationic types; based on different physical-chemical characteristics, it can be alkaline/acidic/polar EPS (Caudan et al., 2012; Pronk et al., 2017). PN and PS with negative charges were verified in AGS-derived EPS (Seviour et al., 2012). Another conception "structural EPS" was proposed and discussed through extracting and characterizing EPS from AGS and anammox GS, and investigating the rheological behaviour of the formed hydrogel, which was considered to be well linked with the strong matrix of granular sludge (Boleij et al., 2019; Felz et al., 2016; Lin et al., 2018; Lotti et al., 2019b).

EPS are dominated by the macromolecular compounds, but with a large broad molecular weight (MW) distribution, typically from a rather low (< 3 kDa) to a large MW (>235 kDa) (Boleij et al., 2019; Feng et al., 2019; Wang et al., 2009). Feng et al. (2019) demonstrated that 77%-96% of PNs in EPS from anammox GS had over 3 kDa WM. Zhu et al. (2015) demonstrated the PN MW range in aerobic or anaerobic granules between 20 and 97 kDa, indicating high MW property favoured the sludge granulation. The highly-broad MW distribution further indicates the complex and diverse characteristics, and the biopolymeric matrix is mainly constituted by PS, PN, glycoconjugates, humic acid, nucleic acids etc. (Felz et al., 2020b; Flemming and Wingender, 2010; Gagliano et al., 2018)

(**Fig. 2**). In most studies, total PN (up to 60%) and total PS (40 – 95%) are demonstrated as majority constituents in extractable extracellular biopolymers (Dubé and Guiot, 2019; More et al., 2014). PN are reported more dominant than PS, with a PN/PS of 3-8 (Feng et al., 2019; Lotti et al., 2019a; McSwain et al., 2005). Thus far, much contribution has been accordingly dedicated to identifying new EPS contents, particularly with regard to PN, PS and heteropolymers with specific/putative functions (Boleij et al., 2019; de Graaff et al., 2019; Lotti et al., 2019b; Wong et al., 2019; Wong et al., 2020). Glycoproteins with a heterogeneous O-glycan structure from anammox GS-based EPS dominated by *Candidatus Brocadia* were identified, perhaps closely associated with system biogranulation process (Boleij et al., 2018). The recognized glycoprotein had a high MW component of over 235 kDa at the acid condition, underlying the presence of acid groups (i.e., –COOH, $-OSO_3^-$) (Boleij et al., 2018). Glycosylated amyloid-like PN (Lin et al., 2018) were extracted from AGS with the dominance of ammonium-oxidizing bacteria. Aromatic PN-like and tryptophan PN-like substances were more abundant in the matrix of AGS/AnGS (Zhu et al., 2015). The secondary structure of PN also contributes to the granulation process and granules stability. Amyloids-like substances were found in anammox GS-derived EPS or hydrogel (Lotti et al., 2019b). Attention should be particularly paid to amino acids. A total of 14 amino acids (glycine, leucine, alanine, isoleucine etc.) were detected in AGS-EPS based on that method used isotope dilution mass spectrometry (Felz et al., 2020b). However, there is a limitation of this method, some amino acids cannot be detected: merely weight fraction of 1.5% of the total amino acids in structural EPS was detected. Recent quantitative proteomics analysis offers a powerful tool to identify extracellular PN with a high throughput. For instance, Chen et al. (2019) pointed out that the main role of the extracellular PN were associated with multivalent cations binding in anammox biofilm through iTRAQ-based quantitative proteomics. This technique is anticipated to be applied to characterize the PN in granular sludge.

As to polysaccharides, two crucial constituents i.e., alginate-like exopolysaccharide (ALE) (Lin et al., 2010; Schambeck et al., 2020) and granulan (Seviour et al., 2010) have been identified as the functional gel-forming constituents in AGS. Despite granulan and ALE display structural hydrogels properties, they are completely distinct. Granulan with a pKa of 9.0 was reported as a complex and highly-novel heteropolysaccharide (Seviour et al., 2010). ALE with a pKa of 4.5 was observed in granules from acetate-fed or mixture of an abattoir and domestic wastewater (Lin et al., 2010). Sialic acids with a nine-carbon backbone were discovered as a component of EPS from seawater-adapted AGS (de Graaff et al., 2019). Sialic acids might distribute in the outer layer of GS to protect galactose against enzyme degradation. Very recently, the linear heteropolysaccharides Glycosaminoglycans (GAGs) i.e., hyaluronic acid-like and sulfated GAGs-like biopolymers were discovered in both AGS and EPS products (Felz et al., 2020b). Interestingly sialic acids (1.6%) and sulfated glycosaminoglycans (2.4%) were also observed in anammox GS-derived EPS (Boleij et al., 2020). GAGs-like polymers and sialic acids might be widely distributed in granules and play an important role for the performance stability. Main sugar monomers in AnGS at high salinity were reported as mannose and N-acetyl galactosamine, indicating the protection role to the methanogenic consortia (Gagliano et al., 2018). Uronic acids (with a MW of > 10 kDa) contain glucuronic, galacturonic and mannuronic acids, which are commonly found in the PS of extracellular matrix, with the roles of supporting the granular structure and preventing bacteria detachment (Al-Halbouni et al., 2009; Jia et al., 2017). Monosaccharides in structural AGS-derived EPS were recently detected using HPAEC-PAD, including some neutral sugars (glucose, galactose, mannose etc.), glucuronic acid, galacturonic acid, amino sugars (galactosamine and glucosamine) (Felz et al., 2020c).

The high diversity of extracellular biopolymers results in the complexities and difficulties to appreciate the EPS components. In particular, PN-glycosylation phenomenon commonly happens in biofilm matrix, which further complicates the analytical process. More efforts by integrating multidisciplinary analyses should be devoted to understanding special EPS components (functions, physicochemical properties, etc. not only measuring the overall PN and carbohydrates contents), to improve the performance stability of GS technology (Felz et al., 2020c; Seviour et al., 2018).

## 3. Granular stability and heterogeneous spatial distribution of GS-derived EPS

GS-based EPS distribution seems quite heterogeneous. Unravelling their spatial distribution is conducive to identify different components' roles for granule formation and provides an overview and theoretical basis for accelerating the formation of granules. Thus, there are intensive researches (*in situ* or *ex situ* analyses) on the investigation of spatial distribution regarding GS-based EPS constituents (Seviour et al., 2018; Seviour et al., 2009a). For AnGS, with fluorescent lectin probes for specific saccharides, most PS were distributed in the external layer (Zhang and Fang, 2004). TB-EPS were found positive for anaerobic granulation process in an expanded granular blanket for treating low-strength domestic sewage (Xu et al., 2018). Regarding AGS-based EPS distribution, two hypotheses including PN-core and non-PN core exist. McSwain et al. (2005) pointed out PN-dominated core and PS-dominated shell contributed to the structural stability of glucose-fed AGS using the fluorescence staining method. Further, β-polysaccharides are mostly responsible for granule structural stability, functioning as a framework to support the surface layer (Adav et al., 2008b; Wang et al., 2005). Nevertheless, β-polysaccharides were witnessed as the granule core in an AGS reactor with the toxic-phenol presence in the influent; whilst α-polysaccharides together with lipids enriched in the surface of granules based on the method used in-situ staining all EPS components (Adav et al., 2008a). Besides, according to Chen et al. (2007), wastewater types affected AGS-based EPS distribution through dye staining combined with the imaging technique (confocal laser scanning microscopy). Regarding acetate-fed AGS, the internal core was composed of PN and β-D-glucopyranose PS; in contrast, the exterior shell was dominated by α-D-glucopyranose PS. However, for phenol-fed AGS spatial distribution of extracellular matrix was vastly different, characterized by a PN core and a α- and β-D-glucopyranose PS shell. It needs to stress that, the importance of EPS property and distribution to granular stability may weigh the biopolymer quantity (Wang et al., 2005). For instance, for AGS with acetate as the substrate, abundant EPS were present in the loose core, almost 6 folds (approximately 240 mg/cm$^3$) of that in the shell, and non-soluble β-polysaccharides accumulated in the outer layer of acetate-fed AGS. Due to that, excellent hydrophobic properties of exterior shell were observed, nearly 4 times (200%) of that in the granule core Wang et al. (2005).

Regarding anammox GS, anammox cells could secrete more EPS (particularly the predominant PN contents) promoting the fast granulation process with both synthetic or real wastewater fed into the reactors (Boleij et al., 2019; Feng et al., 2019; Ni et al., 2010b; Wang et al., 2020). Wang et al. (2020) studied the significance of three sub-classifications of anammox GS-based EPS to adhesion behaviour with a rank of TB-EPS < LB-EPS < S-EPS. Hence, they concluded that the stratified EPS promoted the initial adhesion to abiotic surfaces. Besides, the EPS distribution in anammox GS was demonstrated to be associated with enriched microorganisms (Ni et al., 2015). In high-anammox-enriched GS, the internal core principally consisted of PN and β-D-glucopyranose PS, whilst α-D-glucopyranose PS accumulated in both the inner layer and surface. Whereas in low-enriched granules, α-D-glucopyranose PS primarily gathered in shell margin and the β-D-glucopyranose PS distributed in the inner layer and exterior shell, whilst PN were present throughout the whole granules. Compared with the stability of low- and high- enriched anammox granules, the former endowed higher stability, inconsistent with the granule strength test. In addition, microscopic observation found that the layer thickness of biopolymers embedding cells of anammox GS was higher than AGS and AnGS (Ni et al., 2015).

As mentioned above, the spatial distribution of GS-based EPS shows heterogeneous and diverse. Significant differences are reported in different kinds of granules as well. Notably, there are no universal conclusions, and some results are even controversial, e.g. the theories of PN-core and non-PN core in AGS. The heterogeneous EPS distribution can be attributed to the complexity of EPS components (Section 2) resulting from operational conditions, anaerobic or aerobic environment, microbial communities (e.g., filamentous growth), and the subsequent biological and chemical transformations, etc. (Chen et al., 2010; Zhang et al., 2015). Particularly, the relationship between EPS distribution and microorganisms' distribution is unknown. On the other hand, more powerful analytical techniques are expected to be applied to identify the EPS spatial distribution. Among different approaches, in-situ staining technique has been commonly explored to visualize the 3-D distribution of cells, PN, and PS for many years.

## 4. Extraction and recovery of GS-based EPS with the consideration of general and specific criteria

Prior to understanding the roles of key biopolymers and the application of EPS, the prerequisite is protocol development to extract and recover EPS from different granules. As agreed in intensive literature (Felz et al., 2016; Feng et al., 2019; Sheng et al., 2010), EPS composition and characterisation largely depend on the extraction methods. More exactly, given the high diversity of GS-based EPS in Section 2, distinct extraction and recovery methods can result in a series of differences in terms of quantity (the total yield, EPS components, etc.) and quality (biochemical properties, functional groups, etc.) of the extractable exopolymers. The whole process includes sample preparation, extraction, purification (optional), enrichment, and recovery. Thus far, intensive efforts have been dedicated to EPS extraction (Sheng and Yu, 2006). Techniques for EPS extraction contain physical, chemical, or biological approaches according to **Table 3** (Li et al., 2014; Lotti et al., 2019a; Ma et al., 2012; Ni et al., 2010a; Ni et al., 2015; Sheng et al., 2010; Xing et al., 2016; Xing et al., 2015; Zhang et al., 2016a; Zhang et al., 2016c). The main disruptive physical treatments include centrifugation, sonication, blending, and heating, etc. Physical methods highlight using physical force (shearing, heat, etc.) to disrupt and dissolve polymer from extracellular matrix, allowing softly to extract a small number of EPS but with a guarantee of cell integrity. Chemical extraction techniques include chelating agents, cation exchange resins (CER), alkaline, acid or aldehydic reagents to solubilize polymers. Biological method contains enzymatic treatment. Generally, chemical methods are more effective and appear to yield a relatively higher EPS amount (Sheng et al., 2010). For instance, as verified by a series of physical/chemical tests to extract AGS (Felz et al., 2016) and anammox GS (Feng et al., 2019), the highest effectiveness of EPS yield was derived from the method of heating (80 $^{o}$C) at alkaline conditions, in which pH increase boosted the dissociation of acidic groups in EPS. Using alkaline reagents ($Na_2CO_3$ or NaOH) can solubilise most PN of EPS matrix, while acid method can solubilise most PS components but with a very low yield (Aravinthan et al., 2001; Feng et al., 2019). Alkaline treatment or CER mainly targets ionic biopolymer interactions while aldehydic reagents (e.g., formamide or formaldehyde) function as cell fixation and thus reduce cell lysis during extraction, so non-specific aldehydic methods are widely combined with alkaline treatment. In spite of many efforts, thus far the extraction protocol is still not universal (Seviour et al., 2018).

Critical criteria of polymer extraction are that (Sheng et al., 2010), 1) to extract maximum extracellular biopolymers; 2) not to disrupt the structure of EPS; 3) to minimize cell lysis. In addition to those conditions, a method with the advantages of less time consumption and friendly-operation would be a benefit (Feng et al., 2019). These traditional criteria have been used to evaluate the method effectiveness with the purposes of the fundamental investigation of EPS structure, compositions, characteristics, and function during the water treatment process, which can be classified as general targets. Another important aspect to be considered is the differences between GS and traditional flocculent sludge, extracting GS-based EPS requires more intensive extraction methods than flocs. With this regard, new criteria emerge according to different research purposes. For instance, harsh methods (e.g. heating at high temperature and violent sonication) can be applied to damage cell integrity instead of keeping cells intact, aiming at deciphering the roles of more diverse and unknown biopolymers (Felz et al., 2016). However, the existing extraction and recovery methods, so far, have never considered in terms of the usefulness of the extractable materials for specific practical application and resource recovery. Hence, this is another aspect that requires to be considered when it comes to reusing the recovered EPS-based biomaterial for applications.

Recovering EPS with sufficient purity and amounts is essential to illustrate the structure and function of innovative biopolymers and subsequent analyses (e.g., PN interpretation by proteomics). As shown in **Table 4**, EPS recovery can be achieved by solvent/ethanol precipitation (Li et al., 2014), acid precipitation (Boleij et al., 2019; Lotti et al., 2019a), centrifugal filter device with a membrane

(Feng et al., 2019), and purification by dialysis (Felz et al., 2016; Liu and Fang, 2002) or electrophoretic/chromatographic techniques (Seviour et al., 2010). However, one issue facing currently consists in the diffificulties to compare different recovery methods, considering that the work on comparison and optimization of different recovery methods are seldomly concerned.

## 5. Key parameters affecting EPS production: keeping the balance between 'appropriate' and 'excessive'

Microbial communities and their selective environment are crucial elements to regulate EPS excretion behaviour, in terms of constitutes, structure, and physicochemical properties of EPS extractable products. With this regard, the parameters on EPS production can be divided into two categories: internal factors and external environmental conditions (i.e. operational parameters, feeding substrates, and exogenous substances) (**Fig. 3**). External conditions drive the change of microbial communities and the presence of unique microorganisms at special conditions (Gagliano et al., 2020). The microbiome in different GS systems has diverse metabolism activities, thus triggering the obviously-distinct EPS synthesis and production behavior. Its complexity makes limited information on the functional roles of the microbial communities and specific microorganisms to drive cells aggregation and maintain GS stable. Related contents and yields of EPS derived from distinct GS types and reactor configurations are summarized in **Table A1** in Support Information. PS- and PN-related biopolymers are predominant components of the extracellular matrix. Notably, in AnGS-derived EPS matrix, PN and humic acid were sometimes witnessed as the main constituents, with lower PS concentrations (Guibaud et al., 2012; Métivier et al., 2013). Regardless of granules types, the PN/PS ratios are at a high level, more than 1.0 in most studies. This finding indicates an essential feature with relatively high PN contents for granule formation and maintenance (McSwain et al., 2005). It seems that anammox consortia are more effective in EPS excretion, given that both PN and PS contents in anammox GS-derived EPS are relatively higher compared to AnGS (methanogenic granules) and AGS (Zhang et al., 2016a). The underlying reason can be attributed to the EPS' biodegradability, which means heterotrophic microorganisms can degrade EPS as carbon and energy sources. On the contrary, autotrophic growth bacteria i.e., anammox, are not capable of catabolizing organic substances and therefore accumulate more EPS. Besides, the metabolism of intracellular storages may influence microbial EPS production. For instance, intracellular storage of polyhydroxybutyrate in AGS induced more microbial production of EPS, facilitating the granule formation (Wang et al., 2014).

Typically, GS-based EPS have positive effects on microbial resistance to stress-induced external environments, resulted from substrates, salinity, shear force, toxic compounds, nanoparticles, heavy metals, feast-famine feeding strategy, etc. **Fig. 4** displays the EPS components change at different stressful conditions. Generally, environmental stress stimulated more total EPS production. Particularly PN contents increased in the presence of toxic compounds, but with fewer influences on PS contents. These findings suggest that high extracellular PN contents benefit nucleation and granular formation. Although harsh conditions stimulate biopolymer secretion, granular stability and strength would not improve accordingly. Therefore, it may be deduced the existence of a threshold for extracellular PN contents: within this threshold, more extracellular PN produced would stimulate the granular formation and stability; on the contrary, excessive EPS may adversely affect the performance of granular systems.

### *5.1 Substrates*

Different substrates influence the shift of microbial community due to diverse microbial metabolisms. Accordingly, different types of substrates (e.g., molecular size, category) fed to the reactors influence EPS excretion behavior. For instance, Gagliano et al. (2020) compared the different molecular sizes of substrates i.e., macro proteins and micro amino acids (proline, leucine, and glutamic acid) on EPS production and granulation of AnGS under saline conditions. The replacement of macro tryptone using single amino acids led to the granule disappearance, suggesting that a complex proteinaceous substrate may stimulate granulation process under saline conditions. Similarly, distinct substrate types have proved the discrepancies. For example, high PN contents were found in the PN-grown granule, representing 220 ± 20 mg/g TS, which was 1.6 folds of that in the cannery-fed granules (most all soluble saccharides). Notably, the PN-fed AnGS exhibited very poor bulk features, such as low density, unideal shear strength and weaken settleability (Batstone and Keller, 2001).

The substrates concentration (organic or nitrogen loading rate, referred to as OLR or NLR, respectively) is another essential factor affecting EPS production or morphological shapes of different granules. For instance, Long et al. (2015) highlighted that OLR under 15 kg/($m^3$ d) positively stimulated the maintenance of AGS structure; while granule disintegration occurred with OLR over 18 kg/($m^3$ d). OLR rise promoted anaerobic-core growth in the inner region due to the enlarged granular dimension and restricted oxygen transfer, and further resulted in the granule instability. Batstone and Keller (2001) compared the EPS constituents using two brewery effluents with different OLR of 7 and 7.5 kg COD/$m^3$ d, respectively. EPS yield, PN, and PS contents of brewery-fed AnGS showed an increased tendency with higher OLR. Zhang et al. (2016a) studied NLR effects on Anammox GS-derived EPS production via changing NLR at different levels. Results showed that EPS yield reasonably rose with the NLR lower than 10 g N/(L d), due to bacteria metabolism and stress-induced influences of nitrogen substances. However, a high level of NLR at 20 g N/(L d) resulted in the excessive EPS excretion, leading to granule instability and a deteriorated performance due to biomass washout. Overall, proper extracellular biopolymers excretion benefits granule stability and system performance; it would enhance granule settleability and biomass retention in the reactor units. While excessive EPS trigger negative influences on the granulation process (Zhang et al., 2016a), or even cause granule collapse (Long et al., 2015). Notwithstanding, EPS production behavior should not only take into account EPS amount, but requires to link physicochemical properties (hydrophobicity) with the formation and maintenance of granules.

### *5.2 Salinity*

Exposing to saline/hypersaline conditions leads to a modification of EPS constituents/structure, and granule surface properties (e.g., an increase of hydrophobicity) (Corsino et al., 2017; Wang et al., 2017). As shown in **Fig. 4**, many studies indicated salinity increment resulted in an increased PN secretion (Campo et al., 2018; Corsino et al., 2017; Ou et al., 2018). Such behavior may attribute to the protective mechanism in terms of adjustment osmotic pressure of microorganisms and cation-π interactions of monovalent ions with extracellular PN. By Fourier Transform Infrared Spectrometer analysis, the PN-related peaks (amide I and amide II) largely improved saline-resistance capacity for granules (Ou et al., 2018). Functional PN including porin, periplasmic-binding PN associated with transmembrane transport were found over-expression, further underlying the importance of highly active microorganisms under stressful saline conditions (Wang et al., 2017). It needs to stress that, the increase tendency in EPS yield/ constituents was not always proportional to salt concentration elevated (Corsino et al., 2017; Ou et al., 2018): some studies even obtained controversial conclusions. For example, an increment of salt concentration caused a decrease of PN contents in AGS-EPS (Corsino et al., 2017) or PS contents in anammox GS-EPS (Fang et al., 2018). Li et al. (2017b) observed a drop of overall AGS-EPS yield with an elevation of marine water fraction, whereas PN components in extracellular matrix maintained at a stable level. Interestingly, saline stress stimulated the production of abundant ALE and its enhanced gelling features was proven positive to the granulation process (Li et al., 2017b). Such differences can be due to complex EPS components, seeded sludge, unique bacterial strains, microbial community, and kinds of restricting substances. Therefore, the halophilic microorganisms and EPS production-related microbial communities are supposed to be associated with EPS component variation, however, little information is concerned about it.

### *5.3 Shear force*

Hydrodynamic shear force is an essential factor affecting granule formation (Liu and Tay, 2002) and is closely associated with regulating bacterial EPS secretion from granular sludge (Fernández et al., 2014; Tay et al., 2001; Wu et al., 2009). Researchers conclude that increment of shear force promotes granule formation (Tsuneda et al., 2003) and stimulates more EPS production. However, the change of EPS components in different granules shows different tendencies. For instance, the importance of exopolysaccharides in AGS-derived EPS was highlighted: aeration rate increment led to a PS-content increase but PS-content loss resulting in granule disintegration (Tay et al., 2001). However, in glucose fed-AnGS, PN contents were illustrated to accelerate extracellular PN secretion at high shear force; while PS content fluctuated less than the PN content (Wu et al., 2009). Interestingly, over-produced extracellular PN (over 80.5 mg/g VSS) would be detrimental for nucleation (Wu et al., 2009). Some researchers argued that the shear force may be the unnecessary factor during granulation process. Therefore, the interactions between EPS production behavior, shear force, and granule maintenance are still unknown.

### *5.4 Toxic substances: organic compounds, nanoparticles, and heavy metals*

Toxic exposures can inhibit or even poison microbial growth through interacting, modifying and degrading cell structures together with metabolic functions. Up to now, comprehensive studies have stated the sensitivity of biomass to the presence of toxic compounds. These toxic compounds include emerging contaminants (e.g., Tetracycline (TC) (Shi et al., 2013), Bisphenol A (BPA) (Li et al., 2015b), 4-chlorophenol (4-CP) (Wei et al., 2015b)), nanoparticles (NPs) (He et al., 2020; Li et al., 2015a; Mu et al., 2012) and heavy metals (Zhang et al., 2016b), which are closely associated with the granular stability. As shown in **Fig. 4**, regardless of differences of toxic chemicals and GS types, EPS yield quantities show an increased tendency except for the cases of extremely-high concentration. It is not surprising that granular biomasses have the self-protection behaviour against a poisonous event by producing more EPS acting as the buffer between cells and toxic chemicals. PN contents tend to increase after the exposure of toxic substances, but no significant difference in PS content is observed. This finding suggests PN contents not only play important roles in the GS structure stability (Flemming and Wingender, 2010), but in resistance to adverse conditions resulted from poisonous compound suppression. Further, the functional groups of EPS are modified due to that toxic event happened. For example, Shi et al. (2013) proposed that tetracycline greatly changed PN functional groups of AGS-derived EPS but showed fewer effects on PS contents. Su et al. (2019) investigated the antibiotic amoxicillin effects on AnGS-based EPS. For TB-EPS, there was no C-H bending vibration band, whilst the bands of amide I and C-H stretching vibration were enhanced. In addition to the protective roles of EPS matrix, Zhang et al. (2016b) pointed out that many negatively charged functional groups (-COOH, -OH, etc.) in EPS may electrostatically attract $Cu^{2+}$, by this the electrostatic repulsive force of granules would accordingly decrease.

Nanoparticles (NPs) come to slightly different conclusions, e.g., the presence of nanoscale zero-valent iron (nZVI) caused a significant reduction in AnGS-derived EPS (He et al., 2020). PN, PS, and humic-like substances were reduced by 15%, 27%, and 15% as a result of 1 g/L nZVI present in the EPS solution. On the contrary, high concentration EPS decreased $H_2$ generation rate from nZVI and benefited methanogenesis process by reducing the nZVI inhabitation. Mu et al. (2012) observed that in an AnGS system, no significant effects on EPS secretion and methane generation were observed in case of ZnO NPs with a dosage of under 50 mg/g TSS; but the system experienced adverse performances with a high level of NPs addition (> 100 mg/g TSS). The interactions between EPS and NPs resulted in a fluctuation of system performance, NPs corrosion, the modification of granule surface characterization (surface valence, electrostatic force etc.) and the instability of granules (He et al., 2020). These works are conducive to deeply understand EPS' adsorption property. Comprehensive investigations are expected to dedicate to the following aspects: 1) to identify the potential binding capacity of biopolymers, which is determined by binding sites), 2) to validate priority order of multiple adsorption sites for toxic contaminants, and 3) to characterize thermal dynamics during the NPs adsorption process.

### *5.5 Essential cations*

Mineral cations not only are essential for metabolism for microorganisms, but potentially stimulate the granule formation and bacterial restoration in damaged or suppressed biological systems. Among the cations, Calcium ions ($Ca^{2+}$) are one key parameter for granular formation (Liu et al., 2020), the activity restoration (Zhang et al., 2016b), and bioprocess improvement (Ma et al., 2020), thus resulting in the variation of EPS components and characterisations. For example, Ma et al. (2020) observed a high level of $Ca^{2+}$ (1000-2000 mg/L) not only caused the change of functional groups of AnGS-derived EPS, but simultaneously enhanced EPS production and anaerobic sludge digestion. This finding may attribute to stability damage resulted from the $Ca^{2+}$ addition, leading to microbial consortia to produce more biopolymers against the inhibited situation. Also, $Ca^{2+}$ was reported to play an important role for destructed anammox cells to restore their metabolic growth (Zhang et al., 2016b). Liu et al. (2020) proposed a strategy by combining an external conditioning step with $Ca^{2+}$ addition before reintroducing effluent sludge into SBR, which led to an increased EPS yield and an acceleration of AGS formation.

### *5.6 Feast/famine feeding strategy*

The effects of starvation on both AGS and AnGS have been widely explored. As agreed, a long starvation period leads to low wastewater treatment efficiency and poor treatment capacity. However, it generates a positive effect on microbial granulation as it promotes granules formation and stability. The starvation period is closely interrelated to the selection pressure, concentrations and types of provided substrates. It is therefore difficult to evaluate its contribution. On the other hand, longer starvation time seems to enhance the PN enrichment in the extracellular matrix (Rusanowska et al., 2019). Similarly, as discovered by Kang and Yuan (2017), the yeast-fed AGS generated a higher EPS yield at lower OLR, although granule disintegration was witnessed at the lowest organic load. This phenomenon may attribute to the fast substrates consumption within a short period at a lower OLR, causing a longer famine period for the microorganisms. In this case, more exopolymers are probably produced by microorganisms, serving as a carbon and energy source to support endogenous respiration to survive at the substrate-restricted conditions (Corsino et al., 2017). However, Campo et al. (2018) held a different view that it was mostly in the feast stage that EPS can be taken up by bacteria as an extra substrate. This may be due to the extremely hypersaline environment, which means for the more energy demand for microorganisms to adapt salinity conditions.

### ***5.7 Temperature***

Temperature is a crucial factor for microbial metabolisms and reactor performance. For the anaerobic process, anaerobic digesters can be operated at mesophilic (25°C-40°C) or thermophilic (> 45°C) conditions (Lim & Kim, 2014). In thermostatic anaerobic reactors, the extractable EPS amount of AnGS at thermophilic conditions is usually smaller than that under the mesophilic conditions (Lim & Kim, 2014). Furthermore, the amounts of PN and PS were enhanced by mesophilic conditions while higher lipid content was present in AnGS at long-term thermophilic conditions (Schmidt and Ahring, 1994). The lower PN and PS could be resulted from restricted growths of sensitive methanogens and acetogens, or the accelerated degradation of EPS due to the thermodynamics. It is still unknown that if the short-term temperature shocks would induce the variation of AnGS-based EPS. However, with the increased-temperature shock, different phenomena were observed in an anammox UASB reactor. It is well known that the ideal temperature for the anammox process is mesophilic ranging from 30°C to 37°C (Chen et al., 2020). By short-term temperature shocks (15°C, 25°C, and 55°C), an obvious increase in EPS content of anammox GS was observed, especially after the 55°C shock for 4 h (Chen et al., 2020). It seems reasonable that the sudden change of atmosphere temperature led to the quick response in EPS increase of anammox GS due to the protective mechanisms.

## 6. EPS-based biomaterials and their applications contributing to the circular economy

For practical industrial application, extraction and recovery of enough amount of EPS from sludge samples is the prerequisite. Aerobic granules yield a high ALE content of up to 330 mg/g VSS (Felz et al., 2016; Meng et al., 2019). As calculated, in the Netherlands, the recovered AGS-based ALE is anticipated to achieve 85 kton in the coming decade (Pronk et al., 2015; van Leeuwen et al., 2018). According to the UASB process for a 100,000 inhabitant WWTP (Andreoli et al., 2007), it could yield 150 kg EPS/d (Andreoli et al., 2007). A higher EPS content can be extracted from anammox GS (40% w/w) (Feng et al., 2019; Lotti et al., 2019a). As calculated, the anammox GS-based EPS amounts reached to 185 kg-EPS/d from full-scale partial nitrification/anammox reactors (Lackner et al., 2014). From a circular economy perspective, EPS extracted from waste GS can be considered a resource to be used as far as possible, thanks to their peculiar characteristics. In comparison with the linear economy, the circular economy highlights waste recovery, environmental advantage, and valuation /economic superiority. Several potential applications to recover GS-derived EPS exist, such as paper industry, medical, and construction industry (Kim et al., 2020; van Leeuwen et al., 2018). These industrial solutions of GS-derived EPS, pave the way for further use of “wastes” from WWTP by resource recovery, attributing added-value to sludge, reducing the amount of refuse to be handled and so promoting a perspective shift from wastewater treatment to “Biorefinery” or “Resource Recovery”.

### *6.1 EPS-based hydrogel as coating materials*

GS-based EPS are described to endow the gel formation properties, e.g. AGS-derived ALE (Karakas et al., 2020) and anammox-GS-based hydrogel (Lotti et al., 2019b) to display a rheological performance. The differences of EPS-based hydrogel from AGS and anammox GS consist in the functional components and the gel-forming mechanisms. ALE forms hydrogels with divalent cations solution ($CaCl_2$) at pH 4.5. AGS-derived ALE is composed of neutral sugars, amino sugars, PN, uronic acids, and polyphenolic compounds (Felz et al., 2020c) and somehow resembles standard sodium alginate (Sepúlveda-Mardones et al., 2019). But ALE is different from commercial alginate, characterised by relatively high levels of poly glucuronic acid blocks, up to 69% (Lin et al., 2010). Notably, because of the high complexity of ALE constitutes, gelling mechanism of AGS-ALE can attribute to both ionic cross-linking and interactive reactions of multiple functional groups of structural EPS (Felz et al., 2020a). In contrast, regarding anammox GS-based hydrogel, extracted EPS displayed filming properties without cations addition which is necessary for AGS-derived ALE (Lotti et al., 2019b). Functional amyloid fibrils were observed in TEM images and may be the key component of the complex hydrogel network.

The hydrogel-forming capacity of GS-based EPS implies some potential applications (Karakas et al., 2020; Lotti et al., 2019b). One promising application is used as a raw biomaterial for industrial paper coating to increase the properties of waterproof or grease resistance (Lin et al., 2015; Lotti et al., 2019b). Both AGS-ALE and anammox GS-EPS were homogeneously distributed on papers and proved an obviously-improved water-proof capacity (Lin et al., 2015; Lotti et al., 2019b). Particularly, hydrogel resulted from anammox GS-derived EPS was capable of forming a barrier and acting as a repellent for grease, oil and waxes (Lotti et al., 2019b). The functional groups present in EPS provide abundant binding sites, both hydrophilic and hydrophobic functional groups, which are closely related to the enhanced features. Notwithstanding this, the complex mechanism is still unclear. The roles of PN and other EPS components during coating are less considered and required yet to be determined. With this regard, the research on the extraction of hydrogel-forming exopolymers and understanding physicochemical properties and their interactions with each other or with non-gel-forming EPS is helpful to unveil the EPS roles in both granule formation and their potential application as coating and sizing agents.

### *6.2 Curing of cement*

The curing process is associated with reducing moisture loss from the surface of cement-based materials, which is pivotal in construction engineering (Zlopasa et al., 2014). Retaining cement surface moist is very important to avoid cracking resulted from drying shrinkage. Given the hydrophilic properties and high similarity with alginates, AGS-derived EPS has been applied to improve the curing of cement. Moreover, it has become a commercial material (Karakas et al., 2020). The extension of construction materials' endurance would benefit from the application of eco-friendly and cost-effective EPS-based biomaterials. On the other hand, the use of EPS-based biopolymers could avoid much on-site working load to keep the moisture of concrete, which is time-consuming and needs extra labor investment (Zlopasa et al., 2014). Up to now, only AGS-based EPS was reported to feasibly cure cement in lab-scale tests due to their hydrophilic properties and high similarity with alginates. The reliability of real applications requires to be investigated in the future. Also, more trials are expected to study the protective behavior of different kinds of GS-EPS to construction materials.

### *6.3 Biosorbent materials*

As aforementioned, EPS act as the primary barrier to prevent toxic substances from entering bacterial cells. The extractable EPS products from AGS or anammox GS were found to be able to form hydrogel (Felz et al., 2016; Lin et al., 2013; Lotti et al., 2019b; Seviour et al., 2009b). Hydrogels are types of gel with the swelling (solution adsorption) and de-swelling (solution exuding) characteristics (Shen et al., 2006). Accordingly, GS-derived EPS have been proven to be a cost-effective biosorbent biomaterial for a variety of water treatment. Numerous reports document the capability of GS-derived EPS to bind heavy metal ions ($Ni^{2+}$, $Pb^{2+}$, $Cd^{2+}$) (Guibaud et al., 2012; Li et al., 2017a) or organic pollutants (Suh and Kim, 2000). However, different kinds of granules display distinct removal capacities, e.g., EPS extracted from AnGS possessed a larger contribution to the $Ni^{2+}$ adsorption compared with AGS-EPS (Li et al., 2017a). Similarly, GS-based EPS matrix possesses the capacity of dye pollutant removal, e.g. using AGS-based EPS to remove methylene blue (Wei et al., 2015a). The involved mechanisms for the biosorption process include physisorption (i.e., physical contact and electrostatic attraction), ion-exchange function, the binding sites of EPS, and chemical precipitation (Li et al., 2017a). The primary mechanism attributes to the physicochemical interactions between the adsorbates and functional groups of GS-EPS (Li et al., 2017a; Liu et al., 2015), e.g., the removal of $Pb^{2+}$, $Cd^{2+}$, and $Zn^{2+}$ due to complexation by the functional groups of –COOH and –OH from AGS-derived EPS (Liu et al., 2015); the adsorption of $Cu^{2+}$ due to –COOH of PN from anammox GS-based EPS (Li et al., 2020). It seems amino groups in AGS and AnGS play crucial roles for $Ni^{2+}$ sorption as well (Li et al., 2017a).

GS-based EPS have been demonstrated to endow the capacity of the removal of heavy metals and organic pollutants due to their specific gel-forming properties. It should be noticed that the biosorption effectiveness of EPS largely depends on solution pH, T, conductivity, efficient contacting area/time between EPS and pollutants, EPS sources pollutant structure and concentration (Guibaud et al., 2012). Hence, based on the literature data, a comparison of biosorption capacity of EPS with other biosorbents is still an open research question, due to the variety of biosorbents studied, pollutant considered, experimental techniques used, etc. In addition to the fundamental adsorption behaviour, the performance stability and selectivity in presence of multi-valent cations or organic pollutants with different charges (positive) are seldom addressed. The selectivity may result in the competition between different substances, further inducing the occurrence of unexpected desorption.

### *6.4 Flame retardant materials*

Another advantage would be the reduction of consumptions of halogenated fire retardants. AGS-based EPS were proven to be extinguished bio-based flame retardant materials of flax fabrics due to effective char formation (Kim et al., 2020). Kim et al. (2020) compared Bunsen vertical burning behaviour using two different EPS extracted from AGS and flocculent sludge to cover flax fabric. These two EPS endowed self-extinguishing properties, indicating their feasibility being as coating materials (Kim et al., 2020). Nowadays, halogenated flame retardants cover almost 31% of the market of flame retardants even though they are known as a series of hazardous influences to humans and the environment due to the emissions of poisonous contaminants as dioxins and furans (Kim et al., 2020). With this regard, the EPS extracted from GS could be a nice alternative.

## 7. Conclusions and prospects: where are the bottlenecks?

A better unravelling of the fundamental aspects in terms of EPS composition and the regulation of EPS production has created the foundation to change the critical status of waste sludge management and wastewater treatment in WWTP. Also, it helps to increase sustainable EPS production as value-added biomaterials, causing a technological alternative to sludge management. Meanwhile, many efforts are expected to be dedicated to understanding the functions and properties of complex GS-based exopolymers. Future work can focus on the below aspects:

***EPS-based biomaterials in the circular economy.*** EPS-based materials originating from natural sources are renewable and sustainable, holding the great potential for industrial application. The information on EPS recovery and their conversion into bioproducts with added values gives a unique/new perspective on a less fossil fuel-dependent economy. The investment into capital equipment costs and operational expenses are largely dependent on production scale and extraction methods for EPS production. However, there is still a lack of a comprehensive study to evaluate the whole process in terms of environmental and economic impacts of EPS recovery through life cycle assessments. More work is expected to focus on the industrial applicability, economic and environmental effects during extraction and commercial process of GS-derived biomaterials.

***Identification of complex EPS components.*** The EPS components are far complex, however, one disadvantage of the commonly-used analytical methods (e.g. traditional colorimetric methods) is non-specific. The PN-glycosylation phenomena further increased the difficulty and complexity to depict the glycoconjugates (Felz et al., 2020c). Emerging techniques (e.g., omics, sequencing) are expected to be integrated to analyse biopolymer composition, structural properties, and multiple functionalities. The long period of granule formation and frequent performance fluctuation are typical restricting factors for large-scale application of GS technology (Lin et al. 2020). The mechanisms for EPS synthesis and its contribution to granulation are normally hypothesis-based. Thus, deciphering the EPS generation behaviour during the granulation process should encompass specific attention to the physicochemical structure that can be further exploited in up-cycle product manufacturing.

***EPS extraction and recovery methods.*** The standard for biopolymer extraction should be determined based on different purposes. It must consider whether the traditional standards are applicable to EPS extraction for industrial applications. The considerable yield amounts with an ideal structure are more important than cell lysis. Some GS-EPS components could not be effectively derived according to the traditional extraction protocol (Felz et al., 2020c). Emerging EPS extraction methods should take into account high efficiency, cost-effective, user-friendly and less chemical additives. Besides, purification methods for the extracted EPS are bottlenecks for the current EPS recovery. The optimization protocol for EPS recovery should be adapted based on specific research goals.

***Relationship between different EPS components.*** Most studies investigate the EPS components (e.g. PN and PS) separately. However, the relationship between different fractions is unknown. As far as we know, exopolysaccharides, PN and humic acids in EPS matrix or hydrogel can bind cations, stabilize metal NPs, and be used as sizing reagents due to abundant binding sites. However, information on the interactions of different components of EPS or and other substances (i.e., cations, NP, non-gelling components) are still unclear.

## CRediT author contribution statement

**Cuijie Feng:** Conceptualization, Data curation, Investigation, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Tommaso Lotti:** Investigation, Review & editing. **Roberto Canziani:** Validation, Writing review & editing. **Yuemei Lin:** Validation, Writing review & editing. **Camilla Tagliabue**: Data curation. **Francesca Malpei:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

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## Declaration of interests

All authors declare that there are no competing interests.

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