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Aerobic granular sludge contains Hyaluronic acid-like and sulfated glycosaminoglycans-like polymers

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ABSTRACT

Glycosaminoglycans (GAGs) are linear heteropolysaccharides containing a derivative of an amino sugar. The possibility of the presence of GAGs in aerobic granular sludge was studied by combining SDS-PAGE with Alcian Blue staining (at pH 2.5 and 1), FTIR, mammalian Hyaluronic acid and sulfated GAG analysis kits, enzymatic digestion and specific in situ visualization by Heparin Red and lectin staining. GAGs, including Hyaluronic acid-like and sulfated GAGs-like polymers were found in aerobic granular sludge. The sulfated GAGs-like polymers contained Chondroitin sulfate and Heparan sulfate/Heparin based on their sensitivity to the digestion by Chondroitinase ABC and Heparinase I & III. Heparin Red and lectin staining demonstrated that, the sulfated GAGs-like polymers were not only present in the extracellular matrix, but also filled in the space between the cells inside the microcolonies. The GAGs-like polymers in aerobic granules were different from those produced by pathogenic bacteria but resemble those produced by vertebrates. Findings reported here and in previous studies on granular sludge described in literature indicate that GAGs-like polymers might be widespread in granular sludge/biofilm and contribute to the stability of these systems. The extracellular polymeric substances (EPS) in granular sludge/biofilm are far more complicated than they are currently appreciated. Integrated and multidisciplinary analyses are significantly required to study the EPS.

1. Introduction

The aerobic granular sludge process is a wastewater treatment process which gained increasing popularity as an alternative to the conventional flocculent sludge processes (Pronk et al., 2015). The sludge granules consist of bacteria embedded in a matrix of extracellular polymeric substances (EPS) (Lin et al., 2010; Seviour et al., 2009). The EPS not only provide a structural matrix in which cells can be embedded, but also have various compositions and properties (Flemming and Wingender, 2010).

Recent studies on EPS composition revealed that not only proteins and polysaccharides are the components of EPS, but complex glycoconjugates are present as well. E.g. a highly abundant glycoprotein, carrying a heterogeneous O-glycan structure, was identified in the EPS of anaerobic ammonium oxidation (anammox) granular sludge enriched with Ca. *Brocadia sapporoensis* (Boleij et al., 2018). Glycoproteins were hypothesized to be strongly involved in the structure of anammox granules, having a similar role as glycans in the extracellular matrix (ECM) of multicellular organisms like vertebrates (Boleij et al., 2018; Varki, 2017). In seawater-adapted aerobic granular sludge, sialic acids present as sialoglycoproteins were widely distributed in the EPS. Sialic acids were covering the penultimate galactose unit by this providing stability of the sugar chain (de Graaff et al., 2019). Glycoconjugates have also been found in saline anaerobic granular sludge. The major sugar monomers reported were mannose and N-acetyl galactosamine (Gagliano et al., 2018). The amino sugars galactosamine and glucosamine, the uronic acids glucuronic acid and galacturonic acid and several neutral sugars were identified as monomers in the structural EPS of aerobic granular sludge (Felz et al., 2019). The presence of amino sugars and glucuronic acid, as well as the highly negative charge of structural EPS and their gel-forming ability (Felz et al., 2016) indicate that glycosaminoglycans (GAGs) might be another type of glycoconjugates in the EPS of granular sludge. GAGs are heteropolysaccharides that contain a hexosamine (either glucosamine or galactosamine, generally N-acetylated) and a uronic acid (glucuronic/iduronic acid) or galactose as part of their repeating unit (Esko and Lindahl, 2001; Silbert and Sugumaran, 2002).

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GAGs are building blocks of the ECM in mammalian cells and can be covalently bound to proteins, forming a large and negatively charged proteoglycan (Lindahl et al., 2017; Williams, 1998). By attracting cations and water molecules, these macromolecules form a hydrated gel. Proteoglycans are an important constituent of the connective tissue forming the ground substance which facilitates structural support and enables the diffusion of soluble compounds, e.g. nutrients or signaling molecules, and cell migration within the ECM (Williams, 1998). It is interesting to investigate if there are similar macromolecules present in the EPS of granular sludge. Due to the fact that amino sugars (e.g hexosamines) do not react with phenol-sulfuric acid assay (Felz et al., 2019; Manzi and Esko, 1995), the presence of GAGs might be overlooked by the commonly used colorimetric methods. Thus, it is significantly necessary to establish appropriate methodologies to investigate the possible existence of GAGs in granular sludge or biofilms.

In the current research, it is assumed that there are various GAGs (e.g. Hyaluronic acid, Chondroitin sulfate and Heparan sulfate/Heparin) in the EPS of aerobic granular sludge, which are similar to the polymers forming the ECM in higher organisms. Their possible presence was evaluated by commercial extracellular matrix assays (for use with mammalian cells, tissues and fluids), specific enzymatic digestion and FTIR. The location of highly negatively charged macromolecules (e.g. sulfated GAGs) in granular sludge was visualized by Heparin Red staining.

2. Material and methods

The analysis of GAGs-like polymers (including Hyaluronic acid-like and sulfated GAGs-like polymers) in aerobic granular sludge was summarized in Fig. 1.

2.1. Aerobic granular sludge collection

Aerobic granular sludge was collected from the municipal wastewater treatment plant Dinxperlo in the Netherlands which is operated according to the Nereda® technology. Collected granular sludge was sieved, washed with demi-water, frozen at −80 °C and lyophilized.

2.2. Extraction of structural EPS from aerobic granular sludge and SDS-PAGE (sodium dodecyl sulfate − polyacrylamide gel electrophoresis) analysis

Structural EPS were extracted as described previously (Felz et al., 2019), frozen at −80 °C and lyophilized. The organic and ash fractions were quantified according to the standard methods (APHA, 1998).

Structural EPS were analyzed by SDS-PAGE, as described in Boleij et al. (2018) using NuPage® Novex 4–12% Bis-Tris gels (Invitrogen). The gels were stained by following three different staining protocols. Proteins were visualized with Coomassie Blue staining (Colloidal Blue staining kit, Invitrogen) according to manufacturer's instructions. Glycoprotein staining was performed based on the periodic acid-Schiff (PAS) method using the Thermo Scientific Piercie Glycoproteins Staining Kit, which is specific for glycans containing vicinal hydroxyl groups. For staining of carboxyl and sulfate groups (Shori et al., 2001), Alcian blue staining at pH 2.5 (carboxyl-rich glycoconjugates and/or sulfated glycoconjugates) and pH 1.0 (sulfated glycoconjugates) were performed separately.

2.3. Analysis of Hyaluronic acid content in aerobic granular sludge and the structural EPS

The Hyaluronic acid content in both lyophilized aerobic granular sludge and isolated structural EPS was measured by following the protocol of the Purple-Jelley™ Hyaluronan Assay provided by Biocolor (UK). In brief, the sample (15 mg, dry weight) was digested by proteinase K (0.5 mg/mL) overnight at 55 °C. After centrifugation at 12,000×g for 10 min, the supernatant was collected. Hyaluronic acid in the supernatant was recovered by a sequence of precipitation steps using ethanol saturated with sodium acetate and

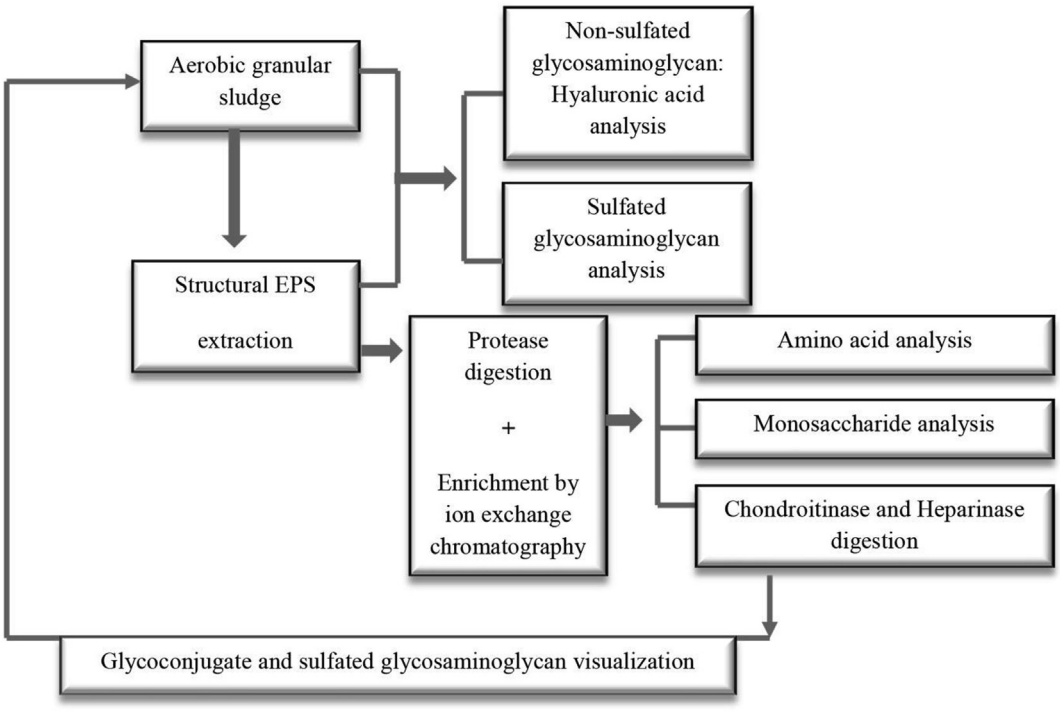

Fig. 1. Schema for analyzing glycosaminoglycans-like polymers in aerobic granular sludge.

cetylpyridinium chloride containing sodium chloride. Afterwards, Hyaluronic acid was precipitated by ethanol (98%) and fully hydrated with 100 μL of water. The extracted Hyaluronic acid gel was quantified by using 3,3′-Diethyl-9-methyl-4,5,4′,5′-dibenzothia carbocyanine bromide supplied in 55% solution of dimethyl sulfoxide.

The Fourier transform infra-red (FTIR) spectrum of the recovered Hyaluronic acid and the Hyaluronic acid standard (extracted from rooster comb) was recorded on a FTIR Spectrometer (Perkin Elmer, Shelton, USA) at room temperature, with a wavenumber range from 600 cm^{-1} – 4000 cm^{-1}.

2.4. Analysis of sulfated GAGs in aerobic granular sludge and structural EPS

The sulfated GAGs content in both lyophilized aerobic granular sludge and structural EPS was measured by following the protocol of the Blyscan™ glycosaminoglycan assay provided by Biocolor (UK). In brief, the sample (1.5 mg, dry weight) was digested by papain extraction reagent overnight at 65 °C. After centrifugation at 10,000×g for 10 min, the supernatant was collected. Total sulfated GAGs were quantified by using the Blyscan™ dye reagent containing 1,9-dimethyl-methylene blue (DMMB) with bovine tracheal Chondroitin-4-sulfate as the standard. In addition, the ratio of O- and N-sulfated GAGs within the test samples was determined by following the nitrous acid cleavage method. Nitrous acid reacts with the N-sulfated D-glucosamine and cleaves the N-sulfated site (Bienkowski and Conrad, 1985). After this reaction, the amount of O-sulfated GAGs was measured again using the Blyscan™ protocol. The difference between the total sulfated GAGs and the amount of O-sulfated GAGs gave the amount of N-sulfated GAGs.

In animal tissue, Hyaluronic acid is a non-sulfated GAG and is not covalently bound to proteins. In contrast, the other GAGs are sulfated and are covalently bound to a protein backbone to form proteoglycans (Lindahl et al., 2017). In order to study if the sulfated GAGs in granular sludge are also covalently bound to proteins, proteinase digestion was performed on structural EPS to remove proteins which are not covalently bound to GAGs.

2.4.1. Proteinase digestion of structural EPS

Structural EPS were digested with papain and proteinase K in sequence. Papain digestion was performed as described in the Blyscan™ protocol (biocolor, UK). Lyophilized structural EPS (200 mg) was digested in 100 mL papain extraction reagent containing 5 mg papain at 65 °C for 24 h 5 mg papain was added afterwards and the mixture was incubated at 65 °C for another 24 h. The mixture was heated to 100 °C for 5 min to deactivate the enzyme, and dialyzed overnight against demineralized water in a dialysis bag (molecular weight cut-off (MWCO) 3.5 kDa).

Subsequently, the dialyzed mixture was further digested by proteinase K according to the manufacturer's instructions with modifications (5 mg proteinase K in 50 mM Tris (pH 8), 4 mM calcium chloride and 10 mM sodium chloride) at 37 °C for 24 h with gentle mixing. After 24 h another 5 mg of proteinase K were added and the digestion continued for 24 h. When the digestion was finished, the mixture was heated to 100 °C for 5 min to deactivate the enzyme, and dialyzed overnight against demineralized water in a dialysis bag (MWCO 3.5 kDa). Consequently, the content inside the dialysis bag was centrifuged at 4,000×g for 20 min and the supernatant was collected and lyophilized.

2.4.2. Enrichment of sulfated GAGs containing polypeptides by ion exchange chromatography

Lyophilized protease digested structural EPS were dissolved in 7 M urea containing 30 mM Tris (pH 8.5) and filtered through a 0.45 μm PVDF filter (Millex®HV Millipore®). The filtrate was separated with DEAE Sepharose® fast flow resin (GE healthcare) in a XK 16 column (GE healthcare) with a column volume of 30 mL using a NGC medium pressure chromatography system (Biorad). The column was washed with three column volumes of 7 M urea containing 30 mM Tris (pH 8.5) prior to introducing the sample. The sample was applied at a flow rate of 3 mL/min. After sample application on the column, the column was washed with 1.5 column volumes of 7 M urea containing 30 mM Tris (pH 8.5). Sample elution was performed with ten column volumes of a sodium chloride gradient from 0 to 2 M. Fractions of 5 mL were collected. Absorbance intensity was measured at 215 nm, 280 nm and 350 nm. After the elution, 50 μL of each fraction was mixed with DMMB reagent to determine the sGAG-like containing fractions (Zheng and Levenston, 2015). Fractions showing precipitation and lower absorbance intensity at both 215 nm and 280 nm which were eluted at sodium chloride concentrations of 0.8–1 M were combined (supplemental material 1), dialyzed against demineralized water in a dialysis bag with 3.5 kDa MWCO, frozen at −80 °C and lyophilized. The reason of collecting these specific fractions is as follows: 1) there is still a signal of the peptide bond which indicates the presence of polypeptides; 2) at the same time the sodium chloride concentration (mainly Cl^-) is high enough to exchange compounds that contain highly negatively charged groups, e.g. sulfated GAGs containing O-sulfate groups and N-sulfate groups. Sulfated GAGs content of the combined fraction was quantified by the Blyscan™ kit (Biocolor, UK).

2.4.3. Monosaccharide and amino acid analysis of the enriched sulfated GAGs containing polypeptides fraction

Monosaccharide analysis of the combined fraction was performed as described previously (Felz et al., 2019). In short, samples were hydrolyzed at a concentration of 10 mg/mL in 1 M hydrochloric acid at 105 °C for 8 h. The hydrolyzed sample was centrifuged at 10,000×g for 5 min and the supernatant was collected. The supernatant was neutralized with 1 M sodium hydroxide, diluted 1:5 with ultrapure water and filtered through a 0.45 μm PVDF filter. The sample was analyzed by a Dionex ICS 5000+ HPAEC-PAD with an AminoTrap pre-column (Dionex) and a PA20 column (Dionex).

The amino acids were analyzed by gas chromatography – mass spectrometry (GC-MS) after acid hydrolysis. Hydrolysis was performed with sample concentrations of 2 mg/mL in 6 M hydrochloric acid at 105 °C for 24 h. After hydrolysis samples were neutralized with 6 M sodium hydroxide and centrifuged at 10,000×g for 5 min. The supernatant was filtered with a 0.45 μm PVDF filter and diluted 1:5 with ultrapure water. Then 100 μL of each diluted sample were mixed with 20 μL of an internal amino acid standard (Wahl et al., 2014) in a GC-vial and lyophilized. Each lyophilized sample was mixed with 75 μL acetonitrile and 75 μL N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), heated for 60 min at 70 °C in a heating block. After cooling down to room temperature, samples were centrifuged at 10,000×g for 2 min. Finally 80 μL of each sample were subsequently analyzed with GC-MS (7890A GC (Agilent) together with a 5975C single quadrupole mass spectrometer (Agilent) as described previously (de Jonge et al., 2011). A sample of 1 μL was injected on a Zebron ZB-50 column (30 m × 250 μm internal diameter, 0.25 μm film thickness; Phenomenex, Torrance, CA, USA) for injection in splitless mode by a programmed temperature vaporizer (PTV; Gerstel, Mühlheim, Germany). Straight glass liners with glass wool were utilized (Agilent). MS was operated in selected ion monitoring mode and the quantification of the amino acids was performed by isotope dilution mass spectrometry.

2.4.4. Enzymatic digestion of the enriched sulfated GAGs containing polypeptides with Chondroitinase ABC and Heparinase I&III

In order to understand if there were Chondroitin sulfate and/or

Heparan sulfate (including Heparin) in the enriched sulfate GAGs, Chondroitinase ABC from *Proteus vulgaris* (Sigma-Aldrich) and a blend of Heparinase I&III from *Flavobacterium heparinum* (Sigma-Aldrich) were used, respectively for digestion (Sigma-Aldrich, 2007). The specificity of the enzymes is described in supplemental material 2. Samples (1 mg/mL) were digested according to manufacturer's instructions, with Chondroitinase ABC (0.1 mg/mL) at 37 °C and pH 8 in 0.01% (w/v) bovine serum albumin (BSA), 50 mM Tris and 60 mM sodium acetate for 24 h. In parallel, samples (1 mg/mL) were digested with Heparinase I&III (0.1 mg/mL) at 25 °C and pH 7.5 in 0.01% (w/v) BSA, 20 mM Tris, 50 mM sodium chloride and 4 mM calcium chloride for 24 h. The amount of sulfated GAGs in the samples after digestion was quantified by using the Blyscan™ glycosaminoglycan assay (Biocolor, UK).

2.5. Visualization of highly negatively charged macromolecules (e.g. Heparan sulfate/Heparin) and other glycoconjugates in aerobic granular sludge

Highly negatively charged macromolecules (e.g. Heparan sulfate/Heparin) were localized in the granular matrix by staining with the fluorescent probe Heparin Red (RedProbes, Münster, Germany). Staining using Heparin Red was done according to the supplier's data sheet. Briefly, 8.8 μL Heparin Red and 1 mL enhancer solution were mixed and added to the granules for 1 h. Other glycoconjugates were examined on the basis of fluorescence lectin barcoding with subsequent fluorescence lectin-binding analysis (Neu and Kuhlicke, 2017). For this purpose all commercially available lectins (FITC, Fluorescein, Alexa488) were screened and individually tested for binding. Granules were stained with lectins (0.1 mg/mL) for 20 min at room temperature in the dark and washed 3 times in order to remove the unbound probe. For both stainings the granules were washed with tap water and mounted in coverwell chambers (Thermofisher) with various spacers (1, 1.7, 2 mm) to keep the original shape of the granule. The samples were examined at a Leica SP5X instrument (Leica Germany) equipped with an upright microscope and a super continuum light source. The microscope was controlled by the LAS AF software version 2.4.1. Confocal images were recorded as single scan or serial scan (step size 1 or 0.5 μm) using a 25x NA 0.95 or a 63x NA 1.2 water immersion lens. Laser excitation was at 480 nm and 567 nm, emission was from 470 to 490 nm (reflection) and 590–650 nm (Heparin Red). For lectins, laser excitation was at 490 nm, emission was from 485 to 495 nm (reflection) and 505–600 nm (lectins). Images were collected with different zoom factors to either get an overview or to match optical and pixel-resolution. In order to optimize image resolution and contrast, the lectin image datasets were subjected to blind deconvolution with Huygens version 18.10.0 (SVI, The Netherlands). Data were finally projected using Imaris version 9.2.1. Heparin Red data sets were loaded in Fiji (https://fiji.sc/) and color coded with the lookup table called "rainbow". For improved color separation of pixel intensities the contrast was set to auto. All image data sets were printed from Photoshop (Adobe).

3. Results

3.1. Structural EPS extraction from aerobic granular sludge and analysis

The structural EPS extracted from aerobic granular sludge represented 253 ± 14 mg/g volatile content of the sludge. The glycoconjugates in the extracted EPS were further characterized using SDS-PAGE in combination with different staining protocols. Following Coomassie Blue staining, a smear was observed with the molecular weight range from 50 kDa to 10 kDa (Fig. 2, lane 1), no clear bands could be seen. Alcian Blue staining was applied with pH 2.5 and pH 1.0 (Fig. 2 lane 2 and 3). At pH 2.5, both carboxyl-rich glycoconjugates ($-COO^-$) and sulfated glycoconjugates ($-SO_3^-$) were stained, while with pH 1.0 only the sulfated glycoconjugates were stained (Shori et al., 2001). A smear appeared at the high molecular weight range (above 235 kDa) with decreasing intensity until 70 kDa at both pH 2.5 and pH 1, with the intensity of the smear at pH 2.5 higher than that at pH 1, implying there are both carboxyl-rich glycoconjugates and sulfated glycoconjugates in the structural EPS. In addition, part of the structural EPS were retained in the wells. It was strongly stained by Coomassie Blue, PAS and Alcian blue (both at pH 2.5 and 1), indicating that it contained proteins/polypeptides, carboxyl-rich glycoconjugates and sulfated glycoconjugates as well.

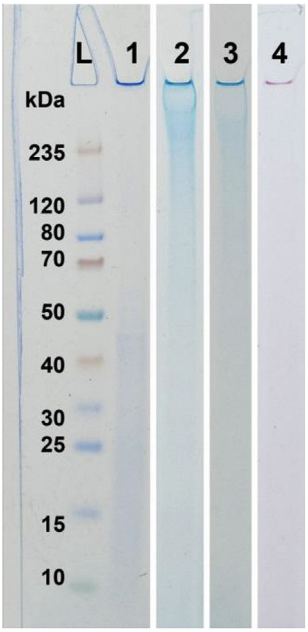

Fig. 2. SDS-PAGE analysis of structural EPS. Visualization of proteins and glycoconjugates. Structural EPS was stained with Coomassie blue (1), Alcian blue at pH 2.5 (2), Alcian blue at pH 1 (3) and PAS (4). The size distribution is illustrated by a broad band ladder (L) of proteins ranging from 10 to 235 kDa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

3.2. Hyaluronic acid content in aerobic granular sludge and extracted structural EPS

The lyophilized granular sludge as well as the extracted structural EPS was treated according to the protocol provided in the commercial kit for Hyaluronic acid (HA) extraction. The extracted polymers turned purple when reacted with 3,3′-Diethyl-9-methyl-4,5,4′,5′-dibenzothia carbocyanine bromide, which is the standard stain to indicate the presence of HA. Based on the calibration curve with the commercial HA standard provided in the kit, the HA content in aerobic granular sludge was determined as 294 ± 22 μg/g. In structural EPS the HA content was much lower with 15 ± 6 μg/g.

In addition, the FTIR spectra of the extracted HA from granular sludge (HA extracted from EPS was not analyzed by FTIR due to its low amount) and the commercial standard were compared. As shown in Fig. 3, they were similar in: The peak at 3447 cm^{-1} and 3150 cm^{-1} are attributed to -NH and -OH stretching region. The peak at 2925 cm^{-1} can be attributed to stretching vibration of -CH. The peak at about 1580 cm^{-1} corresponds to the amide carbonyl of

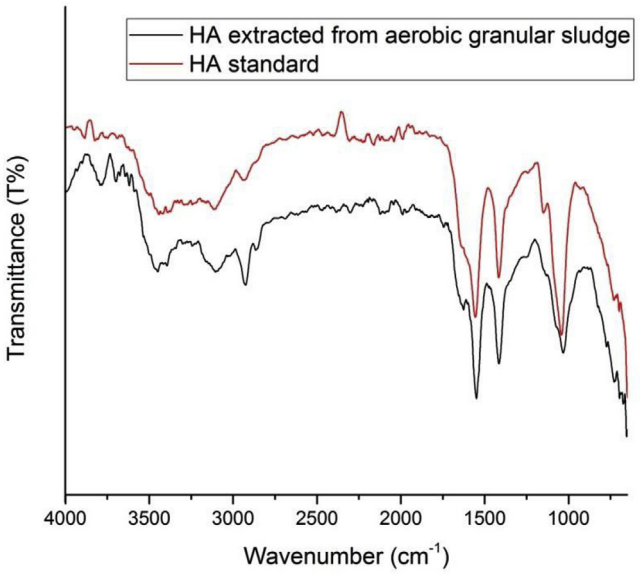

Fig. 3. FTIR spectrum of the extracted Hyaluronic acid (HA) from aerobic granular sludge in comparison to the commercial standard. The two spectra were similar, especially in the wavenumber region 1750 cm^{-1} to 750 cm^{-1}.

amino sugar and the peak at 1420 cm^{-1} is the stretching of COO^-, which refers to the acid group of HA. The peak at 1028 cm^{-1} is attributed to the linkage stretching of -C-OH (de Oliveira et al., 2017). The peaks at around 3800 cm^{-1} and 2750 cm^{-1} which only present in the spectrum of the polymer extracted from granular sludge are probably due to impurities. Therefore, based on the chemical reaction and the FTIR spectrum, it is confirmed that there is Hyaluronic acid-like polymer in aerobic granular sludge.

Hyaluronic acid contains glucuronic acid and N-acetyl-glucosamine as repeating units (de Oliveira et al., 2017). It is reported to be synthesized as an extracellular capsule by a few pathogenic bacteria (e.g. Lancefield group A and C *streptococci*) (Chong et al., 2005). The yield of Hyaluronic acid through fermentation of group C *streptococci* can reach to 0.3 g/g dry weight (Chong et al., 2005). In comparison, the amount of Hyaluronic acid in human extracellular matrix is variable, approximately 15–150 μg/g in lung tissue, 500–2500 μg/g in articular cartilage and 300–500 μg/g in skin (Cowman et al., 2015; Kuo, 2006; Piehl-Aulin et al., 2017). The amount of Hyaluronic acid-like polymer in aerobic granular sludge is comparable with that in the human skin.

3.3. Sulfated GAGs content in aerobic granular sludge and extracted structural EPS

Based on the reaction with the dye 1,9-dimethylmethyene blue (DMMB), the total sulfated GAGs in aerobic granular sludge was determined as 31 ± 5 mg/g in aerobic granular sludge and 87 ± 6 mg/g in structural EPS (Chondroitin-4-sulfate as the standard). If the yield of structural EPS (253 ± 14 mg/g) is considered, the extracted structural EPS contains the major part (approximately 71%) of the total sulfated GAGs in aerobic granular sludge. It is known that, there are five different kinds of sulfated GAGs in mammals: Chondroitin sulfate, Keratan sulfate and Dermatan sulfate which contain O-sulfated hexosamines; Heparin and Heparan sulfate which contain N-sulfated hexosamines. Thus, by determining the ratio between O- and N-sulfated GAGs within test samples, it is possible to detect the presence of Heparin and Heparan sulfate. The ratio between O- and N-sulfated GAGs in granular sludge and in the extracted structural EPS is listed in Table 1. It seems that the major fraction is O- sulfated GAGs, with roughly 10% N-sulfated GAGs in both aerobic granular sludge and the extracted structural EPS.

Similar with Hyaluronic acid, Chondroitin and Heparosan (also called unsulfated Heparin) can be produced by a few pathogenic bacteria (*E.coli* K4, *E.coli* K5, *Pasteurella multoccida* Type F and Type D, *Avibacterium paragallinarum*) (DeAngelis, 2012). However, those microbial Chondroitin and Heparosan are not sulfated. Moreover, neither of them are covalently bound to proteins. In comparison, the Chondroitin and Heparin in mammals are sulfated and are covalently bound to proteins (DeAngelis, 2012).

Aerobic granular sludge and the structural EPS contain sulfated GAGs, which are different from the GAGs produced by pathogenic bacteria reported in literatures. To further understand if these sulfated GAGs are covalently bound to proteins, two different proteases were applied to digest the proteins in the extracted structural EPS in sequence. Nevertheless, after such intense protease digestion, there was still absorbance at both 215 nm and 280 nm in the ion exchange chromatogram (supplemental material 1). Absorbance at both 215 nm and 280 nm are considered as the absorbance of carboxylate groups and aromatic amino acids, respectively. This indicates that there are still polypeptides in the sample, even in those fractions that were eluted out with high concentration of sodium chloride (0.8–1 M). In fact, these fractions were supposed to be highly negatively charged compounds such as sulfated GAGs. To investigate if the sulfated GAGs are bound to polypeptides, the fractions which were eluted out at sodium chloride concentration of 0.8–1 M were collected and combined as one fraction. Its total sulfated GAGs content was determined as 264 ± 5 mg/g (with 77% of O-sulfated GAGs and 24% of N-sulfated GAGs, as shown in Table 1). Therefore, this fraction was considered as enriched sulfated GAGs-containing polypeptides.

Further analysis of the sugar monomers and amino acids provided details of the monosaccharides and amino acids of the enriched sulfated GAGs-containing polypeptides. The detected monosaccharides were: fucose, galactose, glucose, mannose, rhamnose, xylose, galactosamine, glucosamine, galacturonic acid and glucuronic acid. The detected amino acids were: alanine, glycine, isoleucine, leucine, phenylalanine, proline, valine, serine, threonine, tyrosine, aspartate, glutamate, lysine and histidine. Interestingly, the weight percentage of the total amino acids decreased from 22.7% (w/w) in structural EPS to 1.5% (w/w) in the enriched polypeptides fraction, but amino acids could not be

Table 1
The amount of total sulfated GAGs and the ratio between O-sulfated GAGs/N-sulfated GAGs in aerobic granular sludge, structural EPS and the enriched sulfated-GAGs-containing polypeptide.

	Total sulfated GAGs (mg/g) with Chondroitin-4-sulfate as the standard	O-sulfated GAGs (% of the total sulfated GAGs)	N-sulfated GAGs (% of the total sulfated GAGs)	Total amino acids (% w/w)
Aerobic granular sludge	31 ± 5	90 ± 4	10 ± 3	N.A.
Structural EPS	87 ± 6	89 ± 3	11 ± 3	22.7
Enriched sulfated-GAGs-containing polypeptides fraction	264 ± 5	77 ± 2	24 ± 2	1.5

entirely removed by the intense protease digestion. Apparently, with both complex sugar monomers and amino acids existing together, the enriched sulfated GAGs-containing polypeptides are glycopolypeptides.

Therefore, different from the GAGs produced by pathogenic bacteria, the GAGs (except for Hyaluronic acid-like polymers) in aerobic granular sludge were both sulfated and covalently bound to polypeptides.

3.4. Enzymatic digestion of the enriched sulfated GAGs-containing polypeptides fraction

In order to study if there was Chondroitin sulfate and Heparan sulfate/Heparin in the enriched sulfated GAGs-containing polypeptide fraction, the sample was digested with Heparinase I & III and Chondroitinase ABC. After enzymatic digestion, the amount of total sulfated GAGs was decreased by 54% and 43%, respectively, indicating the enriched fraction was sensitive to the digestion with Heparinase I & III and Chondroitinase ABC. Unfortunately, disaccharides were not detected by the follow-up HILIC-MS analysis (data not shown) after the enzymatic digestion, which was different from the property of the Chondroitin sulfate and Heparan sulfate/Heparin from mammals. The reason could be: there are hydrolysis sites which can be recognized by those enzymes (recognition sites of these two enzymes are provided in supplemental material 2), but disaccharides may not be the final product of the enzymatic digestion of the enriched glycopolypeptides fraction. Therefore, there were sulfated GAGs-like polymers, such as Chondroitin sulfate-like and Heparan sulfate/Heparin-like polymers in aerobic granular sludge.

3.5. Visualization of the location of sulfated GAGs-like polymers and other glycoconjugates in aerobic granular sludge

The location of the sulfated GAGs-like polymers in the granules was of interest in order to illustrate their spatial distribution and to understand their function. The visualization was conducted by Heparin Red staining of the whole granule (Fig. 4).

Heparin Red is a fluorescent molecular probe. It is used for direct detection of Heparins in blood plasma. Heparin Red is a polycationic probe. Once there are polyanionic macromolecules (e.g. Heparin), the Heparin Red probe forms supramolecular complex aggregation at the polyanionic template (Fig. 4). In this way, it is possible to visualize the location of the polyanionic macromolecules based on the fluorescence of the probe. Moreover, it is important to note that, the fluorescence intensity of Heparin Red probe is quenched once it forms stable aggregates on the polyanionic chains. Thus, opposite to the normal fluorescence stains, using Heparin Red, a low signal intensity demonstrates a strong binding of the probe (Warttinger et al., 2016a).

As shown in Fig. 5, the distribution of negatively charged polymers in aerobic granular sludge can be mapped by Heparin Red staining. The Heparin Red (HR) images are presented using a look up table in which the pixel intensities are color coded as: very low pixel intensities in blue – no binding of HR, high pixel intensities in red – binding of HR and intermediate pixel intensities in green – strong binding of HR due to quenching. According to literature, the fluorescent signal of Heparin Red is quenched when the negative charge density of the polyanionic macromolecules is higher than −0.81 per monosaccharide (Warttinger et al., 2016a, 2016b). Above this threshold, the higher the negative charge intensity, the lower the fluorescent intensity. For example, the charge density of Chondroitin sulfate in bloodplasma is −0.70 per monosaccharide, while the charge density of Heparin is −1.7 per monosaccharide and Heparan sulfate −1 per monosaccharide (da Costa et al., 2017; Warttinger et al., 2016a). Heparin Red does not bind Chondroitin sulfate in bloodplasma but Heparin and Heparan sulfate, with Heparin resulting in the lower fluorescent intensity (Warttinger et al., 2016b). Therefore, the green colour region in Fig. 5 is where the charge density of the polyanionic macromolucles is higher than −0.81 per monosaccharide; the lower the fluorescent

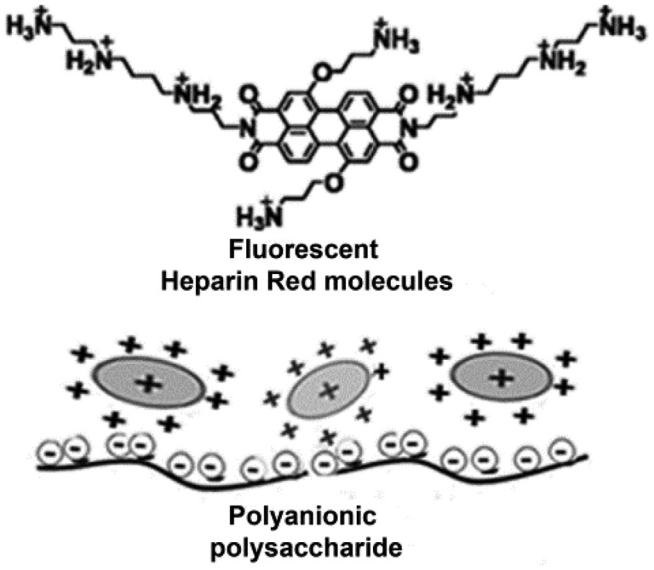

Fig. 4. Molecular structure of the polycationic fluorescent probe Heparin Red (Warttinger et al., 2016a) and Schematic representation of forming aggregation on polyanionic polysaccharides chains.

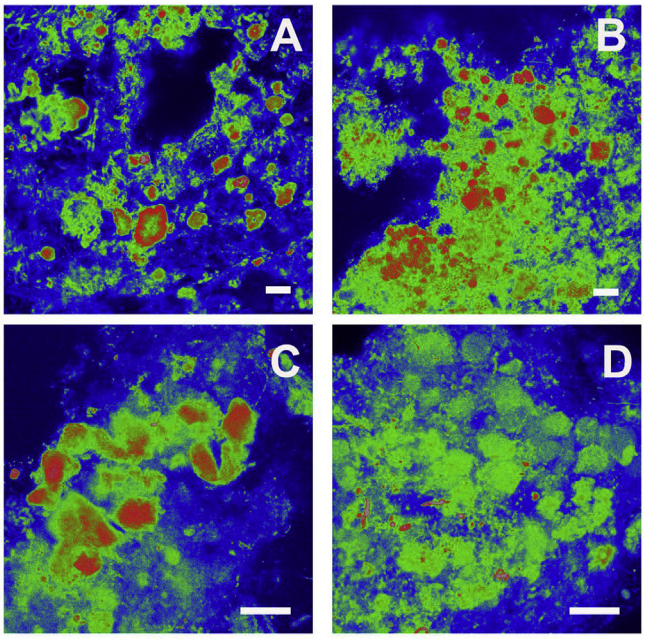

Fig. 5. Distribution of negatively charged polymers in aerobic granular sludge based on Heparin Red staining. Blue: no binding of Heparin Red. Red: Binding of Heparin Red. Green: Strong binding of Heparin Red due to quenching, implying high negative charge density. Scale bars = 10 μm. A and B show the typical globular sub-structures of the granules with areas of low and high binding of Heparin Red, respectively. C and D show that the polymers with higher negative charge density are at the outer boundary of the microcolonies and also in the extracellular matrix in between the colonies. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

intensity, the higher the charge density.

It was observed that the negatively charged macromolecules were not only present in the microcolonies, where the typical globular structures with the size around 10–20 μm can be identified (red), but at the outer boundary of the microcolonies and also in the extracellular matrix in between the colonies (green) (Fig. 5 A, B and C). In the microcolonies, the bacterial cells are not visible, indicating Heparin Red rather stained the capsular region or the space in between capsules (Fig. 5 C and D). On top of that, since the lower the intensity of the signal is, the higher the negative charge density is, the differences in brightness indicated various negative charge levels of the macromolecules. Fig. 5D was representative for areas showing microcolonies with a high degree of quenching (green). As a result, signal intensity and resolution appeared to be rather low, but indicated a strong binding of the Heparin Red probe and a high level of negative charge density.

Furthermore, as GAGs contain a hexosamine (either N-acetyl-glucosamine or N-acetyl- galactosamine) as part of their repeating structure, the locations of glycoconjugates with N-acetyl glucosamine or N-acetyl galactosamine were visualized by staining with different lectins (Fig. 6). It was observed that the hexosamine-containing glycoconjugates were located in the microcolonies (Fig. 6A and 6E) as well as in the extracellular matrix (Fig. 6D and 6F). In the microcolonies, the bacterial cells were not visible, indicating those hexosamine-containing glycoconjugates rather stay in the capsular region or the space in between capsules, which is in line with the result from Heparin Red staining (Fig. 5D).

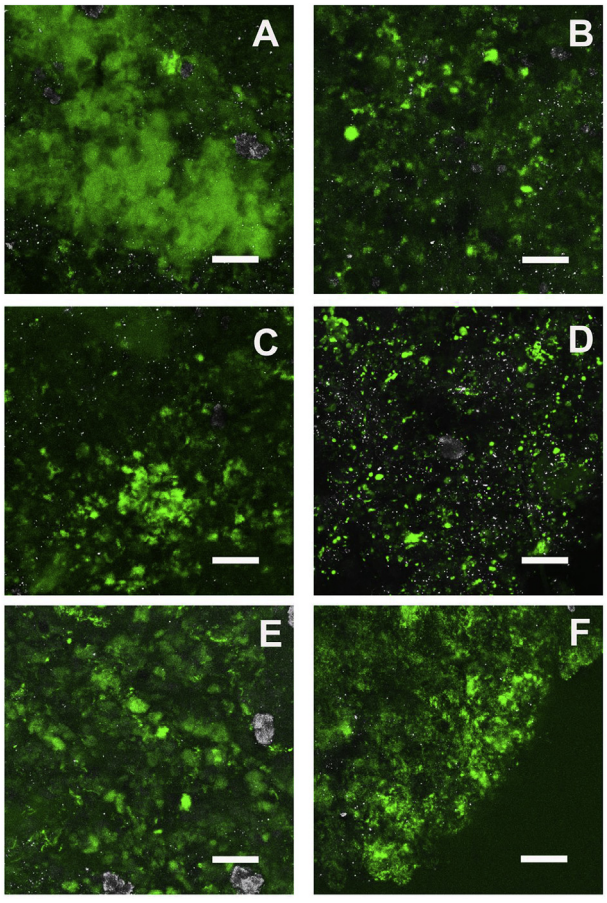

Fig. 6. Confocal laser scanning microscopy showing maximum intensity projections of aerobic granular sludge data sets. Glycoconjugates were stained with N-acetyl hexosamine specific lectins. The different lectins are, A: STA, B: SJA, C: PHA-L, D: LEA, E: ABA, F: SSA. Scale bars = 10 μm. The binding specificity of lectins are listed in Supplemental material 3. Color allocation: lectin signal – green, reflection signal – grey. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

4. Discussion

4.1. GAGs-like polymers in aerobic granular sludge

In the current research, it was confirmed that there were GAGs-like polymers in aerobic granular sludge, such as Hyaluronic acid-like, Chondroitin sulfate-like and Heparan sulfate/Heparin-like polymers. Different from those produced by pathogenic bacteria, the Chondroitin- and Heparan/Heparin-like polymers in aerobic granules were sulfated and covalently bound to polypeptides. Sulfated glycoconjugates were reported in a few EPS related studies in different granular sludge: In anaerobic granular sludge, high molecular weight proteoglycan-like and sulfated proteoglycan-like substance were found in the Bound-EPS (Bourven et al., 2015). In the extracted structural EPS of anammox granular sludge, a glycoprotein with the molecular weight at around 12 kDa contained neutral sugar, carboxyl and sulfated glycoconjugates (Boleij et al., 2018). In aerobic granular sludge enriched with ammonium-oxidizing bacteria, there were glycosylated amyloid-like proteins which have glycocconjugates of neutral sugar, carboxyl groups and sulfate groups in the structural EPS (Lin et al., 2018). The presence of both carboxyl and sulfate groups in those EPSs implies the possible existence of sulfated GAGs-like polymers. Moreover, all those reported glycoconjugates were linked with proteins. Thus, one could reasonably assume that “the existence of sulfated GAGs-like polymers (e.g. Chondroitin sulfate and Heparan sulfate/Heparin) covalently bound to proteins” might be a common phenomenon in granular sludge/biofilms.

4.2. Potential role of GAGs-like polymers in granular sludge

It is interesting to look at the distribution of GAGs in nature:

The microbial production of GAGs has only been studied in certain pathogenic bacteria. The GAGs produced by *Streptococcus, Escherichia* and *Pasteurella* are similar or identical to the backbone of vertebrate polymers, thus the immune system of higher organisms fails to recognize the capsules from those pathogens as a foreign entity, and consequently no antibodies are formed to kill the microbes (Whitfield and Robert, 1999). Basically, the GAGs containing capsule is a camouflage. The GAGs produced by those pathogenic bacteria are neither sulfated nor covalently bound to proteins (DeAngelis, 2002).

Fresh water and land plants do not contain any sulfated glycoconjugates extracellularly. Marine macroalgae produce GAG mimetics (sulfated polysaccharides consist of only neutral sugars instead of hexosamine and uronic acids). These GAG mimetics are not covalently bound to proteins (Vasconcelos and Pomin, 2017).

Invertebrates generally produce the same types of GAGs as vertebrates, except that Hyaluronic acid is not present and the Chondroitin chains tend to be non-sulfated (Lindahl et al., 2017). In comparison, vertebrate cells produce GAGs and secrete them into the ECM or incorporate them into the plasma membrane (Alberts and Johnson, 2002). GAGs are crucial for the pericellular space in which they can define physical properties of tissues or adjust biological functions of cells (da Costa et al., 2017). Within the properties of GAGs, the negative charge – essential to all GAGs – is paramount. In general, the negative charge of GAGs is linked to the sulfate groups. The only exception is Hyaluronic acid. Here the negative charge is only originating from glucuronic acid (da Costa et al., 2017).

The GAGs in the ECM of animals are able to attract cations and to bind water molecules (Prydz, 2015). Hydrated GAG gels have long

been known to play an important role in the absorption of pressure changes in joints and tissues. Proteoglycans with its sulfated GAGs in the extracellular matrix were discovered to influence cellular signaling, largely through electrostatic interactions with charged proteins such as growth factor, morphogens, and other chemokines, acting as a molecular sponge to embed those proteins (Gandhi and Mancera, 2008; Lim and Temenoff, 2013; Prydz, 2015).

Looking at the evolution of GAGs, it seems that the GAGs-like polymers in aerobic granular sludge resemble vertebrates' GAGs due to the presence of Hyaluronic acid-like polymers, the sulfation and the covalent bond with proteins. At this point, it is logical to speculate that, the function of GAGs-like polymers in granules is similar as that in vertebrates, such as attracting cations, forming a hydrogel, binding proteins and signaling in biological processes. Further research should focus on finding out the important functions of GAGs-like polymers in granular sludge/biofilm.

4.3. Development of proper methodologies to analyze GAGs-like polymers in granular sludge/biofilm

GAGs are one of the major components in mammalian extracellular matrix. GAGs-like polymers were found in aerobic granular sludge by combining various analytical approaches: SDS-PAGE with Alcian Blue staining (at pH 2.5 and 1), FTIR, commercial extracellular matrix assays (for use with mammalian cells), monosaccharide and amino acid analysis, enzymatic digestion and specific in situ visualization by Heparin Red fluorescent probe and lectin staining (Fig. 1). The resemblance to vertebrate GAGs implies that, the EPS in granular sludge/biofilm is far more complicated than it is currently appreciated. Thus, appropriate methodologies are needed, aiming at studying EPS components based on their important functions, rather than superficially measuring the relative amount of proteins and polysaccharides. On top of that, as it is gradually clear that protein glycosylation is a widespread phenomenon in EPS, the complexity of glycoconjugates imparts EPS not only various functions but also complicates in the analysis. For example, sulfated GAGs can have variable sulfation patterns. Possible structural variations of sulfated GAGs result in GAGs being one of the most complex groups of macromolecules discovered in nature (Zamfir et al., 2011). It is reported that GAGs' activity is dictated by sulfation patterns (Gama et al., 2006; Tully et al., 2004). Thus, obtaining the detailed molecular structure and the sulfation pattern of the GAGs-like polymers in granular sludge/biofilm will facilitate the understanding of EPS functionality and stability. At this aspect, Integrated and multi-disciplinary analyses such as MS, NMR and metagenomic analysis (Seviour et al., 2019) are significantly required.

5. Conclusion

- Besides pathogenic bacteria, microorganisms in aerobic granular sludge produce Hyaluronic acid-like and sulfated GAGs-like polymers in the extracellular matrix.
- The GAGs-like polymers in aerobic granules are different from those produced by pathogenic bacteria but resemble those produced by vertebrates: there are both Hyaluronic acid-like polymers and sulfated GAGs-like polymers; further the sulfated GAGs-like polymers are bound to proteins.
- The EPS in granular sludge/biofilm is far more complicated than it is currently appreciated. Integrated and multidisciplinary analyses are significantly required.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115291.

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