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Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge

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ABSTRACT

Sialic acids have been discovered in the extracellular polymeric substances (EPS) of seawater-adapted aerobic granular sludge (AGS). Sialic acids are a group of monosaccharides with a nine-carbon backbone, commonly found in mammalian cells and pathogenic bacteria, and frequently described to protect EPS molecules and cells from attack by proteases or glycosidases. In order to further understand the role of these compounds in AGS, lectin staining, genome analysis of the dominant bacterial species, and shielding tests were done. Fluorescence lectin bar-coding (FLBC) analysis showed an overlap with protein staining, indicating presence of sialoglycoproteins in the EPS matrix. Genome analysis gives a positive indication for putative production of sialic acids by the dominant bacteria *Candidatus Accumulibacter*. FT-IR analysis shows upon selective removal of sialic acids a decrease in carbohydrates, extension of the protein side chain, and exposure of penultimate sugars. Enzymatic removal of sialic acids results in the removal of galactose residues from the EPS upon subsequent treatment with β-galactosidase, indicating a linkage between galactose and sialic acid at the terminus of glycan chains. This work indicates the importance of sialic acids in the protection of penultimate sugar residues of glycoproteins in EPS, and provides basis for future research in the composition of EPS from biofilms and granular sludge.

1. Introduction

Aerobic granular sludge (AGS) is an upcoming technology for wastewater treatment, capable of simultaneously removing organic carbon, nitrogen, and phosphorus in a single process unit (De Kreuk et al., 2005a; Yilmaz et al., 2008; Pronk et al., 2015). The sludge granules consist of bacteria embedded in a matrix of extracellular polymeric substances (EPS) (Seviour et al., 2009; Lin et al., 2010). Besides providing a structural matrix in which cells can grow, EPS also serve as a protection against adverse conditions in the bulk liquid (Sutherland et al., 2001; Wang et al., 2007). EPS are found in all kinds of granular sludge, and a multitude of properties and compositions have been described as a result of different operating conditions (McSwain et al., 2005; Sheng et al., 2010).

The application of AGS technology in saline wastewater treatment has been reported before (Figueroa et al., 2008; Pronk et al., 2014; Corsino et al., 2016). Saline wastewater originates from many sources such as sea water (either due to intrusion in sewer networks or due to application of seawater flushing), industrial wastewaters such as from fish canning industries, effluent from mining and mineral processing industries (Lefebvre and Moletta, 2006). Adaptation of AGS to high salinity leads to a change in EPS composition, e.g. higher protein fractions have been reported (Corsino et al., 2017), as well as an increase in hydrophobicity (Wang et al., 2015). Higher concentrations of EPS have been found as a response to growth in saline conditions (Campo et al., 2018). These studies have mainly focused on quantification of carbohydrates and proteins in the EPS by using colorimetric methods. It is noted that not all carbohydrates can be measured with those most commonly used methods. For instance, sialic acids have a low response factor for colorimetric methods such as anthrone assay and phenol sulfuric acid assay, so these sugars cannot be detected and quantified by these methods (Masuko et al., 2005; Le Parc et al., 2014).

Sialic acids are a group of special carbohydrates with a nine-carbon backbone. The most common member is N-

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acetylneuraminic acid (Neu5Ac), which has been observed on the surface of both mammalian cells and pathogenic bacteria (Severi et al., 2007). Sialic acids play important role in the stabilization of molecules and membranes, as well as in modulating interactions with the environment. They can also protect molecules and cells from attack by proteases or glycosidases, extending their lifetime and function (Varki and Schauer, 2009). Information of sialic acids' presence in the EPS of aerobic granules and their function in AGS is significantly important to understand their role in maintaining the stability of granular sludge.

Since sialic acids might be overlooked due to the detection limitation of commonly used colorimetric methods, more effective methods to study EPS components and their specific function are required to provide greater insight into the nature of AGS.

One of the proven effective methods for EPS glycoconjugates analysis is fluorescence lectin bar-coding (FLBC) (Neu and Kuhlicke, 2017). These lectins can bind to specific carbohydrate regions, allowing for screening of glycoconjugates in a hydrated biofilm matrix. This method has been successfully applied for analysis of saline anaerobic granular sludge (Gagliano et al., 2018). Carbohydrates were found to be present as glycoconjugates, with sugar residues including mannose and N-acetyl-galactosamine. Other studies described mannosyl, glucosyl, and N-acetyl-glucosamine residues in anaerobic granular sludge (Bourven et al., 2015). Interestingly, these sugars are often in conjunction with sialic acids, which is similar as commonly found glycoconjugates in the mucin matrix in animals, (Hilkens et al., 1992; Bennett et al., 2012). Lectin staining studies for sialic acids have been described in biofilms before, but a detailed study on their position and function has not yet been conducted (Mlouka et al., 2016).

In this study, glycoconjugates within EPS from seawater-adapted AGS were analysed using lectin staining methods. A genome analysis was performed on the major species that were found in this sludge, to screen for putative production pathways for sialic acids. Selective removal of sugar residues from whole granules have been performed to give insight into the function of sialic acids in granular sludge. A discussion is added about the evolutionary benefit for producing sialic acids in a granular sludge system.

2. Materials & methods

2.1. Reactor operation and dominant microorganisms

2.1.1. Reactor operation

Seawater-adapted aerobic granular sludge was cultivated in a 2.7 L bubble column (5.6 cm diameter), operated as a sequencing batch reactor (SBR). The reactor was inoculated with Nereda® sludge from wwtp Utrecht, the Netherlands, treating municipal wastewater. The temperature was controlled at 20 °C. The pH was controlled at 7.0 ± 0.1 by dosing either 1 M NaOH or 1 M HCl. The dissolved oxygen (DO) concentration was controlled at 3.7 mg/L O_2 (50% saturation). The average sludge retention time (SRT) was 20 ± 2 days. Reactor cycles consisted of 60 min of anaerobic feeding, 170 min aeration, 5 min settling and 5 min effluent withdrawal. The feed of 1.5 L per cycle consisted of 1200 mL of artificial seawater (Instant Ocean®, final concentration 35 g/L), 150 mL of medium A, and 150 mL of medium B. Medium A contained 57.2 mM sodium acetate trihydrate. Medium B contained 42.8 mM NH_4Cl, 4.2 mM K_2HPO_4, 2.1 mM KH_2PO_4, and 10 mL/L trace elements solution (Vishniac and Santer, 1957). The combination of these feed streams led to influent concentrations of 366 mg/L COD, 60 mg/L NH_4^+-N and 9.3 mg/L PO_4^{3-}-P. Electrical conductivity of the influent equaled 40 μS cm^{-1}.

Samples were taken from the reactor every 30 min during aeration, and filtered through a 0.45 μm PVDF filter. Acetate and phosphate concentrations were measured by using a Thermo Fisher Gallery Discrete Analyzer (Thermos Fisher Scientific, Waltham, USA).

2.1.2. Dominant microorganisms visualization by fluorescence in situ hybridization (FISH)

The handling, fixation and staining of FISH samples was performed as described in Bassin et al. (2011). A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) were used for visualizing polyphosphate accumulating organisms (PAO) (Crocetti et al., 2000). A mixture of GAOQ431 and GAOQ989 probes (GAOmix) were used for visualizing glycogen accumulating organisms (GAO) (Crocetti et al., 2002). A mixture of EUB338, EUB338-II and EUB338-III probes were used for staining all bacteria (Amann et al., 1990; Daims et al., 1999). Images were taken with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 5515e565) for Cy5, Cy3 and fluos respectively.

2.2. Sialic acid visualization and quantification

2.2.1. Sialic acid lectin staining

The granules were stained and mounted in coverwell chambers with a 1 mm spacer in order to avoid squeezing of the samples. Glycoconjugates of the granules were examined by means of fluorescence lectin bar-coding. Thus all commercially available lectins (FITC or Alexa488) were applied as an individual probe to one granule. After this glycoconjugate screening, granules were stained specifically for proteins and subsequently with sialic acid specific lectins. Protein stain solution (Sypro red) was directly put on top of the granules. After 3 h' incubation, the sample was washed two times with tap water, and stained with sialic acid specific lectin solution for 1 h. A total of 77 lectins were used for screening of glycoconjugates (Bennke et al., 2013). The binding sites of the lectins that gave the strongest signal are listed in Table 1. Then the sample was washed with tap water for three times. For 3D imaging a TCS SP5X confocal laser scanning microscope (Leica, Germany) was employed. The upright microscope was equipped with a super continuum light source and controlled by the software LAS AF 2.4.1. The confocal datasets were recorded by using 25x NA 0.95 and 63x NA 1.2 water immersion lenses. Excitation was at 490 nm and 550 nm. Emission signals were detected simultaneously or sequentially with two photomultipliers from 505 to 600 nm (lectins) and 600–700 nm (Sypro red). Image data sets

Table 1
Lectins used in the analysis of sialic acids in granular sludge.

Lectins	Abbreviation	Specificity	References
Homarus americanus agglutinin	HMA	Sia, Neu5Ac	Turonova et al. (2016)
Maackia amurensis lectin	MAA	Siaα(2–3)βGal(1–4)βGlcNAc, Siaα(2–3)βGal(1–3)[Siaα(2–6)]αGalNAc	Geisler and Jarvis (2011)
Limax flavus Lectin	LFA	Terminal Sia	Cohen et al. (2012)
Polyporus squamosus lectin	PSL	Neu5Ac α(2–6) Gal	Kadirvelraj et al. (2011)
Wheat Germ Agglutinin	WGA	GlcNAcβ(1–4)GlcNAcβ(1–4)GlcNAc, Neu5Ac	Mlouka et al. (2016)

were projected using Imaris version 9.1.2 (Bitplane, Switzerland). For deconvolution of the image dataset in Fig. 3 the program Huygens version 18.4 (SVI, The Netherlands) was used.

2.2.2. Sialic acid quantification

Quantification of sialic acids (N-acetylneuraminic acid, Neu5Ac) in the seawater-adapted AGS was performed with a Sialic Acid Quantitation Kit (Sigma-Aldrich, USA). Granules were physically crushed and homogenized without disrupting cells, similar to sludge handling for FISH fixation as described in Bassin et al. (2011). Afterwards the crushed granules were washed with Tris-HCl buffer (pH 7.5) and resuspended in demineralized water (15 mg volatile solids granules per 5 mL final volume). The protocol was followed as described in the manual supplied with the quantitation kit for a whole cell assay. 80 μL of homogenized cells were mixed with 20 μL sialidase buffer and 1 μL of α(2 → 3,6,8,9)-neuraminidase, and incubated overnight at 37 °C. Afterwards, 20 μL 0.01 M β-NADH solution, 1 μL of N-acetylneuraminic acid aldolase and 1 μL of lactic dehydrogenase were added, and incubated at 37 °C for 1 h. Absorbance at 340 nm was measured prior and after addition of the last enzymes, and used for calculation of the Neu5Ac concentration. Standards were prepared with Neu5Ac from the same quantitation kit.

2.3. Genome analysis of enzymes for sialic acid synthesis

Forty-eight available metagenome sequences of *Candidatus Accumulibacter* enrichments were obtained from JGI IMG database (listed in Table 2 results). These metagenomes were compared with protein sequences of enzymes from known sialic acid synthesis pathways, obtained from the NCBI protein database. The species from which the reference protein sequences were taken were a range of pathogenic bacteria (*Neisseria meningitides*, *Campylobacter jejuni*, *Helicobacter cetorum*, *Photobacterium damselae*), extremophiles (*Chitinivibrio alkaliphilus*, *Psychrobacter arcticus*, *Salinibacter ruber, Halanaerobium praevalens*), and the common fruit fly (*Drosophila melanogaster*). BLASTp was performed using the on-line BLASTp tool by JGI IMG. Alignment was performed according to the algorithm as described in Altschul et al. (1997) and Schäffer et al. (2001). Lower E-values indicate a lower uncertainty in the presence of certain sequences. Values lower than 5E-25 were set as threshold for positive results (Petit et al., 2018).

Table 2
Genome analysis of enzymes in the Neu5Ac synthesis pathway in multiple *Ca. Accumulibacter* metagenomes (5 highest hits per enzyme shown).

Enzyme *Accession number* *Reference species*	E-value	Metagenome name*
GlcNAc-6-P 2-epimerase	8.00E-125	a
ABW08136.1	2.00E-105	b
Neisseria meningitidis	5.00E-100	c
	6.00E-100	c
	1.00E-98	d
Neu5Ac synthetase	4.00E-118	a
ERP39285.1	5.00E-112	a
Chitinivibrio alkaliphilus	4.00E-112	e
	4.00E-112	f
	4.00E-112	g
Neu5Ac synthetase	4.00E-149	f
WP_011279946.1	1.00E-130	h
Psychrobacter arcticus	1.00E-130	i
	1.00E-130	j
	2.00E-130	k
Neu5Ac synthetase	9.00E-81	f
NP_650195.1	9.00E-81	g
Drosophila melanogaster	1.00E-80	l
	2.00E-79	a
	2.00E-71	m
Neu5Ac synthetase	2.00E-131	l
CBH23620.1	2.00E-82	a
Salinibacter ruber	2.00E-82	e
	2.00E-82	f
	2.00E-82	m
CMP Neu5Ac synthetase	6.00E-44	a
AOW97441.1	2.00E-41	n
Campylobacter jejuni	7.00E-42	n
	2.00E-41	n
	2.00E-42	o
CMP Neu5Ac synthetase	6.00E-50	p
AFI04478.1	1.00E-49	q
Helicobacter cetorum	6.00E-46	p
	1.00E-41	a
	1.00E-41	g
Sialyl transferase	1.00E-115	r
ADO76488.1	2.00E-104	a
Halanaerobium praevalens	6.00E-30	r
	3.00E-23	n
	3.00E-21	n
Sialyl transferase	6.00E-54	e
BAA25316.1	6.00E-54	f
Photobacterium damselae	7.00E-54	a
	6.00E-51	f
	3.00E-51	s

*Metagenome names.
a: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor_6/25/2014_ DNA.
b: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_6/14/2005_ DNA.
c: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_1/10/2011_ DNA.
d: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_9/17/2007_ DNA.
e: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time F- 52min-Aerobic_ RNA (Metagenome Metatranscriptome).
f: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time E −22min-Aerobic_ RNA (Metagenome Metatranscriptome).
g: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time D- 0min-Aerobic_ RNA (Metagenome Metatranscriptome).
h: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI - F_92min_Anaerobic (Metagenome Metatranscriptome).
i: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI - J_51min_Aerobic (Metagenome Metatranscriptome).
j: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/28/2013_ DNA (Hybrid Assembly).
k: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/28/2013_ DNA (Illumina Assembly).
l: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time B −10min-Anaerobic_ RNA (Metagenome Metatranscriptome).
m: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time C-32min-Anaerobic_ RNA (Metagenome Metatranscriptome).
n: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI - Type I.
o: Unknown Accumulibacter genome from EPBR bioreactor metagenome.
p: Candidatus Accumulibacter sp. BA-91.
q: Candidatus Accumulibacter sp. SK-01.
r: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor_6/25/2014_ DNA (SPAdes).
s: Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time I- 292min-Aaerobic_ RNA (Metagenome Metatranscriptome).

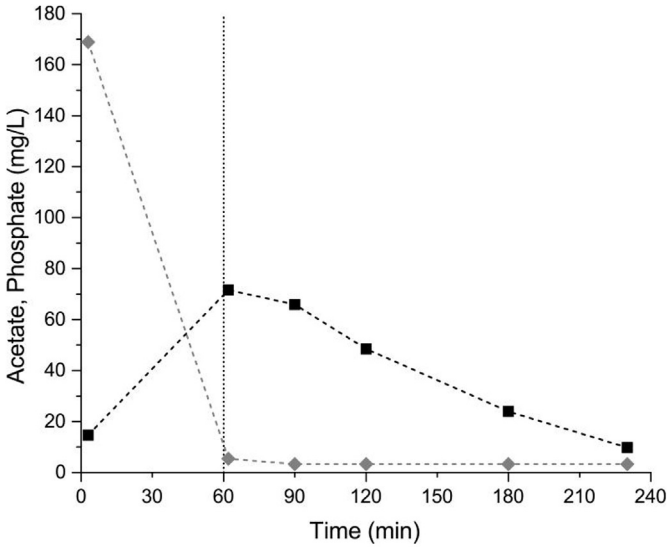

Fig. 1. Concentrations of acetate (diamonds) and phosphate (squares) during a typical reactor cycle with 60 min of anaerobic feeding, followed by 170 min of aeration. Acetate and phosphate values at time interval zero indicate influent plus residual amounts left over from the previous cycle.

2.4. Function of sialic acids in seawater-adapted aerobic granular sludge

2.4.1. Galactose removal tests

Granules were taken directly from the lab-scale seawater-fed reactor, and washed by placing them in distilled water for 2 h. Equal numbers of granules were distributed over 2 Eppendorf tubes, and mixed with 400 µl distilled water and 100 µl sialidase buffer. To the first tube, 5 µl neuraminidase enzyme was added; to the second tube, 5 µl distilled water was added. Both Eppendorf tubes were incubated overnight at 37 °C. Afterwards, the supernatant from both tubes was discarded, and replaced with 450 µL 1x PBS buffer (pH 7.4) and 50 µl β-galactosidase (Roche Diagnostics GmbH, Mannheim, Germany), and incubated overnight at 37 °C. Subsequently, the supernatant was collected from both tubes, and their galactose content was measured with High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection (HPAEC-PAD).

2.4.2. FT-IR analysis

The Fourier transform infra-red (FT-IR) spectrum of granular sludge was recorded on a FT-IR Spectrometer (Perkin Elmer, Shelton, USA) at room temperature, with a wavenumber range from 750 cm^{-1} to 4000 cm^{-1}. The spectra of granules with and without neuraminidase treatment were normalized at 1630 cm^{-1}.

3. Results

3.1. Reactor operation and dominant microorganisms

Seawater-adapted aerobic granular sludge was taken from a lab-scale reactor, performing complete removal of COD (acetate) and phosphate. The reactor was inoculated with Nereda® sludge and it took 8 weeks to reach a stable state as observed from an identical cycle-to-cycle behavior in the online (pH and off-gas) measurements. The reactor was sampled for EPS analysis after operating in stable conditions for three SRTs. The performance of a typical reactor cycle is shown in Fig. 1. Acetate was completely consumed anaerobically within the first 60 min of the cycle, while phosphate was released up to 75 mg PO_4^{3-}-P/L (5.9 net P-mol release). This corresponds to 0.34 P-mol/C-mol of anaerobic phosphate release per carbon uptake, which is in range of values for freshwater lab-scale AGS and only slightly lower than reported for enriched cultures of *Ca. Accumulibacter phosphatis* (Schuler and Jenkins, 2003; De Kreuk et al., 2005b; Welles et al., 2015).

Fluorescence in situ hybridization (FISH) analysis was performed for analysing the relative amounts of polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO). Large numbers of PAO were observed in the seawater-adapted aerobic granular sludge, while GAO-specific probes gave no fluorescent signal in the sludge (Fig. 2a). Distinct and uniform PAO cells were distinguished, and seen in clusters of cells (Fig. 2b). These results indicate dominance of PAO over GAO in the system.

3.2. Sialic acid visualization and quantification

3.2.1. Sialic acid lectin staining

In granular sludge, microorganisms are organized as micro-colonies embedded in the EPS. EPS consists of proteins, polysaccharides, glycoproteins and other substances such as lipids and DNA (Lin et al., 2018). Fluorescently labelled lectins have been widely used to stain extracellular glycoconjugates in various biofilm and sludge (Mlouka et al., 2016). In the current research, to visualize the glycoconjugates in granular sludge 77 different lectins were used for screening (Bennke et al., 2013). From these lectins the LFA lectin gave the strongest signal. A layer of glycoconjugates containing sialic acids on the surface of granular sludge was visualized (Fig. 3). As LFA binds to sialic acids at the termini of the glycan chains of glycoproteins (Cohen et al., 2012), this is an

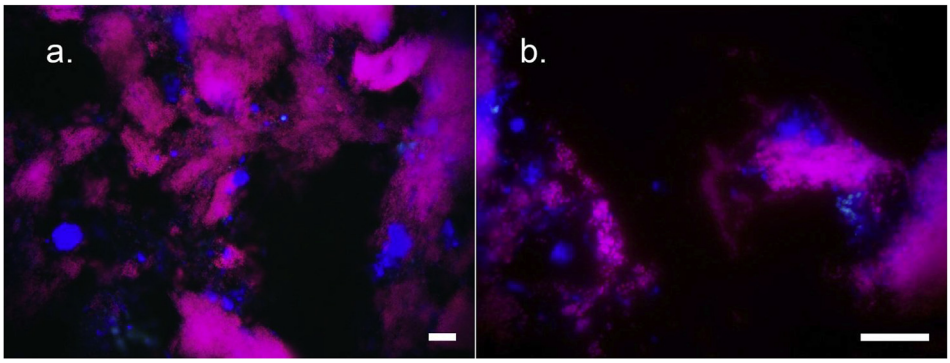

Fig. 2. Fluorescence in-situ hybridization (FISH) images of seawater-adapted aerobic granular sludge, with PAOmix probes (red), GAOmix probes (green), and eubacteria probes (blue) (scale bars equal 10 µm). Magenta colour is an overlap between eubacteria (blue) and PAO bacteria (red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

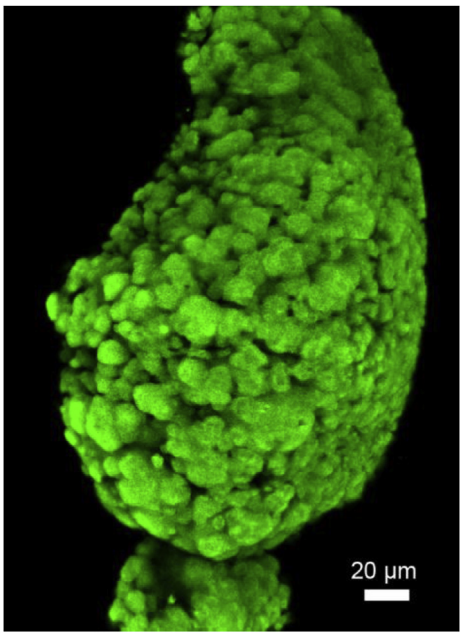

Fig. 3. Lectin staining of sialoglycoconjugates by LFA lectin.

indication that sialic acids are present in the granular sludge associated to glycoproteins (sialoglycoproteins).

To study the distribution of sialic acids inside a granule and investigate if they are associated to glycoproteins, granules were sliced and double stained. The granules were first stained by a protein-specific fluorochrome and then with one of four different sialic acid specific binding lectins (Fig. 4). All four lectins (HMA, MAA, PSL and WGA) gave a strong signal, implying sialic acids are widely distributed in granular sludge. Sialic acid signals originate from both the bacterial cell surface and the extracellular matrix. Interestingly, the locations where lectins for sialic acid stained were overlapping with areas where proteins were visualized. This strongly suggests that the glycan chains containing sialic acids bond to proteins as glycoproteins. This is also in line with the specificity of the lectins MAA and PSL: sialic acids binding to those two lectins are positioned as the terminal sugar residue of glycan chains of glycoproteins. In addition, the fact that binding with both MAA and PSL lectins means the link between sialic acids and the penultimate sugar might be $\alpha(2 \rightarrow 3)$ linkage and/or $\alpha(2 \rightarrow 6)$ linkage (Geisler and Jarvis, 2011; Kadirvelraj et al., 2011). This information of the linkage is important to choose enzymes for sialic acids quantification.

3.2.2. Sialic acid quantification

The result of lectin staining showed that sialic acids are abundantly distributed in granular sludge, and the linkage between sialic acids and the penultimate sugar could be an $\alpha(2 \rightarrow 3)$ and/or $\alpha(2 \rightarrow 6)$ linkage. Thus, neuraminidase, which cleaves N-acetylneuraminic acid (Neu5Ac), the most widespread form of sialic acids, was applied for sialic acid quantification. This enzyme specifically cleaves Neu5Ac that is $\alpha(2 \rightarrow 3,6,8,9)$ linked to the penultimate sugar in the glycan chain. Subsequent quantification yields an amount of 11.33 ± 3.80 mg N-acetylneuraminic acid (Neu5Ac) per gram of volatile solids (VS) (1.1%). Although the presence of sialic acids has been indicated by lectin staining in biofilms (Mlouka et al., 2016), information of their amount is hardly found. In comparison, the normal range for sialic acids in human serum is 0.9–1.4% (weight percentage) (O'Kennedy, 1988).

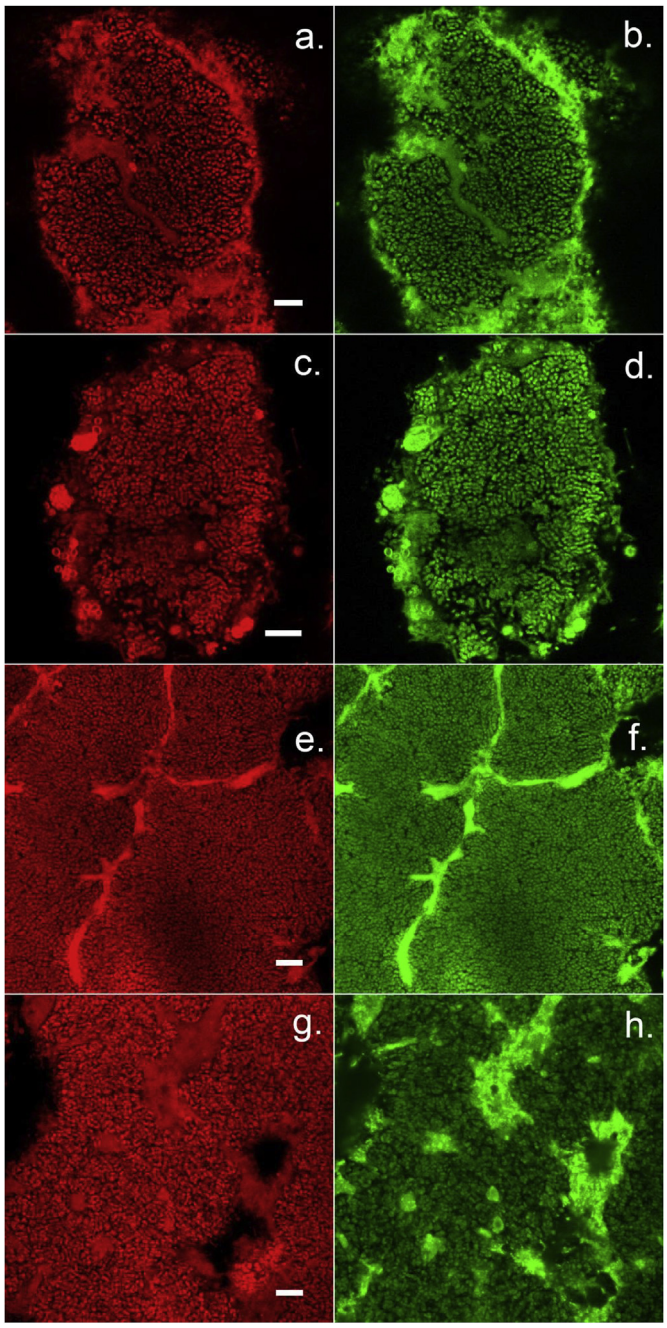

Fig. 4. Lectin staining of granular sludge with SyproRed protein staining (in red: a., c., e., g.) and different sialic acid specific lectins (in green: b. HMA, d. MAA, f. PSL, h. WGA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Genome analysis

The successful cleavage of Neu5Ac raises the question which microorganisms in granular sludge could produce sialic acids. From FISH analysis, PAO are the dominant microorganisms in the granular sludge (Fig. 2). Therefore, the potential of sialic acid (Neu5Ac) synthesis by PAO was assessed by means of *in silico* genome analysis.

As shown in Fig. 5, in the metabolic pathway of Neu5Ac, GlcNAc-6-phosphate is epimerized to ManNAc-6-phosphate by GlcNAc-6-P 2-epimerase. The phosphate groups gets removed to form ManNAc,

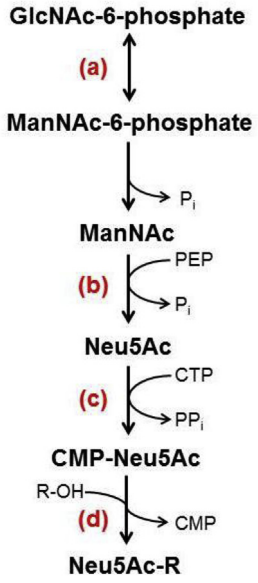

Fig. 5. Pathway of bacterial Neu5Ac metabolism with its respective key enzymes: a. GlcNAc-6-phosphate 2-epimerase, b. Neu5Ac synthetase, c. CMP-Neu5Ac synthetase, d. Sialyl transferase. Adapted from Angata and Varki (2002)..

and Neu5Ac is formed by using phosphoenolpyruvate (PEP), catalysed by Neu5Ac synthetase. The active form CMP-Neu5Ac is catalysed by CMP-Neu5Ac synthetase. This form is finally added to acceptor substrates by sialyl transferase. These key enzymes for Neu5Ac production were analysed with BLASTp on metagenomes of *Ca. Accumulibacter*, which is the dominant PAO in the system. Since *Ca. Accumulibacter* has not yet been isolated in a pure culture, 48 available metagenomes were used in the analysis, which increases accuracy by covering a broad range of clades.

Very low E-values (<1E-100) were found for GlcNAc-6-P 2-epimerase and Neu5Ac synthetase from enzymes from all tested reference species (Table 2). Part of the analysed *Ca. Accumulibacter* metagenomes contained genes that were annotated for Neu5Ac synthetase from *C. alkaliphilus*, *D. melanogaster*, *S. ruber*. CMP Neu5Ac synthetase from *C. jejuni* and *H. cetorum* were also annotated in some *Ca. Accumulibacter* metagenomes.

CMP Neu5Ac synthetase from both *C. jejuni* and *H. cetorum* gave higher E-values than GlcNAc-6-P 2-epimerase and Neu5Ac synthetase, but were still in good range of 1E-40 ~ 1E-50. Sialyl transferase from *Halanaerobium praevalens* gave two very low E-values of <1E-100 and three E-values that are around 1E-20. The sialyl transferase from *P. damselae* gave E-values at around 1E-40 ~ 1E-50.

Overall, the enzymes from the Neu5Ac production pathway show low E-values (<1E-40) during *in silico* genome analysis on *Ca. Accumulibacter*. These results indicate that there is high probability that *Ca. Accumulibacter* possesses genes that can be transcribed into enzymes that are involved in the synthesis of Neu5Ac.

3.4. Function of sialic acids in seawater-adapted aerobic granular sludge

3.4.1. Galactose removal tests

Sialic acids are commonly found in chains of sugars as the terminal residue, rather than directly bound to proteins (Schauer et al., 1984). In line with the specificity of MFA, LFA, and PSL lectins for linkage between sialic acid and galactose (Fig. 4), the shielding effect of sialic acids to underlying galactose residues was analysed.

Whole granules were treated first with neuraminidase and subsequently with β-galactosidase, to selectively cleave off sialic acids and galactose residues, respectively. After this procedure, a galactose peak was measured by HPAEC-PAD analysis, corresponding to a total of 0.37 mg/L galactose (Appendix A). Granules that were treated with only β-galactosidase, and still have sialic acids in the outer layer of their EPS, did not result in a galactose peak in the chromatogram. There was a slight shift in retention time between pure galactose reference samples (7.5 min) and the observed peaks in our samples (7.8 min). Enzyme-treated samples that were spiked with galactose also showed an increased peak height at 7.8 min. Along with the specificity of used β-galactosidase, this indicates that the measured peaks indeed correspond to galactose. These results imply that sialic acids in the outer layer of granular sludge are bound to galactose, and can play a role in protecting galactose from enzymatic hydrolysis.

Cleavage of sialic acids from the granules was verified by FT-IR analysis (Fig. 6). After neuraminidase treatment, the sharp peak at 1730 cm^{-1}, which is assigned to −COOH groups changed to a shoulder peak. Also the intensity of the peak at 1000–1200 cm^{-1} (C-O-C bond in carbohydrates) was decreased. The change of the spectrum is due to the removal of sialic acids, which have −COOH groups and are linked with the penultimate sugar by C-O-C bond (Bonnin et al., 1999; Bramhachari et al., 2007; Lin et al., 2010). Due to the fact that neuraminidase only removed part of the sialic acids,

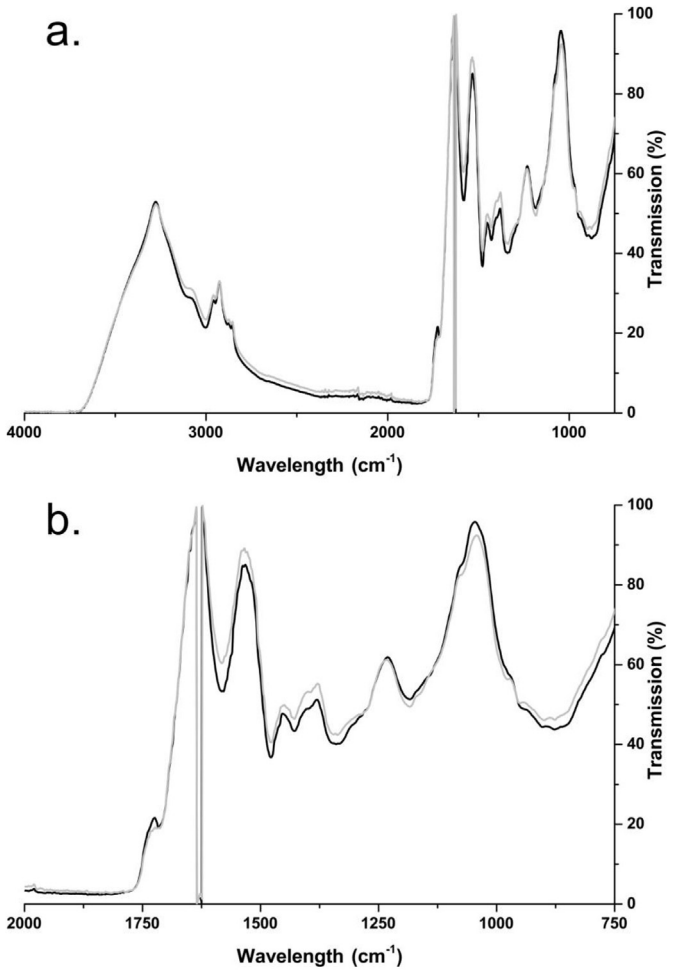

Fig. 6. FT-IR spectrum of aerobic granular sludge after neuraminidase treatment (grey line) and without neuraminidase treatment (black line). a) Full spectrum from 4000 cm^{-1} to 750 cm^{-1}; b) Part of the same FT-IR spectrum as a., but zoomed in on the region between 2000 cm^{-1} and 750 cm^{-1}.

the remaining part is still present as a shoulder peak at 1730 cm^{-1}. Moreover, the removal of sialic acids resulted in the extension of protein side chain (increase of the peak intensity at 1560 cm^{-1}), and the exposure of the penultimate sugar (-CH_2 from C6 sugar with the peak intensity increase at 1450 cm^{-1} and 1370 cm^{-1}), which is in agreement with the masking function of sialic acids to the molecules and glycoproteins (André et al., 2015).

4. Discussion

4.1. Sialoglycoproteins found in the extracellular polymeric substances in seawater-adapted aerobic granular sludge

Sialic acids are unique nine-carbon sugars. They play significant roles in many biological processes of animals and humans, both in the state of healthy cells and tissue (e.g. recognition, ion transport, differentiation) and in many important diseases (e.g. cancer, autoimmune diseases) (Traving and Schauer, 1998). The presence of sialic acids in biofilms has been reported, however, information concerning their function and binding site in biofilms is hardly found.

This paper provides the first evidence for the presence of sialoglycoproteins in aerobic granular sludge. Lectin staining of sialic acids and protein staining have significant overlapping in the locations of their respective fluorescent signal (Fig. 2). Binding with specific lectins (MAA, HMA, PSL and WGA) provides indications that sialic acids locate at the termini of carbohydrate chains of glycoproteins. The penultimate sugar might be galactose or N-acetyl galactosamine.

The presence of glycoproteins in biofilms has occasionally been described, such as in marine biofilms (Ortega-Morales et al., 2007) and full-scale anaerobic granular sludge (Bourven et al., 2015). Interestingly, presence of glycoconjugates has also been found in saline lab-scale anaerobic granular sludge (Gagliano et al., 2018). The major sugar monomers reported were mannose and N-acetyl galactosamine (GalNAc), which is commonly found in conjunction with sialic acids in mammalian glycans and glycolipids (Bennett et al., 2012; Dalziel et al., 2014).

Most bacteria are not described to produce sialic acids (Angata and Varki, 2002). When present, sialic acids are found mostly in capsular polysaccharides and lipopolysaccharides, instead of in glycoproteins. Unlike the situation in animal glycoconjugates, the sialic acids in these bacterial polysaccharides mostly exist as internal residues, rather than terminal residues (Angata and Varki, 2002). However, in the current research, sialic acids are present as sialoglycoproteins, similar to animals. Therefore, the wide distribution of sialoglycoproteins over the granular structure implies not only the importance of glycoproteins, but also a specific function of sialic acids.

4.2. Function of sialic acids

Functionality of sialic acids has frequently been discussed in line of pathogenic bacteria (Severi et al., 2007). Sialylation of the cell surface can modify the interaction with the host, and increase resistance against normal human serum (Bouchet et al., 2003; Vimr et al., 2004). In the current research, sialic acids have now also been found in non-pathogenic bacteria, which would imply a wider range of functions.

Sialic acids show remarkable structural diversity, with the family currently comprising over 50 naturally occurring members. The diversity of sialic acids is reflected by its involvement in a variety of biological functions, many stemming from its unique physical and chemical properties, such as charge and size. The carboxylate group of sialic acid can give a net negative charge, allowing for binding of calcium cations, which is known to be contributing to the stability of aerobic granular sludge (Schauer, 1985; Lin et al., 2013). In addition, sialic acids are nine-carbon sugars, so they have a much bigger size than fine-carbon sugars (e.g. xylose) and six carbon sugars (e.g. glucose) (Varki and Schauer, 2009). Staying on the terminal position in carbohydrate chains, sialic acids can mask penultimate galactose residue and shield it from recognition by β-galactosidases (Traving and Schauer, 1998). It has been found that after the loss of sialic acids, galactose molecules and cells can be bound, or can even be taken up and degraded by naturally occurring proteases (Daley et al., 2008; Varki and Schauer, 2009). This phenomenon has been most extensively studied with serum glycoproteins and blood cells (Karacali, 2017).

In the current research, it is observed that removal of sialic acids from the outer layer of granules results in the hydrolysis of galactose by β-galactosidase, while galactose was not cleaved off without removing sialic acids. This not only shows that sialic acids are linked to galactose, but most importantly indicates that sialic acids play an important role in maintaining the stability of the sugar chain in the EPS of granules by masking penultimate galactose residues. Once sialic acids are removed, the sugar chain is prone to degradation, resulting to the instability of the sialoglycoproteins and the EPS matrix in the end. In comparison, in mammalian tissue, sialic acids are involved in the stability, turnover und function of glycoproteins (Hanisch et al., 2013). Therefore, the function of sialic acids in granules might resemble that in mammalian tissue.

4.3. Evolutionary importance of sialic acids in granular sludge

Resemblance between bacterial and mammalian sialic acid structures and function can also give great insight into the evolutionary benefits. Presence of sialic acids is usually found in pathogenic bacteria, where it is proposed to make the cell walls of the pathogens resemble more the glycocalyx of its host. This would make these cells less detectable for the immune system of the host (Schauer, 1985; Severi et al., 2007; Kajiwara et al., 2010). Granular sludge generally does not contain a significant fraction of pathogens (Winkler et al., 2013). An important similarity between these types of bacterial communities is the necessity of biofilm formation for their survival. Granular sludge is cultivated in an environment that simulates aggregation, while pathogenic bacteria depend on adhesion to their host environment for successful infection. The presence of sialic acids could therefore be a result of requirement for stable adhesion and recognition, not only for pathogens, but also for bacterial aggregates in general.

Production pathways of sialic acids in bacteria have been frequently studied (Angata and Varki, 2002; Lewis et al., 2009). A link with the granular sludge microbiome has never been made in literature. The genome analysis that was performed in this study gives a positive indication for a putative production of sialic acids by *Ca. Accumulibacter* in aerobic granular sludge. A general evolutionary benefit for sialic acid production for adhering bacteria is proposed. Similarly to both *Ca. Accumulibacter* in aerobic granular sludge and pathogenic bacteria, the presence of sialic acids would be expected for other types of adhering bacteria as well. Sialic acids could be more widely present than was previously described in literature.

In the current research, the presence of sialic acids is only studied in saline aerobic granular sludge. It is interesting to examine a broad range of biofilm sample under different conditions (e.g. flocculent sludge, granular sludge, biofilm on carriers under saline and non-saline conditions) to explore if sialic acids present in different environmental samples and what function do they involve in. To this end, it is possible to understand comprehensively the biological roles of sialic acids in prokaryotes.

4.4. Challenge of sialic acids identification and quantification in environmental samples

The sialic acid family comprises more than 50 natural derivatives of neuraminic acid. Unsubstituted neuraminic acid does not occur in nature. The amino group of neuraminic acid is substituted either by an acetyl or glycolyl residue, and the hydroxyl groups may be methylated or esterfied with sulfate, phosphate, acetyl or lactyl groups. Sometimes several of these substituents are present in one sialic acid molecule. Sialic acids are the only natural sugars to show this great variety (Schauer et al., 1984).

In addition, a number of other nonulosonic acids are described in proteobacteria among which the 5,7-diamino-3,5,7,9-tetradeoxy-D-glycero-D-galacto-nonulosonic acid (legionaminic acid (Leg)) and the 5,7-diamino-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (pseudaminic acid (Pse)), which show structural, biosynthetic and functional similarities to sialic acids (Goon et al., 2003; McNally et al., 2007). Therefore, besides natural derivatives of sialic acids, the existence of bacterial sialic acids (e.g. Leg and Pse) contributes to the complexity of sialic acid family identification in environmental samples.

Enzymatic sialic acid quantification method is limited by the recognition of sialidase. If due to the existence of substituents in bacterial sialic acids, the sialidase could not recognize the structure, the result of enzymatic quantification will underestimate the amount of sialic acid in granular sludge or other biofilm systems. Therefore, more specific analytical methods (e.g. mass spectroscopy) which could identify different sialic acid species need to be adjusted towards bacterial sialic acids (Shah et al., 2013).

5. Conclusions

- Sialic acids are discovered and widely distributed in seawater-adapted aerobic granular sludge, both on the bacterial cell surface and the extracellular matrix
- Sialoglycoproteins, with sialic acids as the terminal sugar residue of glycoproteins, are components of the extracellular matrix
- *Ca. Accumulibacter* in seawater-adapted aerobic granular sludge likely contains genes that encode for enzymes that are responsible for sialic acid metabolism
- Sialic acids that are located in the outer layer of the extracellular matrix function as a shield protecting the underlying sugar chain from degradation

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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.2019.02.040.

References

Altschul, S., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

André, S., Kaltner, H., Manning, J., Murphy, P., Gabius, H.-J., André, S., Kaltner, H., Manning, J.C., Murphy, P.V., Gabius, H.-J., 2015. Lectins: getting familiar with translators of the sugar code. Molecules 20, 1788–1823. https://doi.org/10.3390/molecules20021788.

Angata, T., Varki, A., 2002. Chemical diversity in the sialic acids and related r-keto Acids : an evolutionary perspective. Chem. Rev. 102, 439–469. https://doi.org/10.1021/cr000407m.

Bassin, J.P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., van Loosdrecht, M.C.M., 2011. Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with microbial community structure. Appl. Environ. Microbiol. 77, 7942–7953. https://doi.org/10.1128/AEM.05016-11.

Bennett, E.P., Mandel, U., Clausen, H., Gerken, T.A., Fritz, T.A., Tabak, L.A., 2012. Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. Glycobiology 22, 736–756. https://doi.org/10.1093/glycob/cwr182.

Bennke, C.M., Neu, T.R., Fuchs, B.M., Amann, R., 2013. Mapping glycoconjugate-mediated interactions of marine Bacteroidetes with diatoms. Syst. Appl. Microbiol. 36, 417–425. https://doi.org/10.1016/J.SYAPM.2013.05.002.

Bonnin, S., Besson, F., Gelhausen, M., Chierici, S., Roux, B., 1999. A FTIR spectroscopy evidence of the interactions between wheat germ agglutinin and N-acetylglucosamine residues. FEBS Lett. 456, 361–364. https://doi.org/10.1016/S0014-5793(99)00981-3.

Bouchet, V., Hood, D.W., Li, J., Brisson, J.-R., Randle, G.A., Martin, A., Li, Z., Goldstein, R., Schweda, E.K.H., Pelton, S.I., Richards, J.C., Moxon, E.R., 2003. Host-derived sialic acid is incorporated into Haemophilus influenzae lipopolysaccharide and is a major virulence factor in experimental otitis media. Proc. Natl. Acad. Sci. Unit. States Am. 100, 8898–8903. https://doi.org/10.1073/pnas.1432026100.

Bourven, I., Bachellerie, G., Costa, G., Guibaud, G., 2015. Evidence of glycoproteins and sulphated proteoglycan-like presence in extracellular polymeric substance from anaerobic granular sludge. Environ. Technol. 36, 2428–2435. https://doi.org/10.1080/09593330.2015.1034186.

Bramhachari, P.V., Kavi Kishor, P.B., Ramadevi, R., Kumar, R., Rama Rao, B., Dubey, S.K., 2007. Isolation and characterization of mocous exopolysaccharide (EPS) produced by Vibrio furnissii strain VB0S3. J. Microbiol. Biotechnol. 17, 44–51.

Campo, R., Corsino, S.F., Torregrossa, M., Di Bella, G., 2018. The role of extracellular polymeric substances on aerobic granulation with stepwise increase of salinity. Separ. Purif. Technol. 195, 12–20. https://doi.org/10.1016/J.SEPPUR.2017.11.074.

Cohen, M., Varki, N.M., Jankowski, M.D., Gagneux, P., 2012. Using unfixed, frozen tissues to study natural mucin distribution. JoVE. https://doi.org/10.3791/3928.

Corsino, S.F., Capodici, M., Morici, C., Torregrossa, M., Viviani, G., 2016. Simultaneous nitritation-denitritation for the treatment of high-strength nitrogen in hypersaline wastewater by aerobic granular sludge. Water Res. 88, 329–336. https://doi.org/10.1016/j.watres.2015.10.041.

Corsino, S.F., Capodici, M., Torregrossa, M., Viviani, G., 2017. Physical properties and Extracellular Polymeric Substances pattern of aerobic granular sludge treating hypersaline wastewater. Bioresour. Technol. 229, 152–159. https://doi.org/10.1016/j.biortech.2017.01.024.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ. Microbiol. 66, 1175–1182. https://doi.org/10.1128/AEM.66.3.1175-1182.2000.

Crocetti, G.R., Bond, P.L., Banfield, J.F., Blackall, L.L., Keller, J., 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes b. Microbiology 148, 3353–3364. https://doi.org/10.1099/00221287-148-11-3353.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444. https://doi.org/10.1016/S0723-2020(99)80053-8.

Daley, W.P., Peters, S.B., Larsen, M., 2008. Extracellular matrix dynamics in development and regenerative medicine. J. Cell Sci. 121, 255–264. https://doi.org/10.1242/jcs.006064.

Dalziel, M., Crispin, M., Scanlan, C.N., Zitzmann, N., Dwek, R.A., 2014. Emerging principles for the therapeutic exploitation of glycosylation. Science 80, 343. https://doi.org/10.1126/science.1235681.

De Kreuk, M.K., Pronk, M., Van Loosdrecht, M.C.M., 2005a. Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. Water Res. 39, 4476–4484. https://doi.org/10.1016/j.watres.2005.08.031.

De Kreuk, M.K., Heijnen, J.J., Van Loosdrecht, M.C.M., 2005b. Simultaneous COD,

nitrogen, and phosphate removal by aerobic granular sludge. Biotechnol. Bioeng. 90, 761–769. https://doi.org/10.1002/bit.20470.
Figueroa, M., Mosquera-Corral, A., Campos, J.L., Méndez, R., 2008. Treatment of saline wastewater in SBR aerobic granular reactors. Water Sci. Technol. 58, 479–485. https://doi.org/10.2166/wst.2008.406.
Gagliano, M.C., Neu, T.R., Kuhlicke, U., Sudmalis, D., Temmink, H., Plugge, C.M., 2018. EPS glycoconjugate profiles shift as adaptive response in anaerobic microbial granulation at high salinity. Front. Microbiol. 9, 1423. https://doi.org/10.3389/fmicb.2018.01423.
Geisler, C., Jarvis, D.L., 2011. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology 21, 988–993. https://doi.org/10.1093/glycob/cwr080.
Goon, S., Kelly, J.F., Logan, S.M., Ewing, C.P., Guerry, P., 2003. Pseudaminic acid, the major modification on Campylobacter flagellin, is synthesized via the Cj1293 gene. Mol. Microbiol. 50, 659–671. https://doi.org/10.1046/j.1365-2958.2003.03725.x.
Hanisch, F., Weidemann, W., Großmann, M., Joshi, P.R., Holzhausen, H.-J., Stoltenburg, G., Weis, J., Zierz, S., Horstkorte, R., 2013. Sialylation and muscle performance: sialic acid is a marker of muscle ageing. PLoS One 8, e80520. https://doi.org/10.1371/journal.pone.0080520.
Hilkens, J., Ligtenberg, M.J.L., Vos, H.L., Litvinov, S.V., 1992. Cell membrane-associated mucins and their adhesion-modulating property. Trends Biochem. Sci. 17, 359–363. https://doi.org/10.1016/0968-0004(92)90315-Z.
Kadirvelraj, R., Grant, O.C., Goldstein, I.J., Winter, H.C., Tateno, H., Fadda, E., Woods, R.J., 2011. Structure and binding analysis of Polyporus squamosus lectin in complex with the Neu5Acα2-6Galβ1-4GlcNAc human-type influenza receptor. Glycobiology 21, 973–984. https://doi.org/10.1093/glycob/cwr030.
Kajiwara, H., Toda, M., Mine, T., Nakada, H., Wariishi, H., Yamamoto, T., 2010. Visualization of sialic acid produced on bacterial cell surfaces by lectin staining. Microb. Environ. 25, 152–155. https://doi.org/10.1264/jsme2.ME10118.
Karacali, S., 2017. Cell surface sialylated N-glycan alterations during development. Eur. J. Biol. 76, 79–88. https://doi.org/10.5152/EurJBiol.2017.1714.
Le Parc, A., Lee, H., Chen, K., Barile, D., 2014. Rapid quantification of functional carbohydrates in food products. Food Nutr. Sci. 5, 71–78. https://doi.org/10.4236/fns.2014.51010.
Lefebvre, O., Moletta, R., 2006. Treatment of organic pollution in industrial saline wastewater: a literature review. Water Res. 40 (20), 3671–3682. https://doi.org/10.1016/J.WATRES.2006.08.027.
Lewis, A.L., Desa, N., Hansen, E.E., Knirel, Y.A., Gordon, J.I., Gagneux, P., Nizet, V., Varki, A., 2009. Innovations in host and microbial sialic acid biosynthesis revealed by phylogenomic prediction of nonulosonic acid structure. Proc. Natl. Acad. Sci. Unit. States Am. 106, 13552–13557. https://doi.org/10.1073/pnas.0902431106.
Lin, Y., de Kreuk, M., van Loosdrecht, M.C.M., Adin, A., 2010. Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant. Water Res. 44, 3355–3364. https://doi.org/10.1016/j.watres.2010.03.019.
Lin, Y.M., Sharma, P.K., Van Loosdrecht, M.C.M., 2013. The chemical and mechanical differences between alginate-like exopolysaccharides isolated from aerobic flocculent sludge and aerobic granular sludge. Water Res. 47, 57–65. https://doi.org/10.1016/j.watres.2012.09.017.
Lin, Y., Reino, C., Carrera, J., Pérez, J., van Loosdrecht, M.C.M., 2018. Glycosylated amyloid-like proteins in the structural extracellular polymers of aerobic granular sludge enriched with ammonium-oxidizing bacteria. Microbiologyopen e00616. https://doi.org/10.1002/mbo3.616.
Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.I., Lee, Y.C., 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal. Biochem. 339, 69–72. https://doi.org/10.1016/j.ab.2004.12.001.
McNally, D.J., Aubry, A.J., Hui, J.P.M., Khieu, N.H., Whitfield, D., Ewing, C.P., Guerry, P., Brisson, J.-R., Logan, S.M., Soo, E.C., 2007. Targeted metabolomics analysis of Campylobacter coli VC167 reveals legionaminic acid derivatives as novel flagellar glycans. J. Biol. Chem. 282, 14463–14475. https://doi.org/10.1074/jbc.M611027200.
McSwain, B.S., Irvine, R.L., Hausner, M., Wilderer, P.A., 2005. Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. Appl. Environ. Microbiol. 71, 1051–1057. https://doi.org/10.1128/AEM.71.2.1051-1057.2005.
Mlouka, M.A. Ben, Cousseau, T., Di Martino, P., 2016. Application of fluorescently labelled lectins for the study of polysaccharides in biofilms with a focus on biofouling of nanofiltration membranes. AIMS Mol. Sci. 3, 338–356. https://doi.org/10.3934/molsci.2016.3.338.
Neu, T., Kuhlicke, U., 2017. Fluorescence lectin bar-coding of glycoconjugates in the extracellular matrix of biofilm and bioaggregate forming microorganisms. Microorganisms 5, 5. https://doi.org/10.3390/microorganisms5010005.
O'Kennedy, R., 1988. The determination of sialic acid - an experiment that demonstrates many important aspects of spectrophotometric assays. Biochem. Educ. 16, 47–48. https://doi.org/10.1016/0307-4412(88)90024-6.
Ortega-Morales, B.O., Santiago-García, J.L., Chan-Bacab, M.J., Moppert, X., Miranda-Tello, E., Fardeau, M.L., Carrero, J.C., Bartolo-Pérez, P., Valadéz-González, A., Guezennec, J., 2007. Characterization of extracellular polymers synthesized by tropical intertidal biofilm bacteria. J. Appl. Microbiol. 102, 254–264. https://doi.org/10.1111/j.1365-2672.2006.03085.x.
Petit, D., Teppa, E., Cenci, U., Ball, S., Harduin-Lepers, A., 2018. Reconstruction of the sialylation pathway in the ancestor of eukaryotes. Sci. Rep. 8, 2946. https://doi.org/10.1038/s41598-018-20920-1.
Pronk, M., Bassin, J.P., De Kreuk, M.K., Kleerebezem, R., Van Loosdrecht, M.C.M., 2014. Evaluating the main and side effects of high salinity on aerobic granular sludge. Appl. Microbiol. Biotechnol. 98, 1339–1348. https://doi.org/10.1007/s00253-013-4912-z.
Pronk, M., De Kreuk, M.K., De Bruin, B., Kamminga, P., Kleerebezem, R., Van Loosdrecht, M.C.M., 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 84, 207–217. https://doi.org/10.1016/j.watres.2015.07.011.
Schäffer, A.A., Aravind, L., Madden, T.L., Shavirin, S., Spouge, J.L., Wolf, Y.I., Koonin, E.V., Altschul, S.F., 2001. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res. 29, 2994–3005. https://doi.org/10.1093/nar/29.14.2994.
Schauer, R., Shukla, A.K., Schroder, C., Müller, E., 1984. The anti-recognition function of sialic acids: studies with erythrocytes and macrophages. Pure Appl. Chem. 56, 907–921. https://doi.org/10.1351/pac198456070907.
Schauer, R., 1985. Sialic acids and their role as biological masks. Trends Biochem. Sci. https://doi.org/10.1016/0968-0004(85)90112-4.
Schuler, A.J., Jenkins, D., 2003. Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, Part I: experimental results and comparison with metabolic models. Water Environ. Res. 75, 485–498. https://doi.org/10.2175/106143003X141286.
Severi, E., Hood, D.W., Thomas, G.H., 2007. Sialic acid utilization by bacterial pathogens. Microbiology 153, 2817–2822. https://doi.org/10.1099/mic.0.2007/009480-0.
Seviour, T., Pijuan, M., Nicholson, T., Keller, J., Yuan, Z., 2009. Understanding the properties of aerobic sludge granules as hydrogels. Biotechnol. Bioeng. 102, 1483–1493. https://doi.org/10.1002/bit.22164.
Shah, P., Yang, S., Sun, S., Aiyetan, P., Yarema, K.J., Zhang, H., 2013. Mass spectrometric analysis of sialylated glycans with use of solid-phase labeling of sialic acids. Anal. Chem. 85, 3606–3613. https://doi.org/10.1021/ac3033867.
Sheng, G.-P., Yu, H.-Q., Li, X.-Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. Biotechnol. Adv. 28, 882–894. https://doi.org/10.1016/J.BIOTECHADV.2010.08.001.
Sutherland, I.W., 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology. https://doi.org/10.1099/00221287-147-1-3.
Traving, C., Schauer, R., 1998. Structure, function and metabolism of sialic acids. Cell. Mol. Life Sci. 54, 1330–1349. https://doi.org/10.1007/s000180050258.
Turonova, H., Neu, T.R., Ulbrich, P., Pazlarova, J., Tresse, O., 2016. The biofilm matrix of Campylobacter jejuni determined by fluorescence lectin-binding analysis. Biofouling 32, 597–608. https://doi.org/10.1080/08927014.2016.1169402.
Varki, A., Schauer, R., 2009. Sialic Acids, Essentials of Glycobiology. Cold Spring Harbor Laboratory Press.
Vimr, E.R., Kalivoda, K.A., Deszo, E.L., Steenbergen, S.M., 2004. Diversity of microbial sialic acid metabolism. Microbiol. Mol. Biol. Rev. 68, 132–153. https://doi.org/10.1128/MMBR.68.1.132-153.2004.
Vishniac, W., Santer, M., 1957. The thiobacilli. Microbiol. Mol. Biol. Rev. 21, 195–213.
Wang, Z.W., Liu, Y., Tay, J.H., 2007. Biodegradability of extracellular polymeric substances produced by aerobic granules. Appl. Microbiol. Biotechnol. 74, 462–466. https://doi.org/10.1007/s00253-006-0686-x.
Wang, Z., Gao, M., She, Z., Wang, S., Jin, C., Zhao, Y., Yang, S., Guo, L., 2015. Effects of salinity on performance, extracellular polymeric substances and microbial community of an aerobic granular sequencing batch reactor. Separ. Purif. Technol. 144, 223–231. https://doi.org/10.1016/j.seppur.2015.02.042.
Welles, L., Tian, W.D., Saad, S., Abbas, B., Lopez-Vazquez, C.M., Hooijmans, C.M., van Loosdrecht, M.C.M., Brdjanovic, D., 2015. Accumulibacter clades Type I and II performing kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate uptake. Water Res. 83, 354–366. https://doi.org/10.1016/J.WATRES.2015.06.045.
Winkler, M.-K.H., Kleerebezem, R., de Bruin, L.M.M., Verheijen, P.J.T., Abbas, B., Habermacher, J., van Loosdrecht, M.C.M., 2013. Microbial diversity differences within aerobic granular sludge and activated sludge flocs. Appl. Microbiol. Biotechnol. 97, 7447–7458. https://doi.org/10.1007/s00253-012-4472-7.
Yilmaz, G., Lemaire, R., Keller, J., Yuan, Z., 2008. Simultaneous nitrification, denitrification, and phosphorus removal from nutrient-rich industrial wastewater using granular sludge. Biotechnol. Bioeng. 100, 529–541. https://doi.org/10.1002/bit.21774.