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DOI

[10.1016/j.watres.2017.05.068](https://doi.org/10.1016/j.watres.2017.05.068)

Publication date

2017

Document Version

Final published version

Published in

Water Research

Citation (APA)

Pronk, M., Neu, T. R., van Loosdrecht, M. C. M., & Lin, Y. M. (2017). The acid soluble extracellular polymeric substance of aerobic granular sludge dominated by *Defluviicoccus* sp. *Water Research*, 122, 148-158. <https://doi.org/10.1016/j.watres.2017.05.068>

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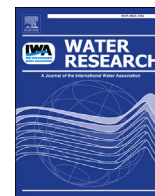
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The acid soluble extracellular polymeric substance of aerobic granular sludge dominated by *Defluviicoccus* sp.

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ARTICLE INFO

Article history:

Received 17 January 2017

Received in revised form

28 May 2017

Accepted 29 May 2017

Available online 30 May 2017

Keywords:

Defluviicoccus

Aerobic granular sludge

Extracellular polymeric substances

Biofilm

EPS extraction

ABSTRACT

A new acid soluble extracellular polymeric substance (acid soluble EPS) was extracted from an acetate fed aerobic granular sludge reactor operated at 35 °C. Acid soluble EPS dominated granules exhibited a remarkable and distinctive tangled tubular morphology. These granules are dominated by *Defluviicoccus* Cluster II organisms. Acetic acid instead of the usually required alkaline extraction medium was needed to dissolve the granules and solubilise the polymeric matrix. The extracted acid soluble EPS was analysed and identified using various instrumental analysis including ¹H and ¹³C Nuclear Magnetic Resonance, Fourier Transform Infrared Spectroscopy and Raman spectroscopy. In addition, the glycoconjugates were characterized by fluorescence lectin-binding analysis. The acid soluble EPS is α -(1 → 4) linked polysaccharide, containing both glucose and galactose as monomers. There are –OCH₃ groups connected to the glucose monomer. Transmission and scanning electron microscopy (TEM, SEM) as well as confocal laser scanning microscopy (CLSM) showed that the acid soluble EPS was present as a tightly bound capsular EPS around bacterial cells ordered into a sarcinae-like growth pattern. The special granule morphology is decided by the acid soluble EPS produced by *Defluviicoccus* Cluster II organisms. This work shows that no single one method can be used to extract all possible extracellular polymeric substances. Results obtained here can support the elucidation of biofilm formation and structure in future research.

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1. Introduction

In recent years, the aerobic granular sludge process is becoming a popular biological wastewater treatment process. It has been successfully applied at several full-scale wastewater treatment plants (Giesen et al., 2013; Pronk et al., 2015c). It is believed that in aerobic granular sludge, similar to biofilms, microorganisms produce a significant amount of extracellular polymeric substances (EPS) to form a hydrogel matrix (Sam and Dulekgurgen, 2016; Seviour et al., 2009). Two kinds of EPS, Granular and alginate-like extracellular polymers (ALE), both extracted under alkaline conditions from aerobic granular sludge, have been reported (Seviour et al., 2012). Roughly, the extractable ALE is 15–25% of the organic fraction in aerobic granular sludge. At present, ALE is the only kind of EPS described as the major structural polymer in

aerobic granular sludge (Felz et al., 2016).

However, whether ALE is always the major structural polymer in aerobic granular sludge remains doubtful due to our recent observations. In the aerobic granular reactor reported previously by Pronk et al. (2015b), it was observed that the white granules dominated by *Defluviicoccus* Cluster II organisms grew separately from black granules dominated γ -GAOs. Most interestingly, very little amount of polymers (<4% of the organic fraction in aerobic granular sludge) could be extracted from white granules when the protocol of ALE extraction was followed. This is in contrast to the black granules where the amount of the extracted polymers is more than 15% of the organic fraction in aerobic granular sludge when the same ALE extraction protocol was followed. Moreover, the granular structure almost remained intact after the extraction was done. Apparently, ALE is not the dominant EPS; neither does it play a role as the structural polymer in these specific granules. Thus, it is reasonable to assume that polymers other than ALE are secreted by *Defluviicoccus* Cluster II organisms that are present as the structural EPS.

In this study, we focus on extracting and characterising the

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structural EPS from granules dominated by *Deffluviococcus* Cluster II organisms. In order to understand the link between the physical structure of these specific granules and the chemical composition of the structural EPS, the acid soluble EPS fraction was recovered using a newly developed method and fully characterized by various instrumental analysis. Furthermore, the link between the acid soluble EPS and *Deffluviococcus* Cluster II organisms was studied by electron microscopy. Different staining techniques were used to examine the location of the EPS in the granular sludge. A new structural acid soluble EPS from aerobic granular sludge is identified.

2. Materials and methods

2.1. Reactor operation

The reactor was seeded with activated sludge performing enhanced biological phosphorus removal (EBPR) from the sewage treatment plant Harnaschpolder, Den Hoorn, the Netherlands. Experiments were conducted in a sequencing batch reactor (SBR) fed with acetate as the sole carbon source at 35 °C. The settling time was gradually decreased from 15 to 3 min over the course of two weeks and was maintained for the duration of the experiment (6 months). Operation of the reactor was similar as described in Pronk et al. (2015b). In short, the 3-h cycle starts with a plug flow feed period of 1 h of anaerobic feeding at 1500 mL h⁻¹ from the bottom of the reactor. The feed consists out of 1200 mL of water at 39 °C and 150 mL of N-source and 150 mL of C-source both at room temperature as to result in a final influent temperature of 35 °C. Feeding is followed by a 112-min aeration period, a settling period of 3 min, and a 5 min effluent period in which the total volume in the reactor is brought back to 1.24 L. The reactor is kept at 35 °C throughout the whole cycle and the dissolved oxygen in the aeration period is set at 60% (3.4 mgO₂ L⁻¹). The pH is controlled between 7.1 and 6.9 by 1 M solutions of HCl and NaOH respectively. Solid retention time is kept at approximately 20 days by removing an appropriate part of the bacterial granules every week. The concentrated C-source medium contained 92 g of sodium acetate per 10 L of demi water. The concentrated N-source medium contained per 10 L of demi water; 11.26 g NH₄Cl, 1.25 g K₂HPO₄, 0.5 g KH₂PO₄, 8.8 g MgSO₄·7H₂O, 3.5 g KCl and 90 g trace element solution as described in Pronk et al. (2015b).

2.2. Morphology of granular sludge

To elucidate the morphology of the granular sludge obtained, samples from the reactor were examined by using Scanning Electron Microscope (SEM) (JSM 6500F, JEOL, USA) and stereo-zoom microscope (Leica Microsystems Ltd, M205 FA, Germany) in combination with Leica Microsystems Qwin (V3.5.1) image analysis software, respectively. For SEM, lyophilized whole granules were mounted on specimen stubs and sputter coated with 10 nm carbon before imaging. A JEM-100C JEOL (USA) Transmission Electron Microscopy (TEM) was performed on thin-sectioned bacterial cells. The cells were first prefixed in 0.1% of OsO₄ for 1 h at 20 °C and then fixed in 1% OsO₄ for 24 h at 4 °C. After removal of the fixative by centrifugation, the cells were mixed with 1% melted agarose, cut in small pieces and dehydrated in a series of EtOH from 50 to 100%. The dehydrated material was embedded in Epon resin and after 3 days solidification the resulting block was subjected to thin sectioning using an ultramicrotome. The thin sections were first stained with 1% uranyl acetate for 1 h and post stained with 1% lead citrate for 40 min (Dawes, 1971). To show the granule structure clearly, slices of the granules embedded in agar were stained with acridine orange overnight before examination under the

microscope. Staining protocol used according to Münch and Pollard (1997).

2.3. Molecular techniques

Samples were taken from the reactor and hybridized at 35% formamide using ALF1b for Alphaproteobacteria (Manz et al., 1992). For *Deffluviococcus* clusters I (DFImix) and II (DFIIImix) species probes developed by Wong et al. (2004) and Meyer et al. (2006) were used respectively. Slides were examined using a Axioplan 2 epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with filter set 26 (bp 575 - 625/FT645/bp 660 - 710), 20 (bp 546/12/FT560/bp 575 - 640), 17 (bp 485/20/FT 510/bp 5515 - 565) for Cy5, Cy3 and fluos respectively. Stringent DNA extraction using liquid nitrogen and subsequent DGGE analysis were performed as described by (Pronk et al., 2015a).

2.4. Extraction of alginate-like extracellular polymers (ALE) and acid soluble extracellular polymeric substance (acid soluble EPS)

ALE extraction was done according to Lin et al. (2013). Granules were collected from the lab-scale reactor. Fresh granules (1 g in dry weight) were added into 100 mL 0.5% (w/v) Na₂CO₃ solution and heated for 30 min at 80 °C. After centrifugation, the supernatant was used for the ALE extraction, and the pellet was used to extract the acid soluble EPS.

The pH of the supernatant was changed to 2.2 with 1 M HCl and centrifuged to get the ALE precipitate.

The pellet which was used to extract the acid soluble EPS was washed 3 times with demineralized water and re-suspended in 100 mL 0.4 M acetic acid. The solution was then stirred and heated in a water bath at 95 °C for 20 h. Then the suspension was centrifuged for 20 min. The supernatant was collected and filtered through 0.2 µm filter. Then ethanol (98%, 1:1 vol) was added to the supernatant. The precipitate was lyophilized and named “acid soluble extracellular polymeric substance” (acid soluble EPS). For the complete extraction scheme (ALE and acid soluble EPS), please see Supplementary 1.

2.5. Chemical composition analysis of the acid soluble EPS

2.5.1. Elemental composition

The elemental compositions of lyophilized EPS were analysed in terms of carbon (C), hydrogen (H) and nitrogen (N) by purge-and-trap chromatography (VARIO Elementar EL, Elementar, Germany), sulphur (S) by ion chromatography (IC 883 Plus, Metrohm, Netherlands). Oxygen was calculated as residual minus the inorganic part.

2.5.2. FT-IR

The Fourier transform infra-red spectrum of the lyophilized acid soluble EPS was recorded on a FT-IR Spectrometer (Perkin Elmer, Shelton, USA) at room temperature, with the wavenumber range from 550 cm⁻¹ to 4000 cm⁻¹.

2.5.3. Raman

Raman spectra of the lyophilized acid soluble EPS over the spectral range 100–3000 cm⁻¹ were obtained with a dispersive-type Renishaw micro spectrometer, (InVia Reflex, UK), equipped with a CCD detector. A Leica microscope with objective magnification ×50 with a numerical aperture of 0.75 was used to focus the laser beam on the sample placed on an X-Y motorized sample stage. Excitation source was provided by a 785 nm diode laser. Near-infrared illumination was chosen to reduce sample intrinsic fluorescence. The spectrum was recorded with 50% (~

30 mW/ μm^2) laser intensity on the sample, with five accumulations using 10 s exposure time.

2.5.4. Nuclear Magnetic Resonance (NMR) spectroscopy

The NMR spectra of the lyophilized acid soluble EPS were recorded using an Avance-400 (Bruker Co., Billerica, MA, USA), equipped with 5 mm NMR tubes at 25 °C in acidified D_2O . The ^1H and ^{13}C spectra were recorded at 100 MHz and 400 MHz, respectively. The chemical shift (δ) was internally related to t-Butanol at 1.2 ppm and 31.2 ppm in the ^1H and ^{13}C spectra, respectively. The carbon spectra were acquired using a 2 s recycle delay, a sweep width of 200 kHz (dwell time of 5 ms), and an acquisition time of 102.4 ms.

2.5.5. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The lyophilized acid soluble EPS (100 mg) was hydrolysed in 10 mL 0.6 M HCl at 100 °C for 12 h, filtered through 0.2 μm filter afterwards and analysed by HPAEC-PAD on a Dionex CarboPac PA20 column (Thermo Fisher, USA) using 200 mM NaOH as the eluent.

2.6. Locating acid soluble EPS in granular sludge

2.6.1. Lugol's staining of the biomass

The aerobic granules were homogenized by a Glass/Teflon Potter Elvehjem homogenizer. The homogenized suspension (200 μL) was smeared on the glass slide and air-dried. Then 1 mL Lugol's solution was added. The excess Lugol's solution was removed and replaced with 1% sulfuric acid. The slide was covered and observed under the light microscope. As a control, the acid soluble EPS extracted from granules (100 mg) were dissolved in 10 mL 10% (v/v) acetic acid. Lugol's solution (1% w/v, 1 mL) was dropped into this EPS solution afterwards.

2.6.2. Glycoconjugates lectin staining

Glycoconjugates were detected in selected aerobic granular sludge samples of the BC-SBR by fluorescence lectin-binding analysis (FLBA), according to (Staudt et al., 2004) and (Zippel and Neu, 2011). After testing a number of lectins, the RCA I lectin of *Ricinus communis* was selected for imaging. Other fluorochromes used comprised Syto60 (nucleic acid specific) and SyproOrange (protein specific). Stained granules were mounted in a cover well chamber with spacer and examined with an upright TCS SP5X confocal microscope (Leica, Germany). The super continuum laser source allowed excitation at optimal wavelength. Images were projected with Imaris ver. 8.2 (Bitplane) and printed from Photo-shop CS6 (Adobe, USA).

3. Results

3.1. Morphology of granular sludge and the dominant microorganism

A detailed description of granule development and performance of the reactor is published in Pronk et al. (2015b). Full nitrification and partial denitrification occurred in the system. No biological phosphorus release and uptake was observed. The most interesting aspect of this reactor is that the aerobic granules have a very special growth morphology that is completely different from what has been reported in literature so far.

Generally, aerobic granular sludge is comprised out of several microbial layers from the core to the fringe. Different layers are sometimes composed of distinct bacterial species with various functional tasks, such as nitrification, denitrification, ammonia oxidation, phosphorus removal, etc. (McSwain et al., 2005; Weber

et al., 2007). In each layer, bacteria grow in micro colonies and single cells embedded in a polymer matrix (Lin et al., 2013; Lochmatter and Holliger, 2014).

The granules grown in this study are built up by a few entangled tubes attached to each other (Fig. 1a–c). The average size of the tube is around 300 μm in diameter and 1000 μm in length (Fig. 1c–d). Apparently, a tube instead of a layer is the building unit of the granules obtained. When a granule was strongly agitated, it fell apart into single tubes, but the structure of every single tube remained intact. This indicates that the structure of a single tube is much stronger than the connection between tubes. This might be due to the differences in the composition and/or chemical linkage between the EPS that contribute to the formation of the tube itself and the connection among tubes. Morphology of the inner structure of the tube can be seen by examining the thin sections shown in Fig. 1e and f. The longitudinal section displays the distribution of the microorganisms inside the tube. Interestingly, the tube is not fully filled with microorganisms; there are still hollow spaces near the centre of the tube. The cross section reveals the morphology of the clusters of microorganisms: the clusters are fully dominated by cubical shaped packets (Fig. 1f). Each packet includes a few cells.

The inner structure of the tubes was studied by SEM as well. The dominating bacteria are coccus-like (Fig. 2a–b). The cells are in a packet configuration of eight or more cells, which is the typical sarcina growth morphology. Those sarcina cocci are piled up through the connection between the packets as seen in Fig. 2d. By means of SEM no EPS matrix in which the packets were embedded was observed probably due to preparation artefacts. The fine structure of both the packet and the coccus cell were investigated by TEM thin section analysis (Fig. 2c–d). TEM showed that the packet is surrounded by a 20 nm fibrous EPS layer (Fig. 2e). This layer serves as an envelope, which holds cells into the many-celled packets. Each cell within a given packet has a 20–40 nm EPS capsule surrounding the cell wall.

3.2. Identification of the dominant microorganism

The dominant microbial species in the granules were further investigated by DGGE and FISH. DGGE extraction was performed to elucidate the variety of species present within the granule. All bands obtained were excised and sequenced to be able to construct a phylogenetic tree presented in Fig. 3 (for full tree see Supplementary 2). Sequences were deposited into Genbank under accession number KX656678–KX656699. Data showed that the abundant microbial species was most likely, *Deffluviococcus* Cluster II related. To validate this observation FISH was performed. Hybridization with the ALFb1 probe showed that the most dominant species present in the granules belonged to the alpha-proteobacteria (Fig. 4c and d). EUB, staining most bacteria, almost completely overlaps with ALFb1 further indicating that the alpha proteobacteria are the dominate species. Moreover, the sarcinae cocci were abundantly present; very few other morphological cell types could be observed microscopically (Fig. 4a). The more specific DF2 probes (Meyer et al., 2006) did not hybridize successfully with the sample from the reactor.

3.3. EPS extraction

In order to understand the role of EPS in the formation of this special granular sludge, the structural EPS was extracted. Since ALE was reported as structural EPS that formed the gel structure in aerobic granular sludge treating municipal wastewater (Felz et al., 2016; Lin et al., 2010), the presence of ALE was investigated first. Extremely different from what is generally reported in the literature, the granules still kept most of the granular shape after being

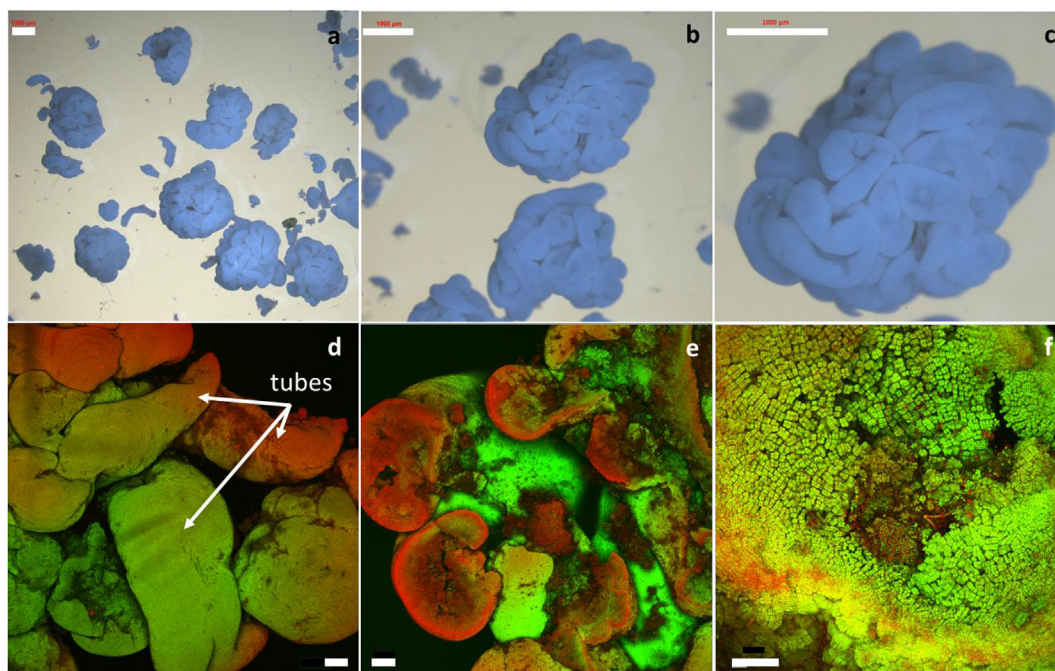


Fig. 1. Structure of aerobic granules obtained from an aerobic granular sludge reactor fed with acetate at 35 °C observed under a stereo-zoom microscope (a–c); acridine orange stained granules to visualize the structure, showing an intact granule (d) and a sliced granule (e and f). Size bars (a, b and c) are 1000 µm, (d, and e) are 100 µm and (f) is 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

heated in 0.5% (w/v) Na_2CO_3 at 80 °C for 30 min (Fig. 5a). The yield of ALE was less than 1% of the organic fraction of the granules, which was much lower than the reported yield of 20% (Felz et al., 2016). As it is emphasized by Felz et al. (2016) that, solubilizing the granular structure (i.e. granular structure is completely disappeared after extraction) is a prerequisite step to extract the structural EPS, while the granules from this study were not solubilized at all with the ALE extraction method, meaning ALE cannot be considered as the structural EPS of the *Defluviococcus* dominated granular sludge. Therefore, polymers other than ALE may represent the structural polymer in the granules obtained in this study.

In contrast, the granules were solubilized when they were put into 0.4 M acetic acid at 95 °C for 20 h after the treatment of Na_2CO_3 . Under these conditions, the granular structure completely disappeared. The polymer extracted was $166 \pm 6 \text{ mg g}^{-1}$ granular sludge (organic fraction). As this polymer was extracted after solubilizing the granules in acetic acid, it is referred to as “acid soluble EPS” and is considered as the structural polymer of the *Defluviococcus* dominated granular sludge.

To compare, aerobic granules from a laboratory reactor and a full-scale aerobic granular sludge reactor treating domestic wastewater were also subjected to the ALE and acid soluble EPS extraction protocols. Not more than 3.9% acid soluble EPS could be extracted from those two kinds of granules (Supplementary 3), indicating that the acid soluble EPS is dominant in the specific granules of this research.

3.4. Chemical composition of the acid soluble EPS

To reveal the chemical composition of the acid soluble EPS, various analytical methods were used. The molecular formula of the extracted polymer was determined by an elemental composition measurement ($\text{C}_6\text{H}_{13.7}\text{O}_{5.7}\text{n}$). No nitrogen was detected in the acid soluble EPS, indicating the absence of proteins and amino sugars. The polymer has a similar molecular formula as that of

carbohydrates ($\text{C}_6\text{H}_{10}\text{O}_5\text{n}$), except that the hydrogen content is relatively high. This could indicate the presence of substitute groups like methyl or ethyl units.

The FT-IR spectrum of the acid soluble EPS is a typical carbohydrate spectrum (Fig. 6a), the peaks at wavenumber 3313 cm^{-1} and 2928 cm^{-1} correspond to hydroxyl group and C–H bond stretching, respectively (Cael et al., 1975). The peaks at 1420 cm^{-1} and 1366 cm^{-1} are attributed to CH_2 and CH bending. The broad band between 1150 cm^{-1} and 950 cm^{-1} are in accordance with the stretching vibration of pyranose rings (Synytsya and Novak, 2014). The peaks at 929 cm^{-1} and 847 cm^{-1} are characteristic peaks for α -linkage between monomers (Synytsya and Novak, 2013).

Raman and IR spectroscopy are complementary to each other. Strong IR bands are related to polar functional groups whereas non-polar functional groups give rise to strong Raman bands (Cho, 2007). In the Raman spectrum of the acid soluble EPS, the peak around 2910 cm^{-1} is a typical peak of $-\text{OCH}_3$ of glucose (Engelsen, 2016) (Fig. 6b). Two peaks ranging from 1420 to 1310 cm^{-1} are attributed to bending vibrations of the CH_2 and CH_3 groups. The peak at around 945 cm^{-1} is characteristic of the stretching of the C–C skeletal backbone structure (Cho, 2007), which is frequently observed in 3,6 anhydro-galactose. The high intensity of this peak is an indication of a crystal structure.

In a ^1H NMR spectrum, the peaks at 5.3 ppm and 4.9 ppm represent the glycoside α -(1 → 4) and α -(1 → 6) linkage type, respectively (Miao et al., 2014). The ^1H NMR spectrum of the acid soluble EPS shows a peak at 5.3 ppm, but no significant peak at 4.9 ppm (Fig. 6d). This indicates that the acid soluble EPS predominantly contains α -(1 → 4) polysaccharide linkages. The band of peaks between 3.2 and 4 ppm are associated with the pyranose ring. The triplet at 1.1 ppm is attributed to the hydrogen atoms in an $\text{O}-\text{CH}_3$ group.

All peaks in the ^{13}C NMR spectrum of the acid soluble EPS fall within the region that is typical for polysaccharides (Fig. 6c). The peak at 99.9 ppm is the typical peak of C-1 in α -(1 → 4) linkage

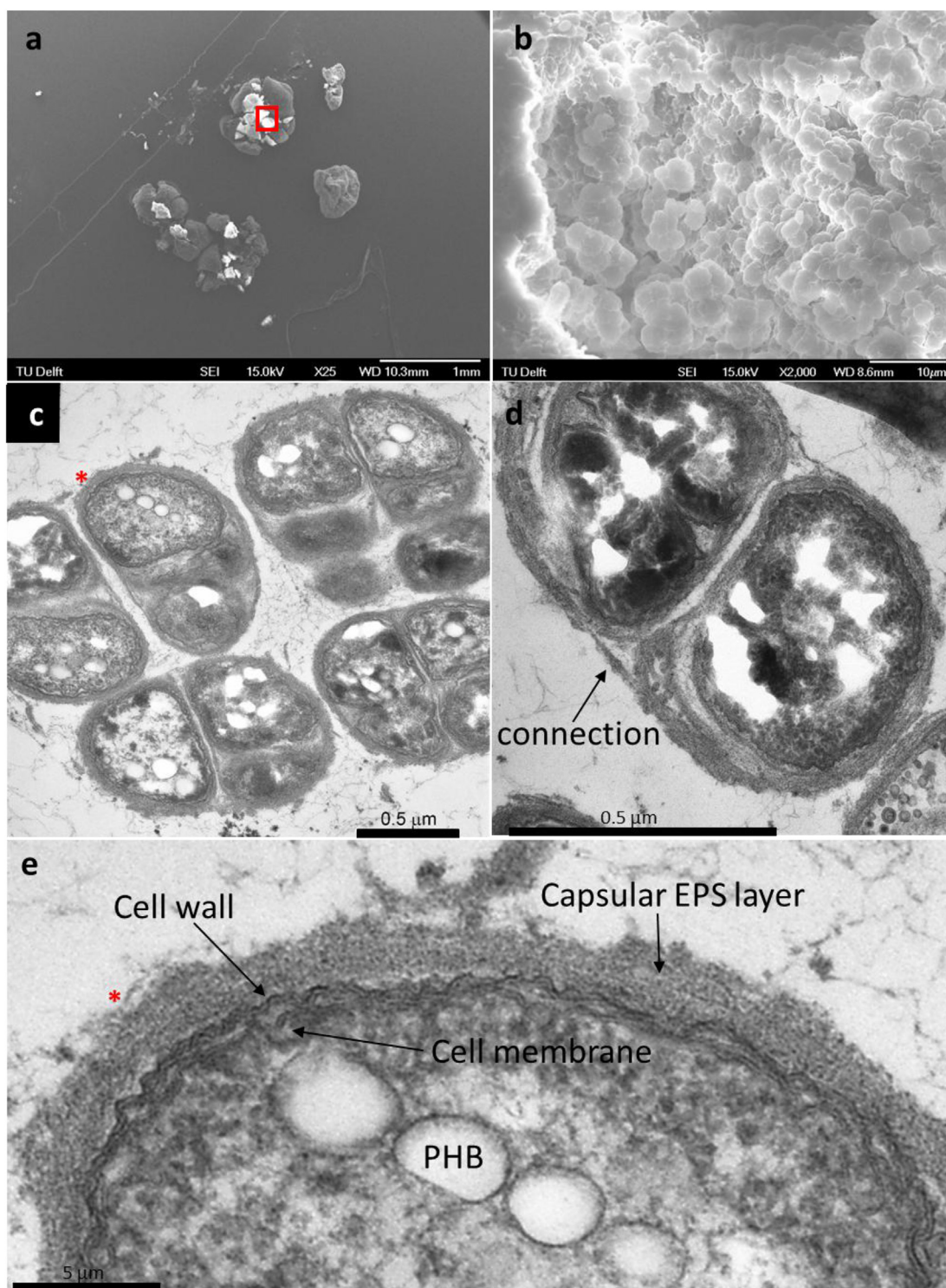


Fig. 2. Scanning Electron Microscope (SEM) picture of the granular sludge dominated by *Defluviicoccus* cluster II species (a–b). Transmission electron microscopy (TEM) of thin sections of pottered aerobic granules (c–e), with asterisk in c (*) showing the enlargement area in e.

(Synytsya and Novak, 2014). The peaks at 71.4, 71.8, 73.1 and 73.6 ppm are attributed to pyranose ring carbons C-4, C-5, C-2 and C-3, respectively. The peak at 61 ppm is attributed to the C-6 of the pyranose ring (Seymour et al., 1979). The peak at 57 ppm is due to the C of an O–CH₃ group. Thus, result of ¹³C NMR completely matches that of the ¹H NMR, the acid soluble EPS is α -(1 → 4) linked polysaccharides with O–CH₃ group as substitution.

According to HPAEC-PAD measurement, the acid soluble EPS contains both glucose and galactose as monomers, with glucose as the dominant monomer.

In summary, the acid soluble EPS is α -(1 → 4) linked polysaccharide, containing both glucose and galactose as monomers. There are –OCH₃ groups connected to the glucose monomer.

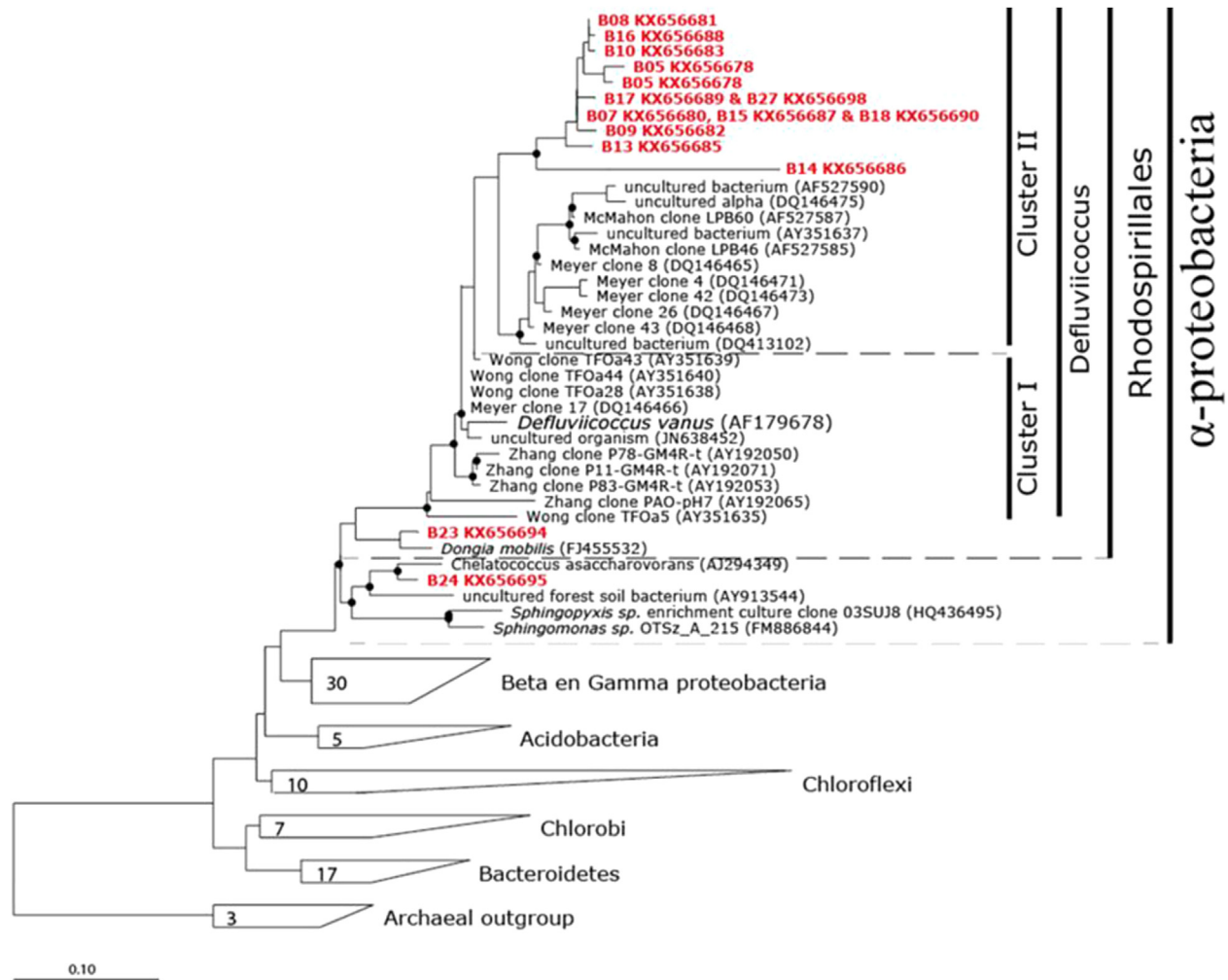


Fig. 3. An excerpt from the phylogenetic tree (left) (full tree in [Supplementary 2](#)).

3.5. Visualization of the acid soluble EPS in granular sludge

3.5.1. Lugol's staining of the biomass

It was found that the acid soluble EPS reacts with Lugol's solution, forming an insoluble purple red complex (Fig. 7). This property was used to locate the EPS in granular sludge by staining a biomass smear. The insoluble purple red complex deposited on the dominant sarcinae coccus bacteria clusters belong to *Defluviococcus* cluster II related species. The more aggregated the cluster was, the more dense the purple colour was, implying that the acid soluble EPS is produced by the *Defluviococcus* as a capsular bound EPS. As no EPS matrix was observed by both SEM and TEM, possibly the acid soluble EPS comes from the fibrous layer surrounding the packets and/or the capsule EPS layer of a single coccus.

3.6. Fluorescence lectin binding analysis (FLBA)

Fluorescently labelled lectins represent an alternative approach for detecting microbial EPS glycoconjugates. Lectins are carbohydrate-binding proteins of high affinity and specificity, which make them suitable probes for detecting specific glycoconjugates in hydrated samples. The lectins tested showed differential binding to the granules. Several lectins bound to microbial structures in between the spherically clustered capsulated bacteria. This morphological characteristic of the granule was also

demonstrated by simple protein staining (Fig. 8 top). The RCA I lectin specific for galactose showed a strong signal from the typical capsulated bacteria (Fig. 8 middle). In combination with nucleic acid staining the granule morphology established with other imaging techniques could be confirmed (Fig. 8 bottom).

4. Discussion

4.1. Extract the structural EPS by solubilizing granules

EPS is responsible for the chemical structure and physical properties of granules and biofilms (Flemming and Wingender, 2010). Various approaches are applied to extract EPS (Adav and Lee, 2008; D'Abzac et al., 2010; Liu and Fang, 2002; Pan et al., 2010). Most of the approaches intended to be an "one procedure fits all" method. However, due to the complexity of EPS, it is almost impossible to extract all the potential EPS components by one single method (Sutherland, 2001). In fact, extraction methods have to be adapted to the specific type of EPS forming the dominant structure in granules and biofilms.

In our previous research, the focus was put on extracting the structural EPS from aerobic granular sludge (Felz et al., 2016). It was assumed that, although there are various components in EPS, only some of them have the function of being responsible for the granular structure (it was proposed to specifically reference to

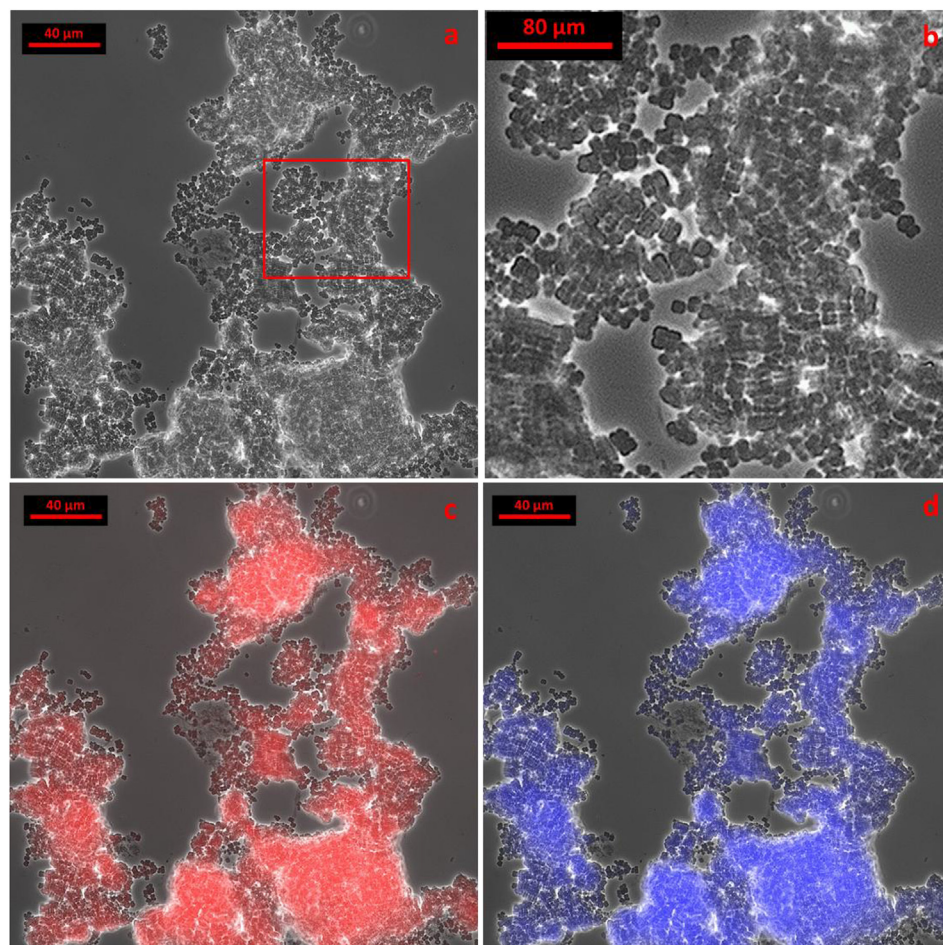


Fig. 4. Light microscope images of homogenized granules; phase-contrast 1000 \times magnification (a), an excerpt and enlargement indicated by the red square (b), epifluorescence image of hybridized sample (c–d), red = ALFb1 probe, blue = EUB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Fig. 5. A comparison between granules treated with sodium bicarbonate to extract ALE fraction (a) and acetic acid at 95 °C to solubilise the acid soluble EPS (b). Scale bar indicates 2 mm.

them as structural EPS); the other compounds are “fillers” so to speak in the granular structure. Only if the granular or biofilm structure is solubilized, the structural EPS will be extracted. Consequently, different structural EPS were extracted from various sources of granular sludge. Alginate-like extracellular polymers

(ALE) were obtained from aerobic granular sludge feeding municipal wastewater. ALE forms ionic gel with Ca^{2+} (Felz et al., 2016; Lin et al., 2010). Glycoproteins containing EPS were isolated from granular sludge containing mainly the anammox bacteria. This EPS may have a comparable function as that in multicellular organisms,

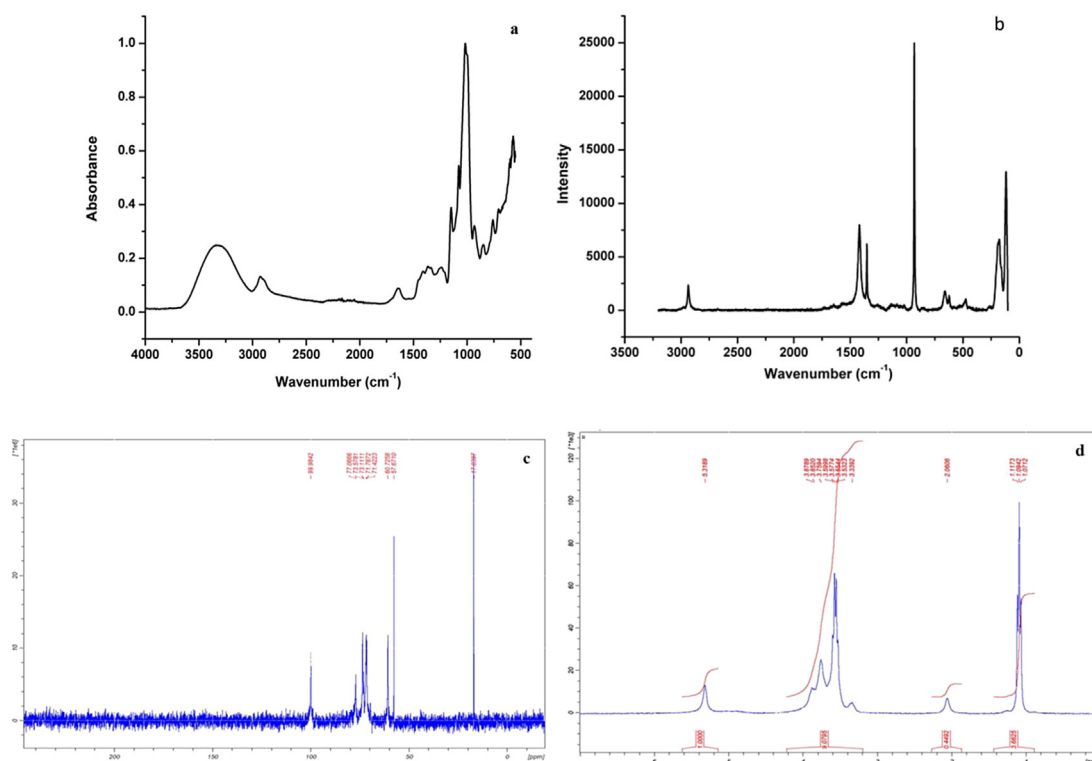


Fig. 6. The FTIR (a), Raman (b), ^{13}C NMR (c) and ^1H NMR (d) spectra of the acid soluble EPS extracted from aerobic granules.

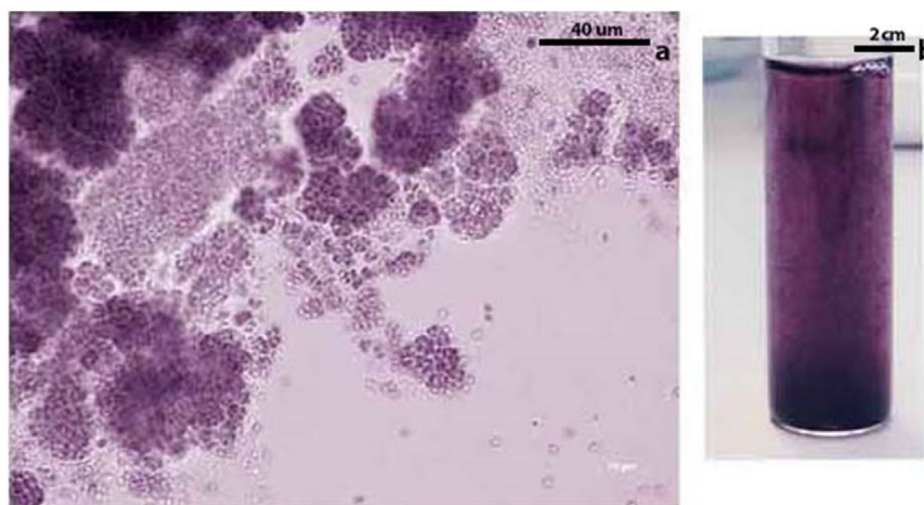
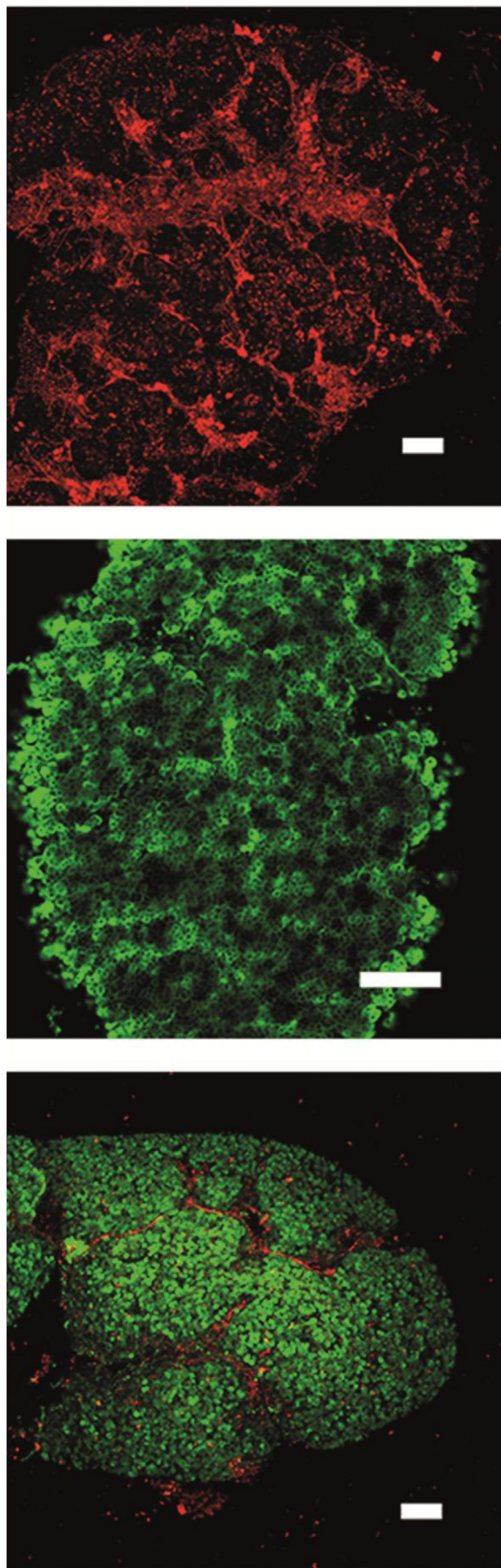


Fig. 7. Biomass smear (a) and extracted EPS (b) stained with Lugol's solution.

e.g. form a gel matrix and be a protective barrier for the cells (Wang et al., 2005).

In the current research, ALE is not the structural EPS, because the granular structure was still intact after following the ALE extraction procedure. The acid soluble EPS is considered as the structural EPS as after extraction the granular sludge was completely solubilized. This EPS is a polymer that consists of O-methylated glucose and galactose as monomers. The composition is completely different from that of ALE, Granulan and glycoproteins containing EPS, respectively (Felz et al., 2016; Lin et al., 2010, 2013; Seviour et al., 2010). The discovery of this acid soluble EPS provides another proof that the structural EPS is different from one kind of

granular sludge to the other. It is therefore necessary to develop specific extraction method that fits for extracting structural EPS from specific granule type. Many EPS extraction methods focus on preventing cell lysis and thus choose for mild conditions ($\sim\text{pH } 7$ and 30°C). However, if the extraction conditions structural of polymers from plants is considered, quite a few polysaccharides (e.g. starch, alginate, cellulose, pectin) will not be solubilized under these conditions and therefore escape extraction. Therefore, for a proper analysis of the structural EPS, the destruction of microbial cells is inevitable.



4.2. Aerobic granular sludge dominated with *Defluviococcus* and its EPS

For the successful extraction of DNA extraction from *Defluviococcus* related organisms adapted extraction protocols were shown to be necessary in previous studies (McIlroy et al., 2008; Meyer et al., 2006; Pronk et al., 2015a). For a successful extraction McIlroy et al. (2008) used a method based on sodium trichloroacetate (Na-TCA) with Ballotini beads (0.1 mm) addition for mechanical disruption of the cells. Meyer et al. (2006) needed stable isotope probing (SIP) to successfully recover DNA. In this study, only a simple liquid nitrogen step was used as a pre-treatment (as proposed by Pronk et al., 2015a, b, c). With the introduction of the liquid nitrogen extra DGGE bands appeared that were not seen with the standard extraction method. The fact that several studies need adaptation from standard extraction protocols may indicate that the acid soluble EPS could be a barrier to the DNA extraction of *Defluviococcus* related organisms. That is, the acid soluble EPS protects the cell from extraction conditions. Standard extraction kits work in the range of pH 8, which would obviously not dissolve the acid soluble EPS and let the reagents penetrate into the cell afterwards. This might also be an explanation for the commonly reported difficulties in recovering the DNA in sludge's that contain *Defluviococcus* related species (McIlroy et al., 2008; Meyer et al., 2006). Furthermore, these findings indicate that metagenome studies might be hampered by limited extraction when the EPS is not properly solubilized.

DGGE and subsequent sequencing showed that the organisms fall within the *Defluviococcus* Cluster II. However, the FISH probes available and specific for this cluster did not hybridize. This might be due to the fact that, the existing probe likely does not work on additional species. New probes have to be developed to identify additional species within the *Defluviococcus* Cluster II (Meyer et al., 2006).

4.3. Connecting granule morphology with the structural EPS

The granular sludge cultivated in the current research this study had a tubular growth pattern with a few entangled tubes connected with each other (Fig. 1). Granules usually grow in spheres with a hydrogel as a matrix (Arrojo et al., 2004; Lemaire et al., 2008). Here the granule morphology is completely different than is normally observed under similar reactor operation and designs (Bassin et al., 2012; de Kreuk and van Loosdrecht, 2004; Wagner et al., 2015; Winkler et al., 2011). Therefore, the growth morphology is most likely connected to the bacterial species obtained rather than other (physical) factors.

No EPS matrix was observed by SEM or TEM. Instead, the sarcinae cocci formed cubical shaped packets. Each packet contains eight or more cells. Packets were stacked in each tube mainly through the connection of the acid soluble EPS, which is tightly bound to the cell surface. The basic cellular architecture was demonstrated by means of FLBA in combination with laser microscopy (Fig. 8).

Fig. 8. Confocal laser scanning microscopy of hydrated granules after staining with different probes. Images are shown as maximum intensity projections. Top – protein staining indicated the cell surfaces of spherically clustered bacteria together with other, often filamentous microbes, linking the clustered packages. Middle – lectin staining showing the specific binding of RCA I to the large capsules of clustered bacterial cells. The cluster-linking bacterial cells are not visible. Bottom – RCA I lectin signal (green) in combination with nucleic acid staining (red). Take notice of the red nucleic acid signal inside the capsulated bacteria. Scale bar = 20 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Similarly, cells from sarcinae packets (e.g. *S.ventriculi* strains) were reported to have a thick, fibrous layer consisting of cellulose or cellulose-like material surrounding the packets (Canale-Parola, 1970). This material, which accounted for up to 19% of the total dry weight of the cells, serves as an intercellular cement that holds the cells together into packets. Another example of organisms with such a growth structure is *Methanosarcina*. A key characteristic of *Methanosarcina* is their multicellular (packet) morphology by aggregates of spatially confined cells that lead to their specific aggregate structure (Milkevych and Batstone, 2014). Those packets are surrounded by methanochondroitin, a heteropolysaccharide, as an outer layer consisting of a repeating trimer of two N-acetylglucosamines and one glucuronic acid (Claus and König, 2010; Kreisl and Kandler, 1986).

The special morphology of *Defluviococcus* dominated granules thus seems to be related to the growth morphology and property of the acid soluble EPS that holds the cells together into the large, many-celled spherical packets. Future research may focus on developing a cell mechanical model to explain the tubular growth pattern and studying the contribution of the acid soluble EPS on environmental tolerances.

5. Conclusion

Aerobic granular sludge dominated with *Defluviococcus* Cluster II species exhibit a special tubular growth pattern instead of a layered structure. Acid soluble extracellular polymeric substances (acid soluble EPS) play a structural role in the formation of this special granule morphology. This new structural EPS is α -(1 → 4) linked polysaccharide, containing both glucose and galactose as monomers. There are $-OCH_3$ groups connected to the glucose monomer. This study shows that there is not a single extraction method for all possible extracellular polymeric substances. Specific EPS extraction methods have to be developed with focus on the particular type of granular sludge and the EPS of interest. If the structural EPS is the target to be extracted, solubilisation of the granular structure is the prerequisite step.

Acknowledgements

The authors would like to thank Kristina Djanashvili for the supply of NMR data, Lijing Xue for the help with FTIR analysis, Jos van Brussel for the elemental analysis, Cor Ras for his insights on the polymer characteristics, and Cristian Picioreanu for the discussion on sarcinae coccus. The Acridine Orange staining was performed by M.K. de Kreuk (Delft University of Technology, the Netherlands and B. Pitts from Montana State University, USA.) The excellent support by Ute Kuhlicke in laser microscopy and image analysis is highly appreciated.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2017.05.068>.

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