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Unravelling gradient layers of microbial communities, proteins, and chemical structure in aerobic granules

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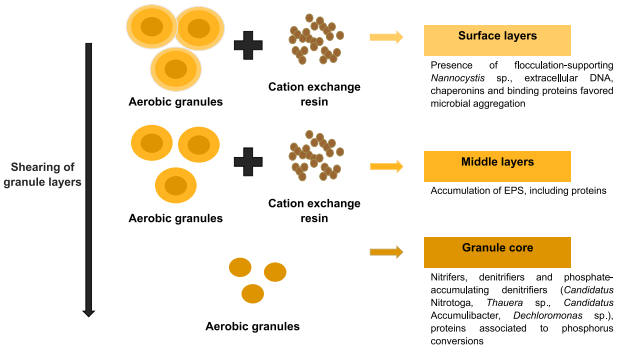
HIGHLIGHTS

- Gentle extraction allowed gradual erosion of granule layers.
- Microbial community, EPS and protein layering were assessed in aerobic granules.
- Versatile EPS-producers were evenly distributed throughout the granule structure.
- *Candidatus* Nitrotoga, *Thauera* sp., and *Dechloromonas* sp. predominated in granule cores.

GRAPHICAL ABSTRACT



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ABSTRACT

Most bacteria live in microbial assemblages like biofilms and granules, and each layer of these assemblages provides a niche for certain bacteria with specific metabolic functions. In this study, a gentle (non-destructive) extraction approach based on a cation exchange resin and defined shear was employed to gradually disintegrate biomass and collect single layers of aerobic granules from a full-scale municipal wastewater treatment plant. The microbial community composition of granule layers was characterized using next-generation sequencing (NGS) targeting the 16S rRNA gene, and protein composition was investigated using metaproteomics. The chemical composition of eroded layers was explored using Fourier Transformed Infrared Spectroscopy. On the surface of the granules, the microbial structure (flocculation-supporting *Nannocystis* sp.) as well as composition of extracellular polymers (extracellular DNA) and proteome (chaperonins and binding proteins) favored microbial aggregation. Extracellular polymeric substances in the granules were composed of mostly proteins and EPS-producers, such as *Tetrasphaera* sp. and *Zoogloea* sp., were evenly distributed throughout the granule structure. The interior of the granules harbored several denitrifiers (e.g., *Thauera* sp.), phosphate-accumulating denitrifiers (*Candidatus* Accumulibacter, *Dechloromonas* sp.) and nitrifiers (*Candidatus* Nitrotoga). Proteins associated with glycolytic activity were identified in the outer and middle granule layers, and proteins associated with phosphorus conversions, in the deeper layers. In conclusion, the use of an existing cation-exchange resin for gradual biomass disintegration, combined with NGS and metaproteomic analysis was demonstrated as a promising approach for simultaneously investigating the identity and functions of microbes in multilayered biofilm structures.

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1. Introduction

Aerobic granular sludge (AGS) is a promising technology for wastewater treatment that is being intensively studied because its unique features enable efficient removal of nutrients and micropollutants from wastewater (Cydzik-Kwiatkowska et al., 2017; Sepúlveda-Mardones et al., 2019). These features are the result of the granules' structure, which provides superior solid-liquid separation capabilities due to their short settling time, and a high density of microorganisms per unit of reactor volume. The granule layers create diffusional gradients of electron donors and acceptors, resulting in a continuum of redox conditions that enables simultaneous removal of carbon, nitrogen, and phosphorus, even in constantly-aerated granular batch reactors. Although granules are now successfully applied in full-scale wastewater treatment plants (WWTPs) (Pronk et al., 2017), the mechanisms underlying aerobic granulation, and the interactions between microorganisms inhabiting AGS are far from understood (Wilén et al., 2018).

In complex microbial structures, microorganisms are embedded in a matrix of extracellular polymeric substances (EPS) that holds them together, protects the consortia from unfavorable environmental conditions and provides carbon during famine conditions (Zhang et al., 2007; Cydzik-Kwiatkowska, 2021). Thus, EPS plays a key role in granule formation but difficulties in extracting these polymers from granules, and the use of divergent methodologies with either high structural resolution or sensitivity have led to contradictory reports regarding the role of EPS in granulation and the operational parameters that influence EPS production. On the one hand, one study indicated that β-polysaccharides are crucial for granule stability, whereas extracellular proteins, lipids, and α-polysaccharides are not (Adav et al., 2008). On the other hand, another study indicated that proteins and α (1–4) glucans were important for aerobic granulation (Zhu et al., 2012).

The composition of tightly bound EPS varies in the individual granule layers, as indicated by staining of cryosectioned granules (Rusanowska et al., 2019). However, the most widely applied methodologies for extracting EPS are of limited use for examining EPS composition between granule layers. Cation exchange resins (CER) bind divalent and trivalent ions (e.g., Ca^{2+}, Mg^{2+} or Fe^{3+}) and thereby destabilize the chemical groups and polymers in the dense EPS matrix (Kończak et al., 2014). As a result, the entire structure of the granule is disintegrated, and a mixture of polymers from throughout the granule is released.

Although granular and conventional activated sludge share a core microbiome, the distribution of microbial populations differs significantly in relative abundance and structural localization in the architecture of granules and flocs (Winkler et al., 2018). Diffusion limitations in granules create different microenvironments that favor microbial biodiversity and increase stability of the treatment process. To date, most studies have visualized the spatial arrangement of microorganisms in granules using fluorescence in situ hybridization, either on total biomass or on cryosectioned granules. More representative and in-depth results can be achieved if next-generation sequencing (NGS) could be applied directly to analyse microbial composition in particular granule layers.

Efficient wastewater treatment is determined by the metabolic activity of the microorganisms and it is therefore important to understand how microbial metabolism varies within the multi-layered structure of aerobic granule. Previous published reports have described the general activity of microorganisms from whole granules (Bassin et al., 2011; Cydzik-Kwiatkowska et al., 2020). Recent reports of shotgun proteomics for investigation of protein composition in AGS revealed that proteins in tightly bound EPS were mainly engaged in metabolic functions and to a lesser extent as dedicated structural components (Yang et al., 2021). However, mass spectrometry has not yet been used to study how metabolic activities change across granule layers.

The present study applied a variation of the CER-based approach for EPS extraction to examine the microbial, proteomic, and chemical characteristics of individual granule layers. In this approach, the mixture of CER and aerobic granules was exposed to continuous gentle shearing forces, to gradually shear off layers of the granules for analysis. The microbial community composition was investigated using 16S rRNA gene amplicon sequencing, metaproteomics was applied to explore the protein composition, and the chemical characteristics of the granule layers were characterized using Fourier-Transformed Infrared Spectroscopy (FTIR).

2. Materials and methods

2.1. Granular sludge samples

The sludge was collected from a municipal WWTP in Jeziorany (Poland). The WWTP was modernised to operate using aerobic granular sludge technology in 2014. The plant conducts organics removal and treats around 500 $m^3 \cdot d^{-1}$ (Cydzik-Kwiatkowska et al., 2018). The concentration of mixed liquor suspended solids (MLSS) in the sample was 6.0 $g \cdot L^{-1}$ with a volatile suspended solids (VSS) content of 65%. The measurement of MLSS was performed using moisture analyser HE53 (Mettler Toledo, USA) while VSS were measured according to APHA (1992). The predominating aerobic granule size was estimated to $Ø = 0.3$ mm based on microscopic examinations. After 5 min of settling, the sludge volume index (SVI) was 91 $mL \cdot g^{-1}$ MLSS, and after 30 min of settling, it was 45 $mL \cdot g^{-1}$ MLSS. The biomass was washed three times with distilled water. Subsequently, 5 g $MLSS \cdot L^{-1}$ of washed granular sludge was used for the shearing experiment.

2.2. Shearing experiment

The experiment was carried out in duplicate in cylindrical plexiglass reactors with a diameter of 10.5 cm and 4 vertical baffles (1 × 13 cm each) (Fig. S1). A single-bladed paddle 12 × 50 mm^2 (heigth × width) placed 4 cm above the bottom of the reactor was stirred at 900 rpm, which corresponded to an average turbulent share rate (G) of 800 s^{-1} as previously determined (Mikkelsen and Keiding, 2002). The preliminary testing research of the shearing of granules was conducted at 500 s^{-1}, 800 s^{-1} and 1100 s^{-1} and indicated that the shearing rate in the investigated range did not affect the biomass integrity and only the biomass loosely attached to the granule structure was released. For all shearing intensities, the turbidity of the samples after 60 min of shearing stabilized at 0.07 OD_{650}/g MLSS. The use of CER at 800 s^{-1} increased the turbidity of samples to about 0.3 OD_{650}/g MLSS, confirming that the disintegration was a result of Ca^{2+} and Mg^{2+} ion removal from the granule structure, a process which did not affect the cell integrity. In the experiment, DOWEX 50 × 8, 20–50 mesh cation exchange resin (CER) was used. It was washed with extraction buffer (2 mM Na_3PO_4, 4 mM NaH_2PO_4, 9 mM NaCl, 1 mM KCl, pH 7) and added to the reactor in the amount of 70 $g \cdot g^{-1}$ VSS as described elsewhere (Frølund et al., 1996). The experiment was carried out continuously at room temperature until turbidity plateaued at 4 h. Turbidity was measured using an UVmini-1240 spectrophotometer (Shimadzu, Japan).

To analyse microbial, protein and EPS composition in individual granule layers, samples were collected from each reactor at the beginning of the experiment and at regular intervals throughout the experiment. The samples were allowed to gravity-settle for 30 min, after which the supernatant was carefully decanted to prevent its mixing with settled suspended solids. It was not possible to distinguish or separate the supernatant from the sample collected at the end of the experiment (240 min) – the biomass was sheared to such an extent that after the assumed time of settling, suspended solids were present in the whole volume of the sample. The samples of supernatant and suspended solids collected from both reactors at a given time-point were pooled and stored at −20 °C until analysis. For FTIR analysis of EPS, the collected biomass was centrifuged at 12,000 × g for 15 min to separate cells and debris before freezing. To evaluate the rate of granule shearing, the size distribution of granules in the suspended solids samples was measured in triplicate using a Laser Diffraction Particle Size Analyzer LS 13 320 SW (Beckman Coulter, USA).

Isolation of total EPS from granules was performed in duplicate as described in Frølund et al. (1996); the Lowry method with bovine serum albumin as a standard and Anthron method against a glucose standard curve

were used to measure concentrations of proteins and polysaccharides in EPS, respectively.

2.3. FTIR

Samples of sheared EPS were lyophilised in a SpeedVac SPD111V (Thermo Scientific, USA). Infrared spectroscopy analysis was performed using a TENSOR II Fourier Transformed Infrared Spectrometer (BRUKER OPTIK GmbH, Germany) equipped with a Platinum ATR accessory. Absorption spectra were recorded between 4000 and 400 cm^{-1} with a spectral resolution of 2 cm^{-1} and 64 scans per sample (n = 4). Baseline correction of the absorption results for each supernatant sample was performed using the ChemoSpec package (https://bryanhanson.github.io/ChemoSpec/) for R version 3.6.2 (R Development Core Team, 2021) in the RStudio environment (https://www.rstudio.com/), version 0.99.

2.4. DNA extraction and 16S rRNA gene amplicon sequencing

Total genomic DNA was extracted from 500 μL of supernatant and suspended sludge samples using a FastDNA SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol. The extracted DNA was quantified using a Qubit dsDNA Broad Range assay kit and a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA). To reduce the effect of PCR drift, amplicon PCR was run in duplicate. The V4 hypervariable region of the 16S rRNA gene was amplified using the well-described F515/R806 primer set (Caporaso et al., 2011). The reaction mix contained 1 × Platinum High Fidelity buffer (Thermo Fisher Scientific, USA), 1.5 mM $MgSO_4$, 400 nM of each dNTP, 400 nM of each primer, 2 mU of Platinum High Fidelity Taq Polymerase (Thermo Fisher Scientific, USA), 10 ng of DNA and water in a final volume of 25 μL. The PCR programme consisted of 2 min at 95 °C; then 35 cycles of 20 s at 95 °C, 30 s at 50 °C and 60 s at 72 °C; and a final elongation for 5 min at 72 °C. The duplicate amplicons were pooled, and the obtained libraries were purified using CleanNGS (CleanNA, The Netherlands), quantity was measured using Qubit dsDNA High Sensitivity assay kit (Thermo Fisher Scientific, USA) and quality was assessed using a TapeStation 2200 with D1000 ScreenTapes (Agilent, USA). The amplicons were barcoded in accordance with the Nextera XT barcoding protocol (Illumina, USA), and subsequently purified and pooled in equimolar amounts. The library pool was sequenced on a MiSeq platform (Illumina, USA) with a MiSeq Reagent Kit v3 (2x300PE) and 20% Phi-X spike-in. The raw sequence data was deposited in the European Nucleotide Archive (ENA) under project accession number PRJEB47090.

The obtained raw sequencing reads were quality checked and processed into amplicon sequencing variants (ASVs) using the AmpProc pipeline version 5.1 (https://github.com/eyashiro/AmpProc), in paired-end mode, using MiDAS version 4.8 as the reference database (McIlroy et al., 2017). For statistical analyses and visualizations, the ampvis2 package (Andersen et al., 2018) for R version 4.0.3 (R Development Core Team, 2021) was used in the RStudio environment, version 1.4.1103.

2.5. Proteome analysis

Protein extraction was performed using a liquid-liquid extraction as previously described (Heyer et al., 2013), changing the resuspension buffer to TEAB (0.05 M TEAB buffer stock, 1.0 $mg{\cdot}L^{-1}$ NaDOC, pH ≤ 8) and the in-gel digestion of extracted proteins were conducted as described elsewhere (Shevchenko et al., 2006). Subsequent desalting and analysis of tryptic peptides by automated liquid chromatograph-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) were performed as previously described (Poulsen et al., 2021).

Q-exactive RAW data files were converted to mzML files using MSConvert (Chambers et al., 2012), and processed using Mascot (v2.5.1) (Matrix Science), searching against the NCBIprot database (2020-12-12), with a peptide tolerance of ±1.2 Da and a MS/MS tolerance of ±0.6 Da.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD028049 and https://doi.org/10.6019/PXD028049.

3. Results

Aerobic granular sludge was collected from the municipal WWTP in Jeziorany (Poland) characterized by removal efficiency of COD, total nitrogen, ammonium nitrogen and total phosphorus of 95%, 91%, 99% and 30%, respectively.

3.1. Shear experiment

The shear experiment was performed to gradually detach exterior layers in the granule structure. Measurement of turbidity of the supernatant showed a linear development during the first 180 min of shearing (Fig. 1a), revealing a disintegration of macrostructure of the granules, and turbidity did not further increase hereafter.

The average granule diameter decreased at a rate of approximately 1 $\mu m{\cdot}min^{-1}$ during the first 180 min of shearing (Fig. 1b). In the final period of shearing (180–240 min), diameter only decreased by an additional 5 μm. Initially the average granule diameter was around 300 μm but at the end of shearing experiment, it had decreased to around 100 μm, which indicates that only very small particles typical for activated sludge remained in the biomass. The size distribution of granules in the suspended solids samples during the course of the experiment is presented in Fig. S2.

3.2. FTIR

Eroded EPS from the various granule layers were analysed by FTIR to identify dominating functional groups. The positions and intensities of respective FTIR peaks indicated that chemical composition of EPS released from particular granule layers was different (Fig. 2). The observed profile in the range of 2000 to 1500 cm^{-1} revealed several peaks associated with proteins and polysaccharides (e.g. carbonyl groups and amides I and II around 1600 cm^{-1}); the fingerprint pattern for proteins was much

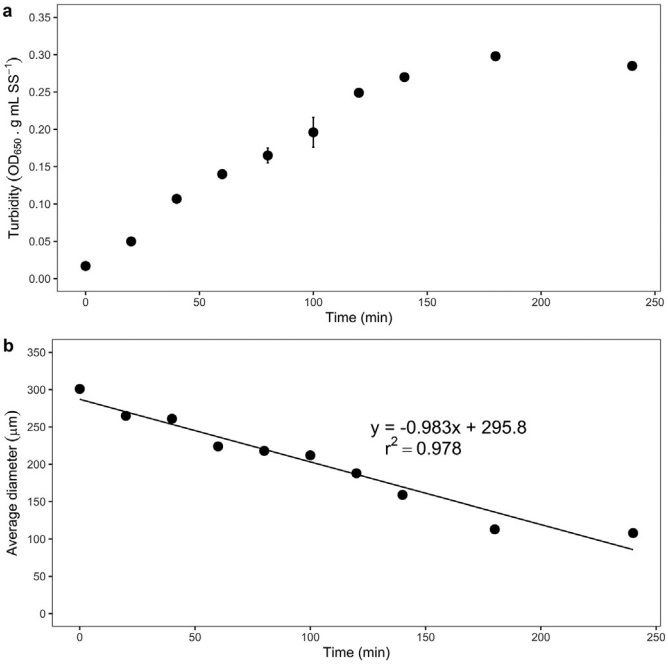

Fig. 1. Development of a) turbidity of the supernatant during the shear experiment with CER addition (shear level of G of 800 s^{-1}); the mean turbidity values of six independent measurements (three per each reactor) are presented with standard deviation and b) an average granule diameter over time during the shearing experiment.

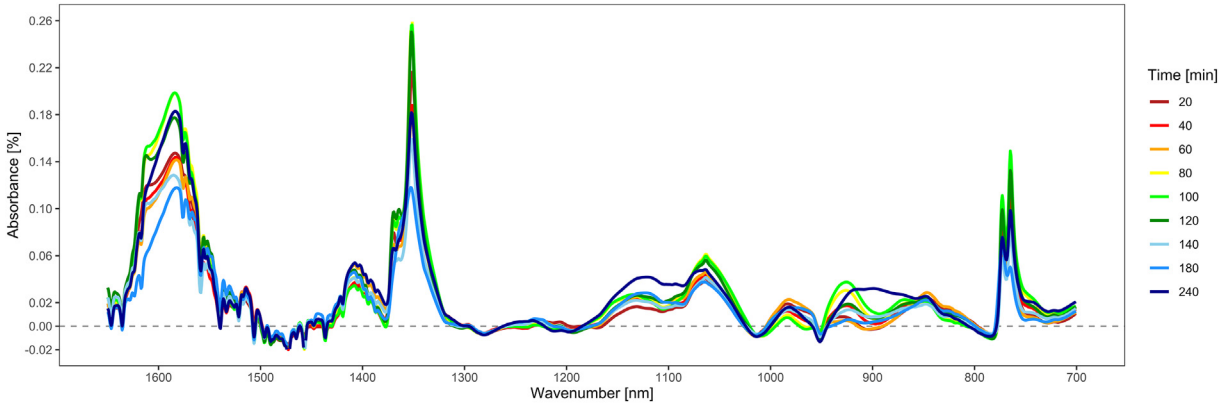

Fig. 2. FTIR spectra of EPS in supernatant at the beginning of the experiment and released after 20, 40, 60, 80, 100, 120, 140, 180 and 240 min of granule shearing; wavelengths from 1640 to 700 cm^{-1}.

stronger than for polysaccharides indicating the predominance of proteins among EPS (Yee et al., 2004). This was also confirmed by the analysis of total EPS isolated from aerobic granules - polysaccharide and protein concentrations were 47.7 ± 4.3 $mg{\cdot}g^{-1}$ MLSS and 143.9 ± 17.2 $mg{\cdot}g^{-1}$ MLSS, respectively. The most intensive bands in the amide II region were 1580 and 1567 cm^{-1} and likely corresponded with deformation vibration of NH in proteins (Nouha et al., 2016).

Weak vibrations from phosphodiester bonds were detected at 984 and 991 cm^{-1}, indicating the presence of extracellular nucleic acids (eDNA) (Badireddy et al., 2010). The highest peak intensity was observed for EPS extracted from 10 to 20 μm under the granule surface and in the granule core. The peaks related to CH_2- and CH_3- bending (~1360 cm^{-1}, Guo et al., 2016) were intense for EPS extracted from 40 to 50 μm below the granule surface with the maximum 40 μm below the surface.

Peaks indicating presence of polysaccharide and nucleic acids (1300–730 cm^{-1}), in particular bands between 1200 and 1000 cm^{-1} (vibrational stretching of OH and CO groups) (Yee et al., 2004; Zhu et al., 2012), showed variations in different granule layers. The intensity of these peaks indicated that they occurred mostly at 40–60 μm below the granule surface and in the granule core. Two very distinctive peaks between 780 and 755 cm^{-1} were assigned to the CH bending in classes of 1,2,3-trisubstituted and 1,2-disubstituted compounds, and were most intensive in a layer 50–60 μm below the granule surface and gradually decreased in adjacent layers.

3.3. Microbial community structure in different granule layers

The composition of the microbial community within AGS was characterized using 16S rRNA gene amplicon sequencing, which resulted in 563,481 high quality reads with an average 29,955 ± 4752 reads per sample in the supernatant samples, and 27,805 ± 5079 average reads per sample in the suspended solids samples. The amount of extracted DNA averaged 257 ± 67 $ng{\cdot}\mu L^{-1}$ and 457 ± 70 $ng{\cdot}\mu L^{-1}$ for supernatant and suspended solids samples, respectively. Sequencing depth was assessed using rarefaction curves (Fig. S3), the number of ASVs (richness) for the analysed samples varied from 5421 to 6384 (Table S1). The Shannon index for supernatant samples increased gradually from 8.04 in sample of supernatant after 20 min of shearing to 8.30 in sample of supernatant after 140 min of shearing (Table S1).

Non-metric multi-dimensional scaling (NMDS) showed that the microbial community composition of the suspended sludge and supernatant samples changed over the course of the shearing, with a trend to convergence of the two sample types toward the end of the experiment time (Fig. 3). The community composition of the supernatant samples clustered loosely together, while the suspended solids samples showed an evolution along the vertical axis as their diameter decreased over time, toward the supernatant samples from taken at 180 min and at the end of the experiment. The most abundantly observed genus across all samples was *Tetrasphaera* (Fig. S4). Other abundantly observed genera included *Candidatus* Accumulibacter, *Nannocystis*, *Haliangium*, and *Zoogloea*. *Candidatus* Microthrix and bacteria belonging to phylum *Chloroflexi* were the most abundant organisms in the middle layers of granules. For such taxa as *Candidatus* Accumulibacter, *Dechloromonas*, *Thauera* sp., *Nitrotoga* sp. or *Lautropia* sp., positive correlations were observed between the time of shearing and their abundance in supernatant samples (Fig. S4).

The distribution of ubiquitously observed organisms throughout the granule structure was investigated by comparing their abundance to the sample of suspended solids collected from 10 μm below the granule surface (Fig. 4). Thirty ubiquitously observed microorganisms were identified, defined by having a relative abundance of at least 0.2% of total reads in all sheared samples. In the outer layers of the granules shorn off at the beginning of the experiment, *Nannocystis* sp. was highly abundant. The largest increase in relative abundance between the granule layers was observed for representatives of genus *Thauera*. In the interior of the granules (240 min of shearing), their abundance was 5.9 times higher compared to 10 μm below the granule surface (20 min of shearing). Similar relative abundance increases of 5.3, 2.5, and 2.4 times were observed for members of the genus *Nitrotoga*, *Candidatus* Accumulibacter, and *Dechloromonas*, respectively.

3.4. Proteins in granule layers

A total of 621 proteins were identified in the 10 analysed granule layer fractions (Fig. 5). Only proteins affiliating with a metabolic function were correlated with the shorn off fractions.

Nitrile hydratase subunit alpha, phosphocarrier protein HPr, phosphopyruvate hydratase (HAP3345843.1) and glyceraldehyde-3-phosphate dehydrogenase were present in the entire structure of the granule except for the outermost layer. The most frequently identified protein in the entire structure was formate C-acetyltransferase, and acetate kinase, although this enzyme was most abundant 10–20 μm below the granule surface. Enzymes predominantly identified 10–20 μm below the granule surface covered ABC transporter ATP-binding protein (WP_141817623.1), phosphate acetyltransferase (WP_0928774 21.1), inorganic diphosphatase, arginine decarboxylase (AAP59882.1), cysteine synthase A (EFA6851913.1), DNA-directed RNA polymerase (CDW59665.1), malate dehydrogenase (HAL1984946.1), pyridoxine 5′-phosphate synthase (KTG63391.1), and universal stress protein D (WP_073544058.1). These proteins were associated to the bacterial genera *Actinomadura*, *Alteromonadaceae*, *Candidatus* Pantoea, *Citrobacter*, *Enterobacterales*, *Izhakiella*, *Kluyvera*, *Microcebus*, *Morganella*, *Olsenella*, *Ornithinimicrobium*, *Trichuris*, *Trueperella* and the families *Enterobacteriaceae* and *Saccharospirillaceae*.

Alpha/beta fold hydrolase (WP_112617616.1), DNA protection during starvation protein (1DPS_A), glucose permease (1IBA_A), HSP70 (DNAK)

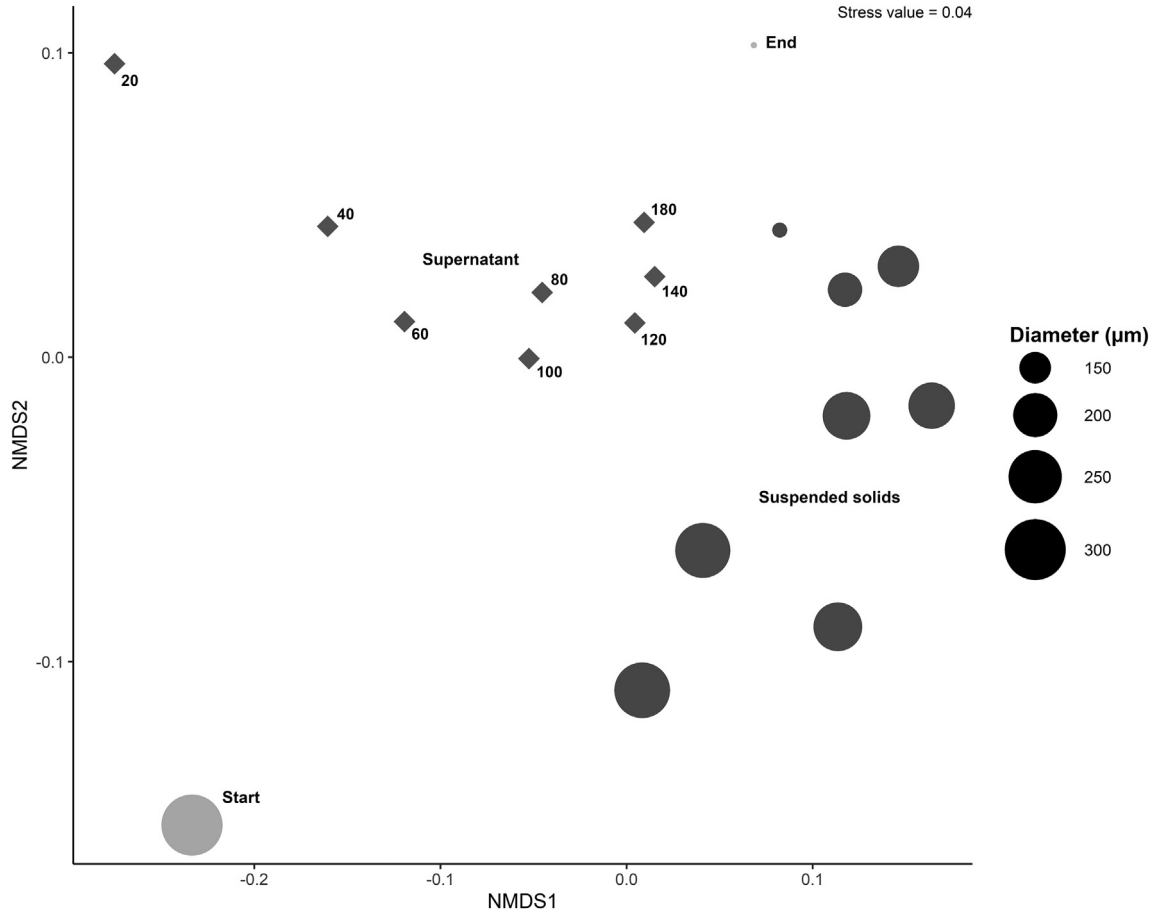

Fig. 3. NMDS ordination plot of the microbial community profiles of the supernatant and suspended solids samples taken during the shearing experiment. Sample type is indicated and for suspended solids samples the size of the points corresponds to the size of the granules at the given time of shearing.

chaperone (1–605) (2KHO_A), osmotically-inducible lipoprotein OsmB (WP_196349102.1), phosphoglycerate kinase (NIH16089.1), and type I glyceraldehyde-3-phosphate dehydrogenase (WP_046288900.1) were mainly present 30–90 μm below the granule surface. These proteins were closely related to representatives of *Anaerolineae*, *Artibeus*, *Blochmannia*, *Brenneria*, *Coriobacteriia*, *Cymothoe*, *Flavobacteriaceae*, *Gibbsiella*, *Nitrospirae*, *Reinekea*, and *Xenorhabdus*.

Proteins that were mainly seen in the granule core (≥180 min of shearing) were glutamate decarboxylase (PAC00150.1), IMP dehydrogenase (WP_110247309.1), and phosphonate ABC transporter substrate-binding protein (WP_133516248.1).

4. Discussion

In the present study, it was demonstrated that the spatial structure of exopolymers and the composition and activity of microorganisms in AGS could be investigated simultaneously to reveal the identity of bacteria and their principal metabolic pathways that are crucial for effective granulation and treatment of municipal wastewater. It has been hypothesized that granule layering affects microbial activity which correlates with physicochemical gradients formed in the granule.

4.1. Granule chemical signature layers

The FTIR analysis indicated that the EPS in different granule layers consisted of different components and that proteins were a major component. Proteins identified in EPS extracts contain functional groups (e.g., -OH, -CO-NH, -COO-, -NH_2 or CO) that provide surface charges, which serve as binding sites and support granule formation (Caudan et al., 2012). A previous study indicated that the level of proteins present in tightly bound EPS is less dependent on fluctuations in substrate availability compared to the level of polysaccharides, suggesting that they are essential for granule stability (Wang et al., 2019). Polysaccharides, although less important for granule formation (Dong et al., 2017), may protect microorganisms from harsh environmental conditions.

Another important EPS component is extracellular DNA (eDNA) released from microbial cells, and recent studies indicate its omnipresent abundance in bacterial biofilms (Campoccia et al., 2021). eDNA content in EPS is strongly correlated with biofilm development (Li et al., 2020), especially in the early stages of biofilm formation (Peng et al., 2020). eDNA interacts with other biopolymers, providing structural integrity to EPS and protecting bacterial cells from physical and chemical challenges (Das et al., 2013). In our study, analysis of EPS extracts showed that eDNA was present mostly in the outermost granule layers, indicating that processes related to granule formation were mostly located in the granule surface. High eDNA levels in the outer granule layers could subsequently reflect that the granules have not yet reached the mature level and undergo renewing of the outer surfaces by continuous granule formation and erosion, as previously described for well-performing granules (Dong et al., 2017). In the present study, the peaks in the FTIR analysis deriving from polysaccharides and nucleic acids in the EPS were highest in the fractions isolated from 40 to 60 μm below the granule surface.

4.2. Granule microbial community layers

Microbial profiling was performed using 16S rRNA gene amplicon sequencing on the eroded AGS layers. Alpha diversity revealed an increase in evenness of microorganisms as the erosion of successive layers of

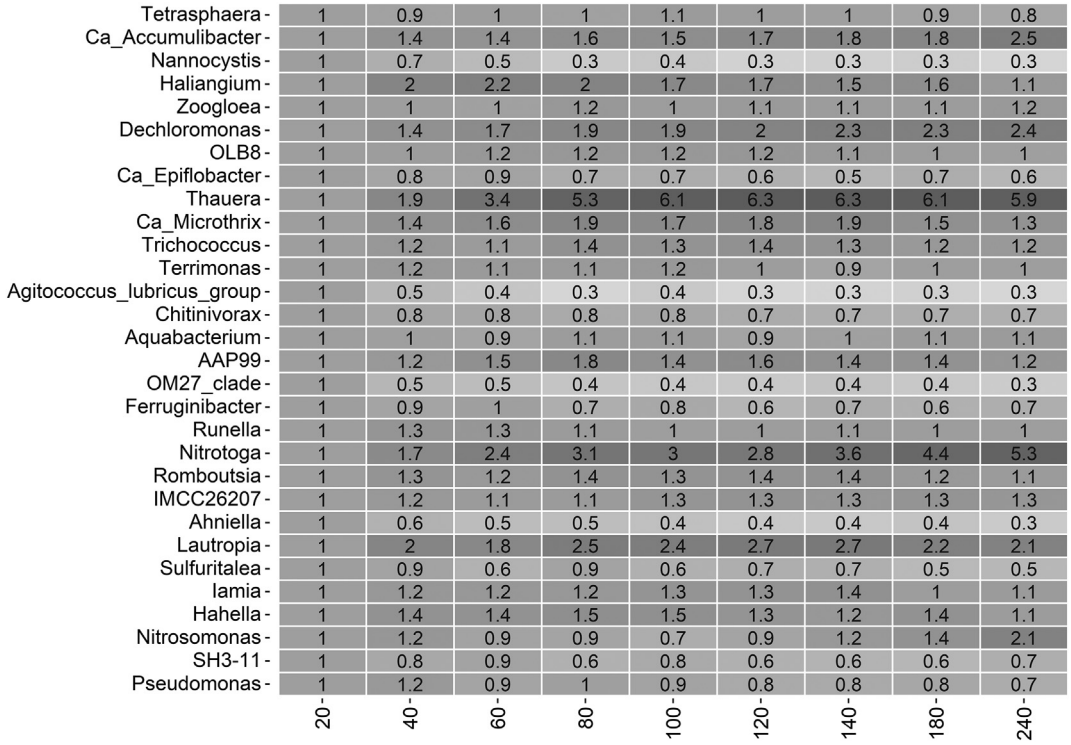

Fig. 4. Heatmap of the relative abundances of ubiquitous microorganisms during the shearing experiment. The supernatant samples has been normalised to the sample collected after 20 min of shearing.

granules occurred indicating that some species that were less abundant (or absent) on the granules surface occurred in its deeper layers. Beta diversity analysis of the obtained microbiome data revealed two distinct clusters of samples stemming from the granules and the supernatant (Fig. 3), indicating a clear differentiation in microbial community composition and functional potential. Furthermore, gradual microbial community changes

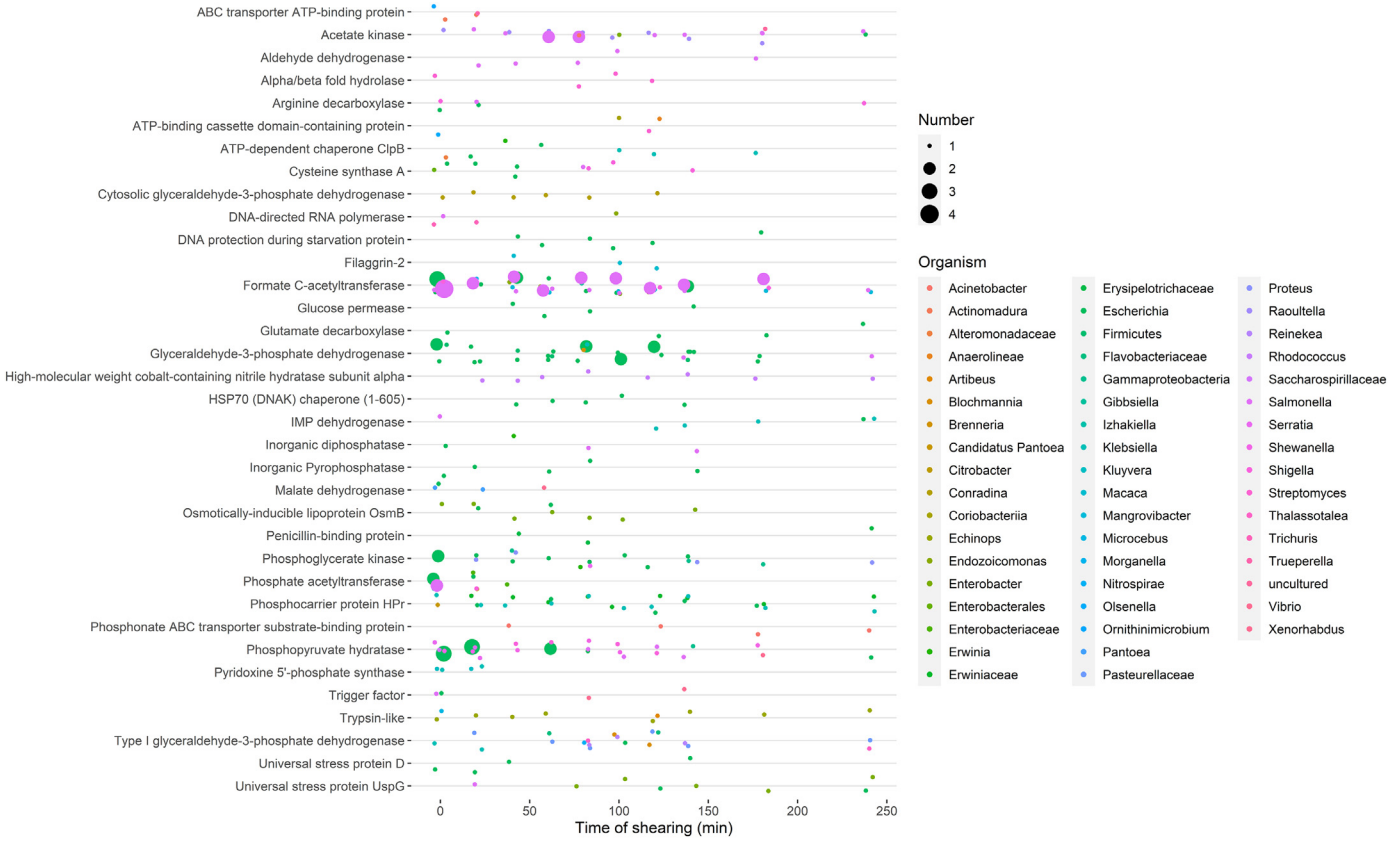

Fig. 5. Relative abundance and their taxonomic affiliations of the proteins identified in the 10 granule fractions (the time of shearing in minutes).

were observed throughout the different granule layers. Near the end of the experiment, the supernatant and suspended solids samples converged, suggesting that the shearing had reduced the granule size to such an extent that a mostly homogenous solution had formed. This is also supported by the average size measurements of the granules throughout the experiment (Fig. S2), which showed that the average diameter of the granules had gradually dropped to below 200 μm after 240 min of shearing. Taken together, these results support the usage of the CER resin as a gentle approach for disintegration of biological structures such as granules.

Tetrasphaera sp. and *Zoogloea* sp. were detected abundantly throughout the entire structure of the granules, likely favoring granule formation due to production of large amounts of EPS and tendency to grow in clusters (Fig. S4). *Tetrasphaera* sp., has been previously reported as a major genus in AGS treating municipal wastewater (Świątczak and Cydzik-Kwiatkowska, 2018). *Tetrasphaera*-related phosphate accumulating microorganisms (PAOs) are important contributors to phosphorus removal and can comprise up to 30–35% of the microbial community in activated sludge systems (Nguyen et al., 2011).

The outer granule layers were relatively abundantly inhabited by *Nannocystis* sp. *Nannocystis* sp. has previously been associated with favoring biomass flocculation (Zhang et al., 2002), and are also beneficial from a technological point of view because it has been shown that high abundance of *Nannocystis* sp. in biomass improves sludge dewaterability (Cheng et al., 2018).

Candidatus Accumulibacter and *Dechloromonas* sp. were highly abundant in the intermediate and inner layers of the granules, respectively. The presence of these genera is desirable because they are important for phosphorus and nitrogen conversions and have a high metabolic versatility, which increases their chances of survival in a multispecies consortium of AGS. *Dechloromonas* sp. cells have the ability to accumulate phosphate with dynamic levels of poly-P and polyhydroxyalkanoates during feast-famine cycling (Petriglieri et al., 2019). Both Ca. Accumulibacter and *Dechloromonas* sp. were important components of biomass in sequencing batch reactors operated in anaerobic-anoxic and anaerobic-oxic modes, which indicate that they can use both O_2 and NO_3^- as electron acceptors (Yun et al., 2019). Moreover, several studies (e.g., Han et al., 2019; Wang et al., 2020) indicate that they can also fully denitrify to N_2, which, as in the case of *Thauera* sp., limits greenhouse gas emissions from wastewater treatment systems.

Filamentous microorganisms belonging to *Candidatus* Microthrix and *Chloroflexi* sp. occupied mostly the inner granule layers and potentially constituted a backbone for AGS formation (Liu and Liu, 2006).

Denitrifiers, nitrifiers and PAOs formed stratified gradients within the AGS and were relative more abundant in the granule core. Among denitrifiers, a large number of representatives of *Thauera* sp. were identified. *Thauera* sp. are able to effectively produce EPS (Wan et al., 2015), thus strengthening the granule core. Moreover, microorganisms belonging to *Thauera* sp. possess *nosZ* genes and are able to fully denitrify to N_2 (Zhao et al., 2013).

Candidatus Nitrotoga was the predominant nitrite-oxidizing bacteria (NOB) in the biomass and its relative abundance was over 5 times higher in the core compared to the outer parts of the granules. *Candidatus* Nitrotoga strains are highly important for efficient wastewater treatment because they can tolerate high nitrite concentrations (20 mM) and grow efficiently even in the presence of organics (Ishii et al., 2020). Aerobic nitrifiers have previously been found to be more abundant in the outer parts of granules, while denitrifiers and PAOs were observed relatively more abundant in the granule core (Guimarães et al., 2017). However, another study found that nitrifiers grow both at the oxygen-rich surface and also in inner locations of the granules to which oxygen and ammonia are transported across the pores and water-filled voids in the granule structure (Szabó et al., 2017). Our study supports the hypothesis that *Candidatus* Nitrotoga grows abundantly in granule cores, which is in agreement with genomic predictions suggesting that it can be metabolically active in low oxygen or anoxic conditions (Boddicker and Mosier, 2018).

4.3. Granule functional and protein layers

In this study, the proteomic profile of the individual AGS layers was investigated for the first time. Ten shallow proteomes revealed a gradient of different proteins with diverse functions within the granule structure (Fig. 5). Although cautious interpretations should be drawn from the shallow proteomes, they demonstrate the potential to investigate differential presence of functions through the granule gradient.

A number of proteins that were identified throughout the entire granule structure were associated to the glycolysis pathway, including phosphoglycerate kinase, cytosolic glyceraldehyde 3-phosphate dehydrogenases and acetate kinase. In activated sludge systems, P removal performance has been observed to be better in full-scale WWTPs with higher glycolysis activity (Lanham et al., 2013). Furthermore, nitrile hydratase subunit alpha, which catalyzes the hydration of various nitrile compounds to the corresponding amides, was observed throughout the entire granule except for the outermost layers.

Proteomic analysis revealed a composition of versatile metabolisms in the surface granule layers. Enzymes such as arginine decarboxylase, phosphate acetyltransferase, aldehyde dehydrogenases and malate dehydrogenases were observed up to the 20 μm below the granule surface. In the outer granule layer also pyroxidine 5′-phosphate synthase was present, which is involved in the metabolism of nitrogen compounds.

Chaperonins and binding proteins have been shown to be crucial for sludge granulation promoting microbial aggregation and polymer assembly (Dong et al., 2017). An ABC transporter ATP-binding protein was observed in the outer parts of the granules. This could potentially be related to previous evidence that this type of protein supports microbial colony adhesion and cell reunion, which all are surface processes (Higgins and Novak, 1997). About 20–70 μm below the granule surface, chaperonins, including HSP70, were present. Hsp70 is a ubiquitous, ATP-dependent molecular chaperone that is an important component of the cellular machinery that is involved in protein homeostasis and virulence of many pathogenic bacteria (Genest et al., 2019, Mayer, 2021). Chaperonins are regarded as a biomarker of cellular stress (Yusof et al., 2021), which explains their abundance in surface granule layers exposed to variable environmental conditions.

An increasing number of unique proteins involved in P conversions such as a substrate-binding protein of phosphonate ABC transporter and IMP dehydrogenase was observed deeper inside the granule structure and corresponded well with the observed increasing relative abundance of PAOs in the same layers (Fig. 4).

Based on the observed protein groups throughout the granules, it can be suggested that processes such as carbohydrate transport and anaerobic glucose metabolism were present throughout the whole granule structure, while processes relating to phosphorus metabolism were more active toward the core of the granules.

5. Conclusions

A gentle extraction approach based on a CER and defined shear was employed to collect single layers of AGS and simultaneously characterize their microbial, proteomic and chemical composition. On the surface of the granules, the microbial community (highly abundant *Nannocystis*), EPS (eDNA) and proteome composition (chaperonins and binding proteins) favored microbial aggregation. Versatile EPS-producers such as *Tetrasphaera* and *Zoogloea* were evenly distributed throughout the whole granule structure. Denitrifiers and PAO were abundant in the granule core. The most abundantly observed nitrifier, *Candidatus* Nitrotoga, inhabited the inner parts of granules. Proteins associated with the glycolysis pathway were present in all granule layers, while proteins associated with phosphorous conversions were present in the deeper layers of the granules.

CRediT authorship contribution statement

Agnieszka Cydzik-Kwiatkowska: Investigation, Formal analysis, Validation, Data curation, Methodology, Software, Visualization, Writing – original draft.

Nadieh de Jonge: Investigation, Formal analysis, Validation, Data curation, Software, Methodology, Writing – original draft.

Jan Struckmann Poulsen: Formal analysis, Data curation, Software, Visualization, Methodology, Writing – original draft.

Jeppe Lund Nielsen: Writing – review & editing, Conceptualization, Methodology, Funding acquisition, Supervision, Validation, Resources.

Declaration of competing interest

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

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Appendix A. Supplementary data

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