Influence of the bioreactor operating mode and wastewater composition on the structure of microbial communities in activated sludge and abundance and activity of polyphosphate and glycogen accumulating organisms

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ABSTRACT

Two lab-scale sequencing batch reactors were seeded with flocculent activated sludge from a full-scale wastewater treatment plant, fed with synthetic wastewater with acetate as the only source of organic carbon and operated in anoxic-anaerobic-aerobic (SBR1) and truly aerobic modes (SBR2). In SBR1 granules were formed, while flocs in aerobic SBR2 were overgrown with filamentous bacteria. FISH showed that synthetic wastewater equally influenced the abundance of bacterial phyla and classes in both SBRs regardless the operating mode: Proteobacteria increased their abundance and outcompeted Chloroflexi and Actinobacteria, Betaproteobacteria was the dominant class, abundance of Alphaproteobacteria decreased, and Gammaproteobacteria remained stable. The anoxic-anaerobic-aerobic conditions and acetate favoured growth of polyphosphate (PAO) and glycogen accumulating organisms (GAO) in SBR1, whereas the aerobic conditions in SBR2, along with the acetate, triggered growth of filamentous *Thiothrix*/021N. Despite the large differences between the abundance of PAO/GAO in both SBRs, the composition of their populations did not differ significantly between reactors, and the type of organic substrate was a decisive factor shaping the structure of their communities. *Tetrasphaera* PAO, predominant in the seed, were outcompeted in both SBRs by *Accumulibacter*. Clades IA and IIA, C, D together constituted 78% and 73% of the *Accumulibacter* lineage in SBR1 and SBR2, respectively, whereas 97% of *Accumulibacter* in the seed was not targeted by any of the clade-specific probes. *Defluviicoccus vanus* cluster 2 was partially replaced by cluster 1 and *Competibacter* in both SBRs. The substantial abundance of PAO in SBR1 was reflected in the high enhanced biological phosphorus removal activity in anaerobic batch tests and high presence of intracellular polyphosphate granules at the end of the aerobic period. Despite the significant abundance of *Accumulibacter*, the biomass from SBR2 neither released P nor took up C_{org} in anaerobic batch tests, which indicates that the activity of ecophysiological groups was largely influenced by the mode of operating bioreactors.

Keywords: Biodiversity; Ecophysiological groups; Biological nutrients removal; Polyphosphate accumulating organisms; Glycogen accumulating organisms; Filamentous bacteria

1. Introduction

Municipal wastewater is usually treated with activated sludge in wastewater treatment plants (WWTPs). This method relies on biodegradation of organic pollutants and biotransformation of inorganic compounds by various ecophysiological groups of bacteria under 3 conditions: anaerobic, anoxic and aerobic. This is achieved by separation of electron acceptors in space (separate tanks in continuous flow systems) or over time (successive phases in

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sequencing batch reactors (SBRs)) [1]. Most organic compounds in wastewater are macromolecules that require hydrolysis. Numerous *in situ* studies (reviewed by [1,2]) revealed the importance of filamentous bacteria that secrete surface associated hydrolytic enzymes, indicating their involvement in the breakdown of complex organics. Important protein degraders in activated sludge are filamentous bacteria belonging to *Curvibacter*, Chloroflexi, phylum TM7 and epiflora on various filamentous bacteria (*Epiflobacter*). Most polysaccharides hydrolyzing organisms belong to *Tetrasphaera* and some filamentous *Bacteroidetes* and Chloroflexi, lipids are hydrolyzed by filamentous Microthrix and Mycolata. Soluble organic substances that are generated by hydrolyzers or were originally present in wastewater, serve as substrates for anaerobic fermenting bacteria producing acetate and other short-chain volatile fatty acids (VFAs) [3,4]. VFAs are anaerobically taken up by polyphosphate (PAO) and glycogen accumulating organisms (GAO) and stored as polyhydroxy-alkanoates (PHA) and/or glycogen [3,5], or consumed anoxically together with other readily biodegradable substrates (e.g., amino acids, sugars) by denitrifying bacteria that reduce nitrate and nitrite [6]. Under aerobic conditions, nitrification (the oxidation of ammonia via nitrite to nitrate) is the main process driven by ammonia-oxidising bacteria and archaea, nitrite-oxidising bacteria, and comammox (complete ammonia oxidizer) [7,8], however, some heterotrophic activity also takes place in these conditions [1].

The overall effectiveness of wastewater treatment process is thus determined by the structure and activity of microbial communities in activated sludge [1]. However, recent studies [9,10] show that despite decades of intensive research on microbial ecology of activated sludge systems, many important microorganisms remain unidentified. The development of sequencing technologies revealed that while complex microbial communities are responsible for unit operations, many bacterial lineages are still not described at the taxonomic and functional levels. By applying the new MiDAS 4 taxonomy, including placeholder names for novel taxa generated using AutoTax, it has been shown that among the ecophysiological groups the same genera are found in activated sludge of WWTPs across the world, and relatively few species are abundant worldwide [10]. Differences in community structure are due to external factors such as wastewater composition, temperature, process type and plant operation, rather than to geographical factors, and therefore research results can be transferred from one geographical region to another. On the other hand, Dottorini et al. [11] suggest that microbial communities in activated sludge of WWTPs may be strongly influenced by immigration from incoming wastewater, but the extent of this impact has not yet been fully elucidated.

Enhanced biological phosphorus removal (EBPR) is a widely used method for efficient phosphorus removal from wastewater without the use of chemical precipitation. The process operates in alternating anaerobic and aerobic conditions and is mediated by PAO. Wastewater is fed into the system under anaerobic conditions, PAO take up VFAs and store them as PHA, using the energy from the cleavage of the internally stored polyphosphate (poly-P).

The PHA synthesis requires reducing power, which is provided by the hydrolysis of intracellular glycogen and/or the anaerobic operation of the tricarboxylic acid (TCA) cycle. The release of orthophosphate into bulk liquid is observed with a simultaneous increase in internally stored PHA and a decrease in the intracellular glycogen pool. Under subsequent aerobic conditions (famine conditions), PAO use the stored PHA to support growth, replenish the glycogen pool, as well as generate energy for orthophosphate uptake and poly-P synthesis. PAO accumulate poly-P in excess of cellular demand, and phosphorus is eliminated from wastewater by wasting excess sludge with high poly-P content [12,13]. The second group of bacteria important for the EBPR process are GAO, which have a metabolism like PAO but do not accumulate poly-P. Instead, GAO use glycogen as an energy source for the anaerobic uptake of VFAs and therefore these bacteria were initially believed to deteriorate the EBPR process because they outcompete PAO in lab-scale reactors for organic substrates [14]. However, some studies reviewed by Nielsen et al. [5] challenge this assumption as there are no well-documented cases of EBPR deterioration in full-scale systems which could be clearly induced by GAO. This can be explained by significantly higher acetate concentrations in synthetic wastewater in lab-scale studies (promoting GAO growth) compared to the low concentrations of VFAs in full-scale WWTPs that favour PAO.

Candidatus Accumulibacter (hereafter referred to as *Accumulibacter*) is a widely accepted canonical model of PAO, which is the predominant organism mainly in lab-scale reactors fed with acetate or other low molecular weight VFAs as sole carbon sources [14]. Members of *Accumulibacter* are diverse, the phylogeny and taxonomy of this genus have been recently re-evaluated by Petriglieri et al. [13]. Based on the phylogeny of polyphosphate kinase genes (*ppk1*), the *Accumulibacter* lineage is comprised of two major types (I and II), and each type contains multiple clades IA-IH and IIA-II_i, respectively. These clades differ in their metabolism under different environmental conditions, including the ability to reduce nitrate or use the TCA cycle to obtain additional reducing power for PHA synthesis [12,13]. Another important PAO, especially in full-scale WWTPs with significant contribution of industrial wastewater, are members of the actinobacterial genus *Tetrasphaera* [3,15–17]. Due to the high level of phylogenetic diversity, based on the 16S rRNA gene *Tetrasphaera* PAO were organized into 3 distinct clades [15]. However, recent genomic analyses [18] revealed that members of *Tetrasphaera* clade 3 belong to two genera of *Candidatus* Lutibacillus and *Candidatus* Phosphoribacter, the latter being the most abundant *Tetrasphaera* PAO in WWTPs worldwide [10]. *Tetrasphaera*-related PAO are more versatile in substrate uptake capabilities, can utilise glucose and amino acids, ferment complex organic substances, and accumulate fermentation products [1,3,15]. Only some members are able to take up VFA anaerobically, but none of them synthesize and store PHA. Apart from *Accumulibacter* and *Tetrasphaera*, other PAO have been identified in full-scale WWTPs, including "*Candidatus* Accumulimonas phosphatis" (hereafter referred to as *Accumulimonas*), *Microlunatus phosphovorus*, *Dechloromonas* spp., *Candidatus* Obscuribacter, *Tessaracoccus* spp. [5,14,19,20]. However, very limited information is

currently available about their abundance and further research is needed to investigate their role and metabolism in full-scale WWTPs. Nevertheless, different groups of PAO in full-scale systems can use diverse carbon sources, allowing them to occupy different ecological niches and providing ecophysiological redundancy in microbial communities, which contributes to a robust and stable EBPR.

There are 4 groups of GAO that can occur in activated sludge of EBPR WWTPs, where biomass is subjected to alternating anaerobic/aerobic conditions: the gammaproteobacterial "*Candidatus* Competibacter phosphatis" (hereafter referred to as *Competibacter*), the alphaproteobacterial Defluviicoccus–related GAO, the betaproteobacterial "*Candidatus* Propionivibrio aalborgensis" (hereafter referred to as *Propionivibrio*), and the actinobacterial *Micropruina* spp. [5]. First two GAO are phylogenetically highly diverse based on their 16S rRNA sequences. *Competibacter*, which was considered a single genus, due to phylogenetic diversity has recently been proposed as the family *Competibacteraceae*, and consists of 2 lineages: *Competibacter* and *Plasticicumulans* [21]. The latter does not show the GAO phenotype, the former comprises 14 clades that may compete with PAO for various VFAs. Different clades often coexist in activated sludge of WWTPs, but the structure of their community can vary considerably between and temporally within EBPR systems, which reflects the phenotypic variability and niche partitioning. As with *Competibacter*, considerable phylogenetic and metabolic diversity exists also between individual members of the genus *Defluviicoccus*, which were proposed to fall into 5 distinct clades [22,23]. Clades I, II, IV and V have the characteristic morphology of cocci arranged in tetrads and are also known as tetrad-forming organisms (TFOs). However, members of clade III are typical filamentous bacteria with the *Nostocoida limicola* II morphotype [24]. Bessarab et al. [22] showed considerable variations in the presence and numbers of genes encoding key substrate assimilation and metabolic pathways between the *Defluviicoccus* clades. All of them can anaerobically assimilate propionate at a higher rate than acetate, but some members are also able to take up butyrate, amino acids and/or glucose, which could be a key feature that allows *Defluviicoccus*-related GAO to compete not only with *Accumulibacter* but also with *Tetrasphaera*-related PAO. However, the ecophysiological niche similar to *Tetrasphaera* is occupied rather by members of *Micropruina* GAO, which utilize and store sugars and amino acids generated by fermentation [25]. On the other hand, *Propionivibrio* possesses the same anaerobic phenotype as *Accumulibacter* and is phylogenetically closely related to this PAO [26]. The different physiology patterns of GAO groups have potentially important implications for EBPR as GAO can efficiently compete for various substrates with PAO under changing environmental conditions.

It is postulated that PAO and GAO may play an important role in the formation and structure of aerobic granules in activated sludge systems [21,27–30]. Aerobic granular sludge (AGS) is an innovative biological wastewater treatment technology that has great advantages over the conventional activated sludge systems: compact and dense biomass aggregates which settle much faster than flocculent sludge, resulting in a higher biomass concentration in the reactors and a better solid–liquid separation [31,32]. AGS systems have received widespread interest as a promising technology for wastewater treatment, including removal of nutrients and recalcitrant organic, due to the regular and dense aerobic granules that can withstand high organic loads or toxic effects of substrates present in the reactor influent [33–37]. However, despite the advances in understanding the granulation process [34,38,39], its comprehensive mechanism has not yet been fully established. The formation of dense and mature granules is often linked with the bacterial community structure, especially the presence and activity of bacterial species capable of secreting large amounts of extracellular polymeric substances (EPS), such as PAO and GAO [38–41].

The microbial community structure in activated sludge is shaped by a number of factors, including wastewater composition, the configuration of WWTP and mode of bioreactor operation. However, how combined changes in organic carbon source and bioreactor operating mode can affect community composition and select for specific ecophysiological bacterial groups has not been experimentally studied in detail. Moreover, it remains still unclear how the presence of certain functional groups of bacteria and specialized species among them may influence the granulation process in similar bioreactors fed with the same wastewater, but with different operational modes. The present study, therefore, investigated the influence of the bioreactor operating mode and the composition of wastewater on the structure of microbial communities in activated sludge. Furthermore, the abundance and activity of selected ecophysiological groups that are important for efficient granulation, such as PAO, GAO and filamentous bacteria, were examined to explore their effect on the process. For this purpose, the performance of AGS sequencing batch reactor (SBR), operated in anoxic-anaerobic-aerobic conditions, was tested in parallel with a flocculent sludge SBR that was operated in a truly aerobic mode.

2. Materials and methods

2.1. Wastewater and reactors

Two identical laboratory-scale sequencing batch reactors (SBR1 and SBR2) with a working volume of 6.9 L were seeded with activated sludge collected from the municipal WWTP with A2O configuration (Biogradex® activated sludge technology) located in Stare Babice near Warsaw (detailed plant characteristics can be found elsewhere [42]. SBR1 was operated in an anoxic-anaerobic-aerobic mode for over 100 d, as previously described by Muszyński et al. [43]. Briefly, the reactor cycle consisted of an anoxic/anaerobic period of 120 min (including 10 min of filling), an aerobic period of 190 min, a settling period of 40 min, and a decantation period of 10 min. This resulted in a 6 h cycle and a hydraulic retention time of 12 h. SBR2 was operated in a truly aerobic mode, its cycle was similar to SBR1, with the difference that instead of the anoxic/anaerobic phase, the aerobic period was extended to 310 min. At the end of the aerobic periods of SBR1 and SBR2 the excess sludge was withdrawn once a day as mixed liquor to maintain solids retention time of 8–18 d, and mixed liquor suspended solids (MLSS) of 3–4 g/L. Both SBRs were fed with synthetic wastewater used

in the previous study [43], containing acetate (770 mg/L) as the organic carbon source, NH_4Cl (153 mg/L), K_2HPO_4 and KH_2PO_4 (112 and 88 mg/L, respectively), which corresponded to approximately 600 mg/L of chemical oxygen demand (COD) and COD:N:P ratio 15:1:1. Dissolved oxygen concentration during the aerobic periods in both SBRs and pH was controlled and maintained within the range of 2.0–2.5 mg/L and 7.5–8.0, respectively. Both reactors were operated at room temperature of 19°C–23°C.

2.2. Batch tests

The activity of PAO and GAO was examined by performing anaerobic batch EBPR tests after adding excess electron donor in accordance with the EBPR.ANA.3 protocol [44]. Tests were carried out for 6 h after the addition of modified synthetic wastewater (as described above but excluding peptone and yeast extract) with sodium acetate (a carbon source) in excess (without causing total acetate depletion). This allowed for the determination of the maximum concentration of released $P–PO_4^{3-}$ and the maximum concentration of carbon consumed in order to estimate the maximum activity of EBPR communities under non-limiting carbon conditions. The tested activated sludge was collected at the end of the aerobic stage of each SBR to minimize the presence of the carbon source originally present. The tests were executed after a washing procedure, under truly anaerobic conditions in the absence of any electron acceptor (molecular oxygen, nitrate, or nitrite) to exclude the activity of ecophysiological groups of bacteria other than PAO or GAO. Samples for the determination of dissolved organic carbon (DOC) and $P–PO_4^{3-}$ were collected every 5 min for the first 30 min of the batch test, and then every 15 min thereafter until the end of the test. The EBPR activity was determined from the C_{org} uptake and $P–PO_4^{3-}$ release profiles through the calculation of the P_{rel}/C_{upt} ratio (P-mol/C-mol Hac).

The fractions of PAO and GAO populations present in SBRs were quantified using the method of López-Vázquez et al. [45]. The PAO fraction (f_{PAO}), as a fraction of the total PAO and GAO community, was calculated using a formula:

$$f_{PAO} = \frac{P_{rel}/C_{upt}}{0.51}$$

whereas the GAO fraction (f_{GAO}) was calculated as the remaining fraction of the total PAO and GAO population:

$$f_{GAO} = 1 - \frac{P_{rel}/C_{upt}}{0.51}$$

2.3. Microbiological analyses

The microbial abundance was examined using quantitative fluorescence *in situ* hybridization (qFISH), as described by Muszyński and Załęska-Radziwiłł [16]. When required, cells were permeabilised to improve the penetrability of oligonucleotide probes, using lysozyme (360,000 U/mL), achromopeptidase (60 U/mL) and 0.1 M HCl solutions. A 6-Fam labelled EUBmix oligonucleotide probe (equimolar mixture of EUB338, EUB338II, and EUB338III) was used to target the entire bacterial community. The specific probes were labelled with Cy3. The probes ALF968, BET42a, GAM42a, DELTA495a, SAP-309, CFXmix (equimolar mixture of GNSB-941 and CFX1223), HGC69a, LGCmix (equimolar mixture of LGC354A, LGC354B and LGC354C), and Ntspa662 were used to target bacterial phyla, classes and families that usually dominate in activated sludge communities: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, *Saprospiraceae*, Chloroflexi, Actinobacteria, Firmicutes, and Nitrospira, respectively. The oligonucleotide probes G123T, PAOmix (equimolar mixture of PAO462, PAO651 and PAO846), Hal180, GB, Prop207, DF1mix (equimolar mixture of TFO_DF218 and TFO_DF618), DF2mix (equimolar mixture of DF988 and DF1020) and DF198 were used to detect specific ecophysiological bacterial groups (soluble substrate-dependent filamentous bacteria, PAO and GAO): *Thiothrix*/021N group, most *Accumulibacter*, *Accumulimonas phosphatis*, most *Competibacter*, *Propionivibrio aalborgensis*, *Defluviicoccus vanus* clusters 1, 2 and 3, respectively. Probes Tet1-266, Tet2-174, Tet2-892, Tet3-654 were used to target clades 1, 2 and 3 of *Tetrasphaera*-related PAO. Abundance of Type I *Accumulibacter* (clade IA and others) and Type II *Accumulibacter* (clade IIA, IIC and IID) was determined directly by qFISH using Acc-I-444 and Acc-II-444 oligonucleotide probes labelled with Cy3. The abundance of other *Accumulibacter* clades, not targeted by either of the clade specific probes, was calculated by subtracting abundance of Type I and II *Accumulibacter* from the total abundance of the whole lineage. The more broadly specific Pse136 probe was applied in a hierarchal approach to decrease the possibility of false positives for probe Hal180 as suggested by Nguyen et al. [19]. When necessary, the oligonucleotide probes were used in combination with their corresponding unlabelled competitors and/or helpers. Detailed information about the probes used in the study is given in probeBase [46], except for Acc-I-444 and Acc-II-444 (both described by Flowers et al. [47]), and Prop207 [26].

The quantification of bacteria was performed in a similar way to that described by Muszyński et al. [48]. Twenty separate images for each probe were captured with a Nikon Eclipse 50i Microscope (60x objective) and analysed using ImageJ software [49]. The microbial abundance (biovolume, expressed as a percentage of EUBmix), which was relative to the pixel area of cells positive for the specific probe, was then quantified as a percentage of the pixel area for all bacteria positive for the EUBmix (a mean of 20 separate measurements). Standard error was calculated as a standard deviation of the percentage abundance of specific bacteria divided by a square root of 20 measurements.

The presence of poly-P granules in bacterial cells was tested by DAPI (4′,6-diamidino-2-phenylindole) staining (1 µg/mL, 1 h at 4°C) and examined by excitation at 364 nm and emission at >537 nm as described by Zilles et al. [50] and Muszyński and Miłobędzka [51].

2.4. Chemical analyses

Total phosphorus (TP) and nitrogen (TN), soluble orthophosphate ($P–PO_4$) and COD were determined spectrophotometrically using standard LCK vial test kits (Hach-Lange).

MLSS and pH were determined in accordance with Standard Methods [52]. DOC was measured by a TOC-5000 analyzer (Shimadzu, Japan).

3. Results and discussion

3.1. SBRs performance

The efficiency of COD removal in SBR1 and SBR2 is presented in Fig. 1. No significant differences were found between the two reactors in the elimination of organic pollutants during the study. It shows that bioreactor operating mode did not affect the efficiency of wastewater treatment, and the elimination of COD was similar both under anoxic-anaerobic-aerobic and truly aerobic conditions. Moreover, practically from the very beginning of the experiment, a high efficiency of wastewater treatment was observed, on average 91%–92% (ranges of 87%–95% and 87%–93% for SBR1 and SBR2, respectively). It demonstrates that changes in wastewater composition by replacing real wastewater containing a variety of substrates with the synthetic feed containing only acetate as an organic carbon source did not affect the efficiency of COD removal and no sludge adaptation in both SBRs was required. However, these changes strongly influenced the microbial community structure, which is discussed in the next section.

A definitely different situation was observed in terms of P removal (Fig. 1). The anoxic-anaerobic-aerobic operational strategy for SBR1 resulted in more effective P elimination compared to aerobic SBR2, which was in line with our expectations. The P removal efficiency in SBR2 was only 50% on average, and an ascending trend was observed in the phosphorus concentration in effluent from this reactor (from 16 to 26 mg·P/L). Contrary to this reactor, in SBR1 over 99% efficiency was achieved, and P concentration in the effluent dropped from 12 mg/L at the beginning of the experiment to 0.1 mg/L. However, it required over 3 months

of sludge adaptation and was probably associated with the selection of an appropriate ecophysiological group of PAO, which is discussed below.

Both SBRs were seeded with the same flocculent activated sludge from a full-scale WWTP and fed with identical synthetic wastewater. The activated sludge flocs in the seed were medium-sized, rather loose, but not overgrown with filamentous bacteria (Fig. 2A and B). During the experiment, significant changes in the morphology of the sludge flocs in both reactors were observed. After 35 d granules began to form in anoxic-anaerobic-aerobic SBR1 (Fig. 2C), while in aerobic SBR2 there was an excessive growth of filamentous bacteria forming the characteristic rosettes (Fig. 2D). It should be noted that in SBR1, no special physical processes were applied to intensify the granulation process, and after 3 months the mature granules reached a diameter of 0.5 cm (Fig. 2E).

Progress has been made in understanding how granulation occurs, however, a comprehensive granulation mechanism is yet to be established and it is well known that no single mechanism is responsible for formation of aerobic granules [34,38,39]. The process is strongly influenced by small ranges of operational parameters including short settling time washing out fine aggregates, volumetric exchange ratio, reactor geometry, aeration intensity and hydrodynamics responsible for the shear forces, wastewater composition, food to microorganisms ratio (organic loading rate), feeding strategy (feast and famine periods) [33,35,38]. Aerobic granular sludge is generally realised in SBRs, however, research on achieving aerobic granules demonstrate that continuous flow full-scale WWTPs can also successfully cultivate granules [31,32].

Both SBRs in this study were operated in a similar manner, except that the operational cycle of SBR1 included anoxic/anaerobic phase, whereas SBR2 was operated under truly aerobic conditions. Therefore, anoxic/anaerobic phase
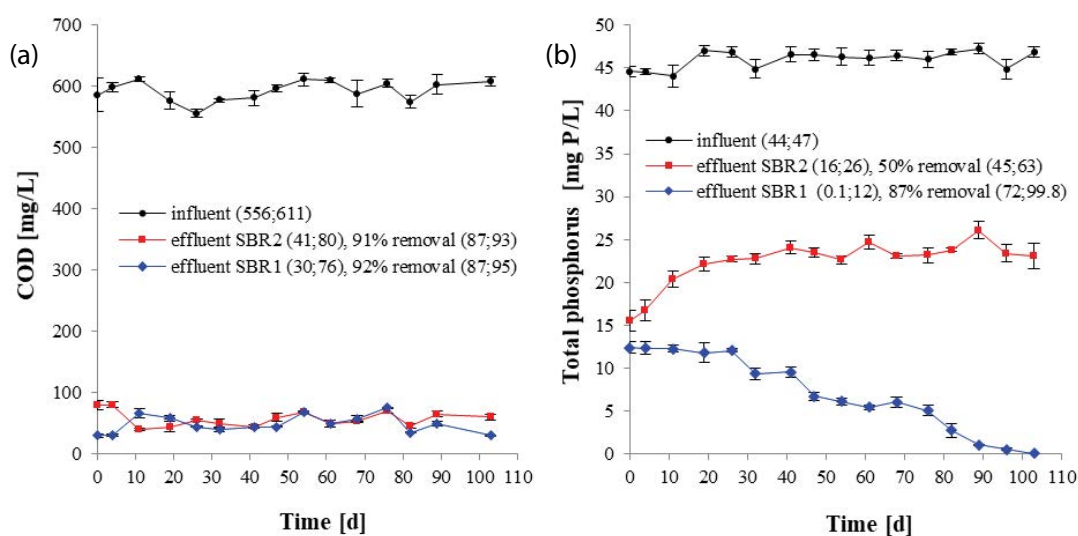

Fig. 1. Comparison of the removal efficiency of organic compounds (expressed as chemical oxygen demand) (a) and total phosphorus (P_{tot}) (b) in SBR1 and SBR2 (anoxic-anaerobic-aerobic reactor and truly aerobic reactor, respectively). Means of triplicates are presented, ranges (minimal and maximum values) for chemical oxygen demand, P_{tot} and removal efficiency are shown in parentheses, error bars illustrate standard errors. Error bars that are smaller than the marker symbols are not displayed.
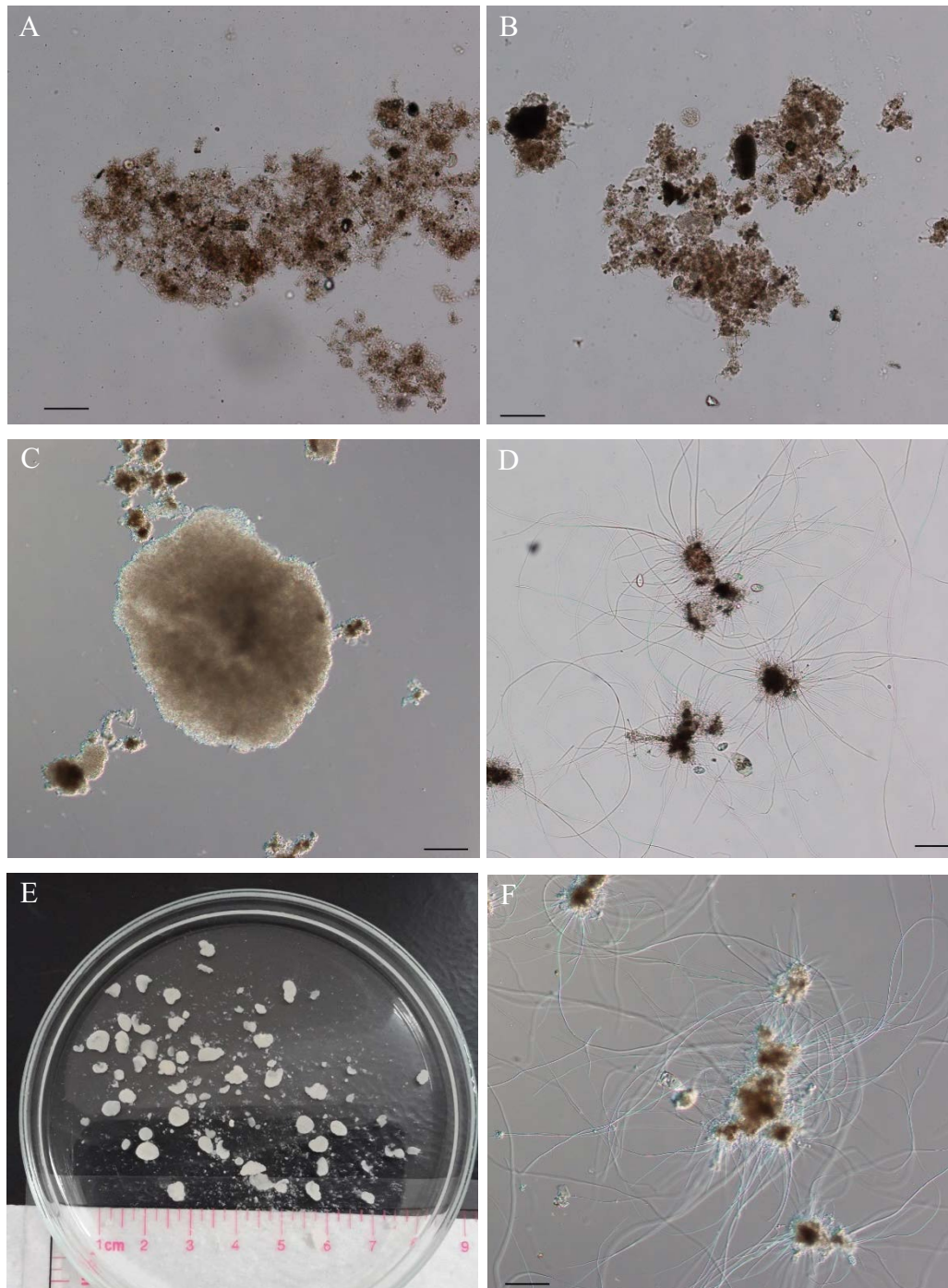
Fig. 2. Morphology of activated sludge flocs in anoxic-anaerobic-aerobic SBR1 (left panel) and truly aerobic SBR2 (right panel) after 0 (A and B), 35 (C and D) and 97 (E and F) days of the experiment. Scale bars (photos A–D and F) represent 100 μm.

(including feeding) was the key factor for successful granulation in SBR1. De Kreuk and van Loosdrecht [53] found that anaerobic feed improved the stability of the granules by outcompeting filaments and selecting slow growing polymer-storing bacteria. Batch feeding strategy and feast/famine conditions favour the growth of the latter microorganisms, which become hydrophobic. The impact of feast/famine operation on granulation process, however, is still not well understood, but it is hypothesized that cell hydrophobicity accelerates microbial aggregation [54]. Many studies, reviewed by Zahra et al. [39], showed that the bioreactor operating mode, especially a short anoxic phase, may strongly intensify the production of EPS, which are considered key determinants in the granulation process. However, Rocktäschel et al. [55] demonstrated that the typical for SBR gradient of the substrate between bulk phase and biomass

is not absolutely necessary for the successful obtaining of aerobic granules, while Kosar et al. [56] highlighted the importance of seed origin for both the granulation process and the nutrients removal performance of aerobic granular sludge reactors.

3.2. Microbial communities

In order to investigate whether changes in the floc morphology were related to the microbial population dynamics, the abundance of the main types and classes of bacteria was examined using qFISH. We found that the applied oligonucleotide probes allowed the identification of only 82% of the bacteria present in the seed, but the number of identified microorganisms increased the longer the experiment was conducted (Fig. 3).

Unidentified bacteria accounted for nearly 1/5 of the whole community in the sludge from the full-scale WWTP, while after 3 months of the experiment, all bacteria in both lab-scale SBRs were assigned to a phylum, a class or a family. This can be attributed to a decline in biodiversity of sludge in both laboratory reactors compared to the seed from the full-scale WWTP. The variety of substrates present in municipal wastewater from full-scale WWTPs creates more niches for bacteria, and temporal fluctuations in wastewater composition ensure the availability of different forms of carbon to various ecophysiological groups [1,57,58]. Abundant natural organic macromolecules and polymers such as proteins, polysaccharides, lipids, and nucleic acids, are converted by hydrolizing and fermenting bacteria (so called "flanking communities") into soluble and readily biodegradable substrates that can then be utilized by specific ecophysiological groups, such as PAO, GAO, or denitrifiers [1,5]. On the other hand, lab-scale systems are operated under artificially controlled conditions and fed with synthetic wastewater, usually containing a single carbon source, as was the case in this study. Microorganisms experience a strong selective pressure that is significantly different from that in full-scale WWTPs, and well-defined operational conditions reduce the number of ecological niches available to bacteria [58]. Thus, the microbial diversity in lab-scale bioreactors fed with a single carbon source is rather narrow, as it was observed in this study. The obtained results demonstrate that much poorer composition of the synthetic wastewater in comparison to municipal sewage is an important factor controlling the microbial composition of the sludge.

Bacterial community structure in the seed of SBRs was typical for municipal full-scale WWTPs. Over 40% of the whole community identified by the EUBmix probe belonged to the phylum Proteobacteria. This value falls within the ranges (33%–78%) typically reported for Polish, German, Australian, and Japanese WWTPs [59–62], but is higher than in Danish plants (27%) tested by Nielsen et al. [63] or the average value (23%) reported by Wu et al. [9] for 269 WWTPs in 23 countries on 6 continents. However, the results of the latter work were obtained by 16S rRNA amplicon sequencing and can differ from the results of qFISH analyses.

The most abundant proteobacterial class in the seed was Betaproteobacteria (17% of all bacteria detected with the EUBmix probe), less numerous were Alpha- and Gammaproteobacteria (13% each). These results are close to the values reported for municipal WWTPs in Australia (1%–17%, 10%–66% and 5%–17%) [61], Germany (18%–51%, 9%–16% and 4%–11%) [60], and Japan (20%–30%, 9%–12% and 6%–9%) [62], respectively. The seed in this study contained also a significant percentage of Actinobacteria and Chloroflexi (20% and 17%, respectively). These phyla are usually included among the dominant ones in activated sludge of municipal WWTPs all over the world [10,63–67]. Bacteria belonging to Deltaproteobacteria, *Saprospiraceae*, Firmicutes and Nitrospira accounted for a minor fraction in this study (<1% each). However, Kondrotaite et al. [4] performed a detailed analysis of the V1–V3 amplicon data from the recent Global MiDAS survey on 929 samples from
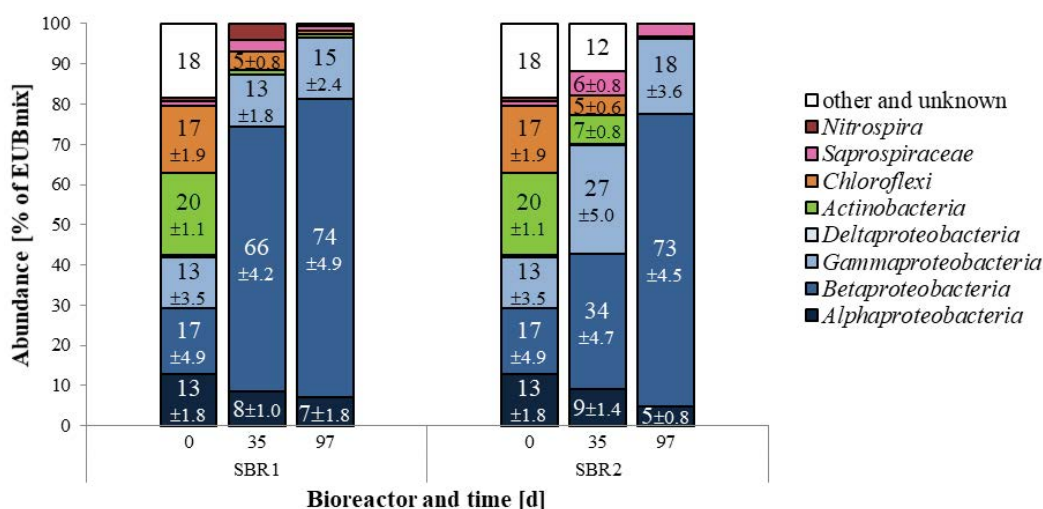

Fig. 3. Microbial community structure of seed (time 0) and activated sludge in SBR1 and SBR2 (anoxic-anaerobic-aerobic reactor and truly aerobic reactor, respectively) after 35 and 97 d of the experiment. The abundance of a given bacterial group is expressed as a percentage of the whole community identified by the EUBmix probe ± standard error. Labels that are lower than 5% are not displayed.

30 countries [10] in order to determine the most abundant genera of *Saprospiraceae*. They showed that Bacteroidota was the second most abundant phylum in the examined WWTPs, with *Saprospiraceae* being the most abundant and diverse family, comprising 162 genera. There was a huge variation in their abundance from plant to plant, which can be overlooked when using the widely applied family-level SAP-309 probe due to the insufficient coverage.

The synthetic wastewater has greatly affected the microbial community structure in both SBRs. However, after 3 months of the reactors' operation, no clear differences were found between them in terms of the bacterial phyla, classes and families, despite the different operating modes of the SBRs. The percentage of Proteobacteria in both SBRs has more than doubled from 43% to 96% (Fig. 3) and the majority of bacteria belonged to the betaproteobacterial class, which accounted for 74% and 73% of the biovolume in SBR1 and SBR2, respectively. The abundance of Alphaproteobacteria dropped from 13% in the seed to 7% and 5% in SBR1 and SBR2, respectively, Gammaproteobacteria remained relatively stable, while Deltaproteobacteria were not detected in any of the reactors. Proteobacteria practically outcompeted Chloroflexi and Actinobacteria. Most of Chloroflexi belong to the group of filaments specialized in the breakdown of complex organics contained in real municipal wastewater, such as polysaccharides and proteins [2,66,68]. The ecophysiology of Actinobacteria is very complex, apart from starch degraders, this phylum also encompasses bacteria capable of assimilating substrates when available, storing them intracellularly as carbon and energy sources, and thus easily coping with feast and famine conditions typical of EBPR systems. However, despite the broad substrate assimilation profiles and the ability to utilize a wide range of both hydrophilic and hydrophobic substrates aerobically, anoxically and anaerobically, in most cases acetate is not assimilated by members of this phylum [2,69]. It should be noted that the synthetic wastewater fed to both SBRs contained only acetate as an organic carbon source, and Actinobacteria were easily outcompeted by other ecophysiological groups. *Saprospiraceae* abundance detected by the SAP-309 probe fluctuated without a clear trend during the experiment within the range 1%–6% of the whole bacterial community. Most genera of this family are still poorly characterized, with unknown functions. Nevertheless, some members of the *Saprospiraceae* were shown to break down proteins, lipids, and other macromolecules, while others are often found in activated sludge systems with nitrogen removal, indicating their potential role of nitrogen converting bacteria [4,70].

Lv et al. [38] linked the successful process of sludge granulation with the bacterial community structure and revealed that mature granules have a spherical core with anaerobic *Rhodocyclaceae* covered by an outer spherical shell with both aerobic and anaerobic strains. During granulation, the microbial community decreased in diversity, as it was found in this study. Li et al. [71] also showed that the formation of granules was accompanied by the reduction of microbial diversity and Beta- and Gammaproteobacteria were the dominating classes in glucose granules. Lv et al. [38] demonstrated that the flocculated flocs were first transited to young granules with increased abundances of *Flavobacteriaceae*, *Xanthomonadaceae*, *Rhodobacteraceae* and *Microbacteriaceae*. Significant changes in the bacterial community structure were also observed in both SBRs in the present study. However, it was interesting and somewhat surprising that no clear differences were found between the reactors with regard to the microbial communities, and the formation of matured granules in SBR1 cannot be attributed to the abundance of the main bacterial phyla and classes. Likewise, Liu et al. [72] did not confirm either that the flocculated flocs and the aerobic granules had different microbial community structures. Authors suggested that granulation did not exert any microbial selection pressure, and granules and flocculated flocs co-existed in a full-scale reactor treating real wastewater.

Secretion of EPS is recognized as the key factor for assisting efficient granulation [38,39,73]. However, the formation of EPS is strongly associated with the presence of specific species, or even strains and clades in activated sludge communities. Zhao et al. [74] noted that *Thauera* and *Zoogloea* were dominant genera in aerobic granules cultivated with piggery wastewater. Also, Weissbrodt et al. [75] showed that *Zoogloea*, which is widely known for producing EPS, was enriched in dense granules, whereas filamentous Burkholderiales dominated in loose granules. PAO and GAO usually form dense monocolonies due to their ability to secrete large amounts of EPS, and both ecophysiological groups are often detected in aerobic granular sludge [40,41,51]. In this study a significant increase in PAO and GAO abundance was observed in SBR1 from 23% and 2% in the seed to 40% and 20% on day 35, to finally reach 68% and 16% on day 97, respectively (Fig. 4).

A clear change in the composition of the PAO group was observed; *Tetrasphaera*-related PAO were predominant in the seeding sludge from the full-scale WWTP, whereas in both lab-scale reactors these bacteria were completely outcompeted by *Accumulibacter* after just 35 d. *Tetrasphaera*-related PAO are more versatile compared to *Accumulibacter* in terms of substrates to be assimilated under anaerobic conditions. Depending on the clades, they are capable of taking up glucose and various amino acids, fermenting glucose, and expressing extracellular surface-associated amylases for degradation of starch [3,15]. Therefore, *Tetrasphaera* were shown to be highly abundant PAO worldwide in full-scale WWTPs with EBPR [5,10,15,16]. However, hardly any of *Tetrasphaera* clades can utilize acetate that was the only source of organic carbon in the wastewater used in this study. These results support the widely accepted postulation that in contrast to well-controlled lab-scale SBR conditions, the availability of various organic substrates in actual wastewater in full-scale WWTPs provides greater niche partitioning for the coexistence of multiple species, strains, and clades of PAO and GAO [1,12,58].

The qFISH analysis of the structure of *Accumulibacter* lineage (Fig. 5) performed on day 97 revealed that clades IA and IIA, C, D constituted 78% and 73% of the *Accumulibacter* population in SBR1 and SBR2, respectively. On the other hand, 97% of *Accumulibacter* cells in the seed from the full-scale WWTP were not targeted by any of the clade-specific Acc-I-444 and Acc-II-444 probes, reflecting the greater diversity of the *Accumulibacter* lineage. It should be stated
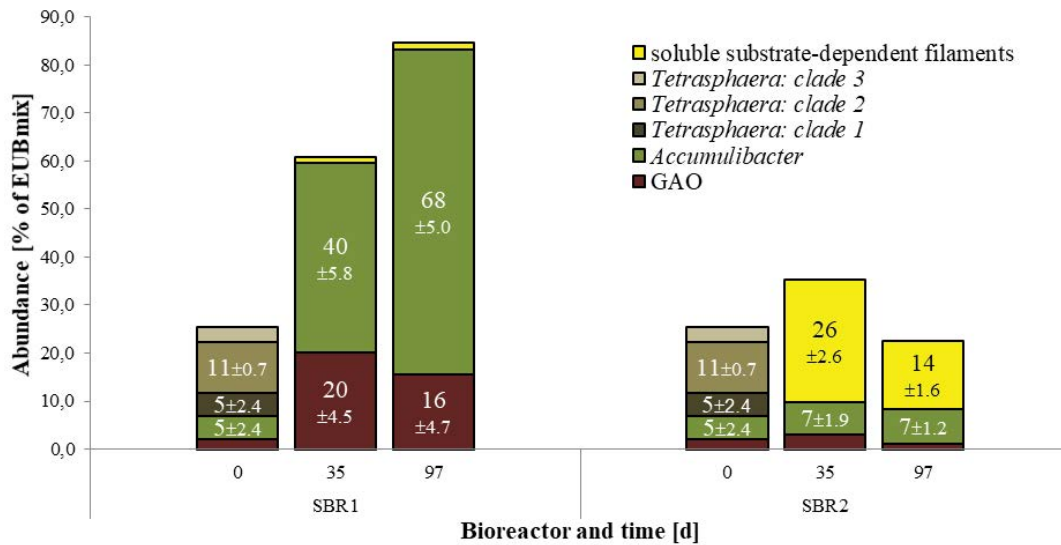
Fig. 4. Changes in the abundance of soluble substrate-dependent filaments (*Thiothrix*/021N group), polyphosphate accumu-lating organisms (*Accumulibacter phosphatis* and *Tetrasphaera* clades 1, 2, 3), and glycogen accumulating organisms (sum of *Competibacter phosphatis* and *Defluviicoccus vanus* clusters 1, 2 shown together) in activated sludge of SBR1 and SBR2 (anox-ic-anaerobic-aerobic reactor and truly aerobic reactor, respectively) after 0 (seed), 35 and 97 d of the experiment. *Accumuli-monas phosphatis, Propionivibrio aalborgensis,* and *Defluviicoccus vanus* cluster 3 were not detected. The abundance of a given bacterial group is expressed as a percentage of the whole community identified by the EUBmix probe ± standard error. Labels that are lower than 5% are not displayed.
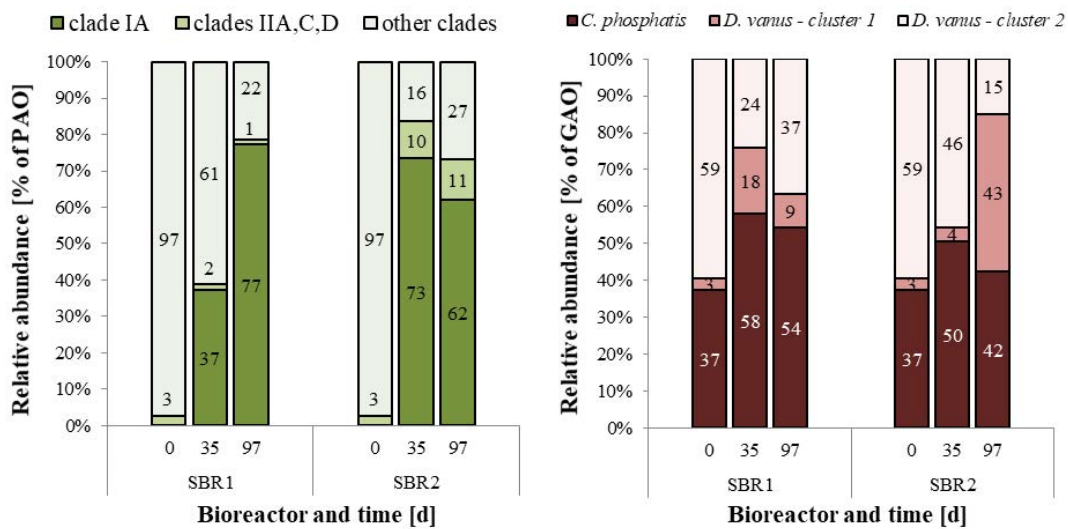

Fig. 5. Relative abundance of clades within *Accumulibacter* lineage (left chart) and the glycogen accumulating organisms community structure (right chart) in activated sludge of SBR1 and SBR2 (anoxic-anaerobic-aerobic reactor and truly aerobic reactor, respec-tively) after 0 (seed), 35 and 97 d of the experiment. The abundance of *Accumulibacter* clades is expressed as a relative percent-age of the *Accumulibacter* lineage identified by the PAOmix probe. The abundance of *Competibacter phosphatis,* and clusters 1 and 2 of *Defluviicoccus vanus* (right chart) is expressed as a relative percentage of the glycogen accumulating organisms community identified by probes GB, DF1mix and DF2mix. *Propionivibrio aalborgensis* and *Defluviicoccus vanus* cluster 3 were not detected.

that these probes were designed to distinguish Type I from Type II in well-characterized laboratory-scale bioreactors [47]. Recently, Petriglieri et al. [13] used the full-length 16S rRNA gene sequences to re-evaluate the existing probes and to design a set of new species-level probes that can be used to better resolve the *Accumulibacter* phylogeny. They identified 15 novel *Accumulibacter* species and substantially

resolved the complex phylogeny of this lineage. It should also be emphasized that PAOmix probes have been shown to be inadequate to distinguish *Accumulibacter* from spe-cies belonging to *Propionivibrio*, a well-known GAO [26]. However, we did not detect *Propionivibrio aalborgensis* by using the Prop207 probe, hence hybridization of PAO462 and PAO846 probes with this GAO in the present study can

be ruled out. Instead, *Competibacter phosphatis* and clusters 1 and 2 of *Defluviicoccus vanus* were detected in the seed and activated sludge of both SBRs, with the abundance of these GAO in SBR2 being significantly lower (below 3% in total) compared to SBR1 (20% and 16% after 35 and 97 d, respectively). It is noteworthy that in both SBRs fed with acetate as the sole source of organic carbon, the relative percentage of the *Defluviicoccus vanus* cluster 2 was significantly lower than in the seeding sludge from the full-scale WWTP. Instead, an increase in the contribution of cluster 1 and *Competibacter* in the GAO community was observed (Fig. 5). Interestingly, despite the large differences between the abundance of PAO and GAO in SBR1 and SBR2 (Fig. 4), the community structures of both ecophysiological groups did not differ significantly from each other between the reactors (Fig. 5). However, McIllroy et al. [21] suggested that the *Competibacter* lineage is too diverse (>89% 16S rRNA sequence similarity) to be considered a single genus. It can therefore be speculated then that more specific probes designed by McIllroy et al. [21] to detect members of the family *Competibacteraceae* would have distinguished the abundance of specific *Competibacter* clades. Similarly, new species-specific probes [13] would provide insight into the differentiation of the *Accumulibacter* genus in SBR1 and SBR2. Nevertheless, for the time being only the *ppk1* marker gene allows for resolving the *Accumulibacter* phylogeny into clades for a finer-scale than 2 specific probes Acc-I-444 and Acc-II-444.

PAO and GAO communities play a key role in the formation of aerobic granules [21,29,30]. The formation of alginate-like exopolysaccharides, which are the major constituents of EPS, is strongly associated with the presence of *Rhodobacteraceae* and *Competibacteraceae* members [39,40,76], mainly *Accumulibacter* and *Competibacter*, which dominated the microbial communities in SBR1 in this study. Zou et al. [77] reported that *Accumulibacter* increased its abundance from 0.4% to 10% after 60 d of operation of an aerobic granular sludge system for nitrogen and phosphorus removal and significantly contributed to the formation and stabilization of aerobic granules. In addition to *Competibacter*, other GAO, such as *Defluviicoccus*, which were detected in SBR1 with an average volume fraction close to 9%, also participate in the formation of dense granules [41]. In this study, the abundance of *Accumulibacter*, *Competibacter* and

Defluviicoccus in SBR1 increased from 5%, 1% and 1%, respectively, to 40%, 12% and 8% after 35 d (Figs. 4 and 5). Microbial community in SBR1 after 97 d of the experiment was completely dominated by the PAO and GAO populations, which together accounted for 84% of all bacteria detected by EUBmix probe (Fig. 4). This was in response to the anoxic-anaerobic-aerobic operating mode known to effectively enrich microbial communities important to the EBPR process due to the similarities of both bacterial group in their metabolic abilities to survive under cyclic redox conditions. In contrast to SBR1, the abundance of both PAO and GAO in the truly aerobic SBR2 was relatively stable throughout the experiment, except for three clades of *Tetrasphaera*-related PAO which were not detectable in the sludge of both SBRs after only 35 d. The flocs in SBR2 exhibited a characteristic filamentous outer structure (rosettes) due to the massive growth of *Thiothrix*/021N (Figs. 4 and 6). Several studies reviewed by Nielsen et al. [2] showed that the filamentous bacteria of the *Thiothrix*/021N group are aerobic, nutritionally very versatile and flexible, do not secrete hydrolytic exoenzymes and prefer soluble substrates for growth. They are classified to the versatile, soluble substrate-dependent filaments, may cause serious bulking problems in industrial WWTPs, but they are rare in reactors with denitrification [2,78,79]. Representatives of this ecophysiological group are able to assimilate a wide range of short-chain fatty acids, including the acetate used in the wastewater in this study, as well as sugars, amino acids and alcohol under aerobic conditions, as was the case in SBR2. The results of our research confirmed that the presence of acetate as a soluble substrate is essential for the persistence and excessive proliferation of the *Thiothrix*/021N group.

3.3. Activity of PAO and GAO

Epifluorescence microscopy revealed apparent co-existence of PAO and GAO communities in anoxic-anaerobic-aerobic SBR1, however, with a significantly higher abundance of the former compared to the latter. To check whether the high PAO abundance (nearly 70% of the whole bacterial community identified by the EUBmix) was reflected in the EBPR activity, activated sludge from both SBRs was subjected to the anaerobic batch tests. Under anaerobic conditions

Table 1
Comparison of P_{rel}/DOC_{upt}, and polyphosphate accumulating organisms (*Accumulibacter*) and glycogen accumulating organisms (*Competibacter* + *Defluviicoccus*) fractions (relative abundance of each bacterial group in the whole PAO + GAO community) quantified from P_{rel}/DOC_{upt} and by qFISH analysis in anoxic-anaerobic-aerobic SBR1 and truly aerobic SBR2 after 97 d of the experiment

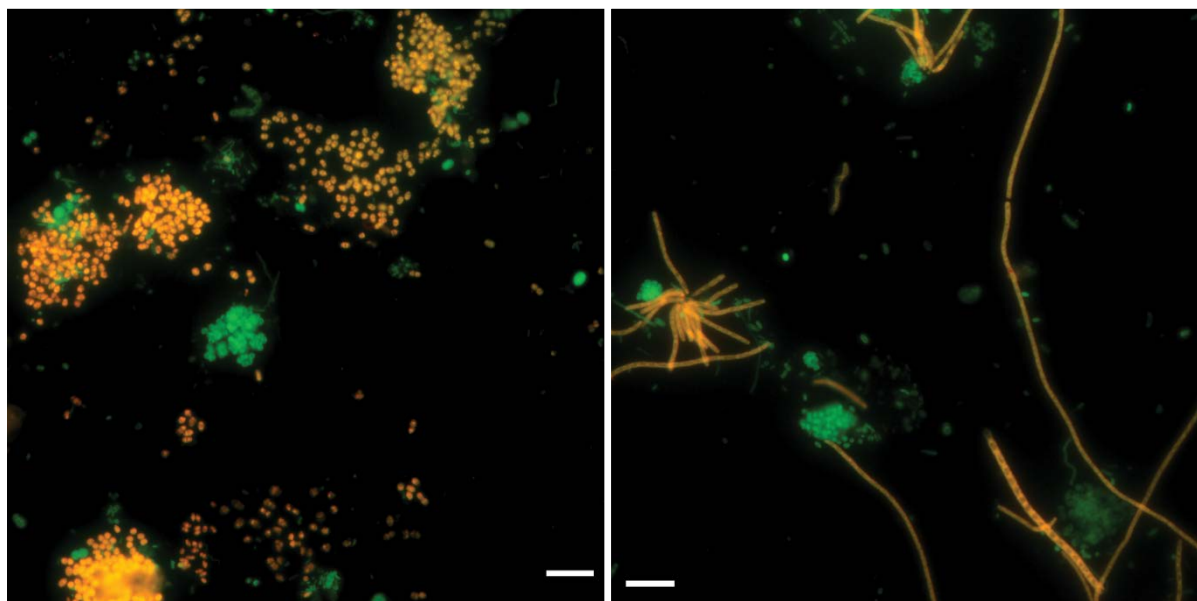| Parameter	SBR1	SBR2
P_{rel}/DOC_{upt} [P-mol/C-mol]	0.44	0.43
PAO fraction quantified from P_{rel}/DOC_{upt} (f_{PAO})	0.86	0.84
GAO fraction quantified from P_{rel}/DOC_{upt} (f_{GAO})	0.14	0.16
PAO [% of EUBmix]	68	7
GAO [% of EUBmix]	16	1.2
PAO fraction quantified by qFISH (PAO/(PAO + GAO))	0.81	0.85
GAO fraction quantified by qFISH (GAO/(PAO + GAO))	0.19	0.15

Fig. 6. FISH micrographs of polyphosphate accumulating organisms in aerobic granules (left photo) and *Thiothrix*/021N group in activated sludge flocs (right photo) sampled from anoxic-anaerobic-aerobic SBR1 and truly aerobic SBR2 on day 97, respectively. Photos are merges of those taken with the specific probe (PAOmix or G123T, respectively) labelled with Cy3 (red), combined with those taken of the same field of view with EUBmix, labelled with 6-Fam (green). Yellow cells are overlay of red and green. Scale bars represent 10 μm.

both PAO and GAO take up acetate from wastewater, but only PAO hydrolyze intracellular poly-P to obtain energy for this purpose and release $P–PO_4^{3-}$ into bulk liquid. This results in a decrease in the concentration of organic carbon in the mixed liquor (expresses as DOC) and a simultaneous increase in the concentration of orthophosphate. Chemical profiles of extracellular DOC and $P–PO_4^{3-}$ concentrations for activated sludge sampled from SBR1 on day 97 were consistent with this typical PAO phenotype (Fig. 7). The P_{rel}/DOC_{upt} ratio (0.44 mol/mol) was relatively high and typical for acetate and *Accumulibacter* enriched cultures (0.30–0.50 mol/mol), as reported in the literature [14,44]. It implied a high activity of PAO in SBR1 at the end of the experiment, which was also confirmed by DAPI staining of the sludge sampled at the end of the aerobic period. Orthophosphate released under anaerobic conditions was taken up by PAO in the next aerobic phase and stored as poly-P, as shown by positive staining results (Fig. 8A and B). The activated sludge from SBR2 did not show the EBPR activity typical of PAO cultures in the anaerobic batch tests, despite 7% *Accumulibacter* abundance at the end of the experiment (Fig. 7). It should be stated that a prerequisite for the effective C_{org} uptake by PAO under anaerobic conditions is the presence of poly-P granules in bacterial cells as a source of energy available for PAO in the absence of oxygen. DAPI staining did not confirm the presence of poly-P in PAO cells at the end of the aerobic period, as presented in Fig. 8C and D. This suggests that *Accumulibacter* culture may require acclimatisation to the anaerobic/aerobic conditions after long-term aerobic exposure in order to restore their metabolism to that of EBPR.

Fractions of the PAO/GAO populations in SBR1 and SBR2, calculated from the P_{rel}/DOC_{upt} ratios (0.44 and 0.43 mol/mol, respectively) were 0.86/0.14 and 0.84/0.16
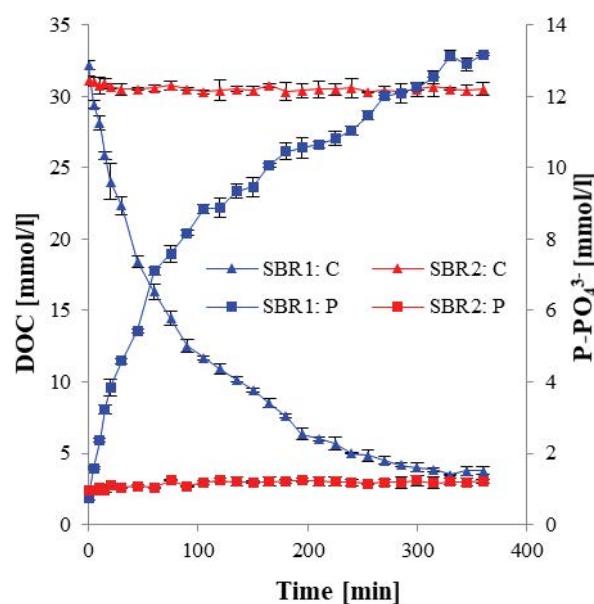

Fig. 7. Profiles of extracellular $P–PO_4^{3-}$ and DOC during the anaerobic batch EBPR tests of activated sludge taken from SBR1 and SBR2 after 97 d of the experiment. Batch tests were carried out after adding excess electron donor (under non-limiting carbon conditions). Means of triplicates are presented, error bars illustrate standard errors. Error bars that are smaller than the marker symbols are not displayed.

(Table 1), and were close to the values obtained from the FISH analyzes (0.81/0.19 and 0.85/0.15, respectively). This shows the compatibility of both methods used for quantification of PAO and GAO fractions, when the former is
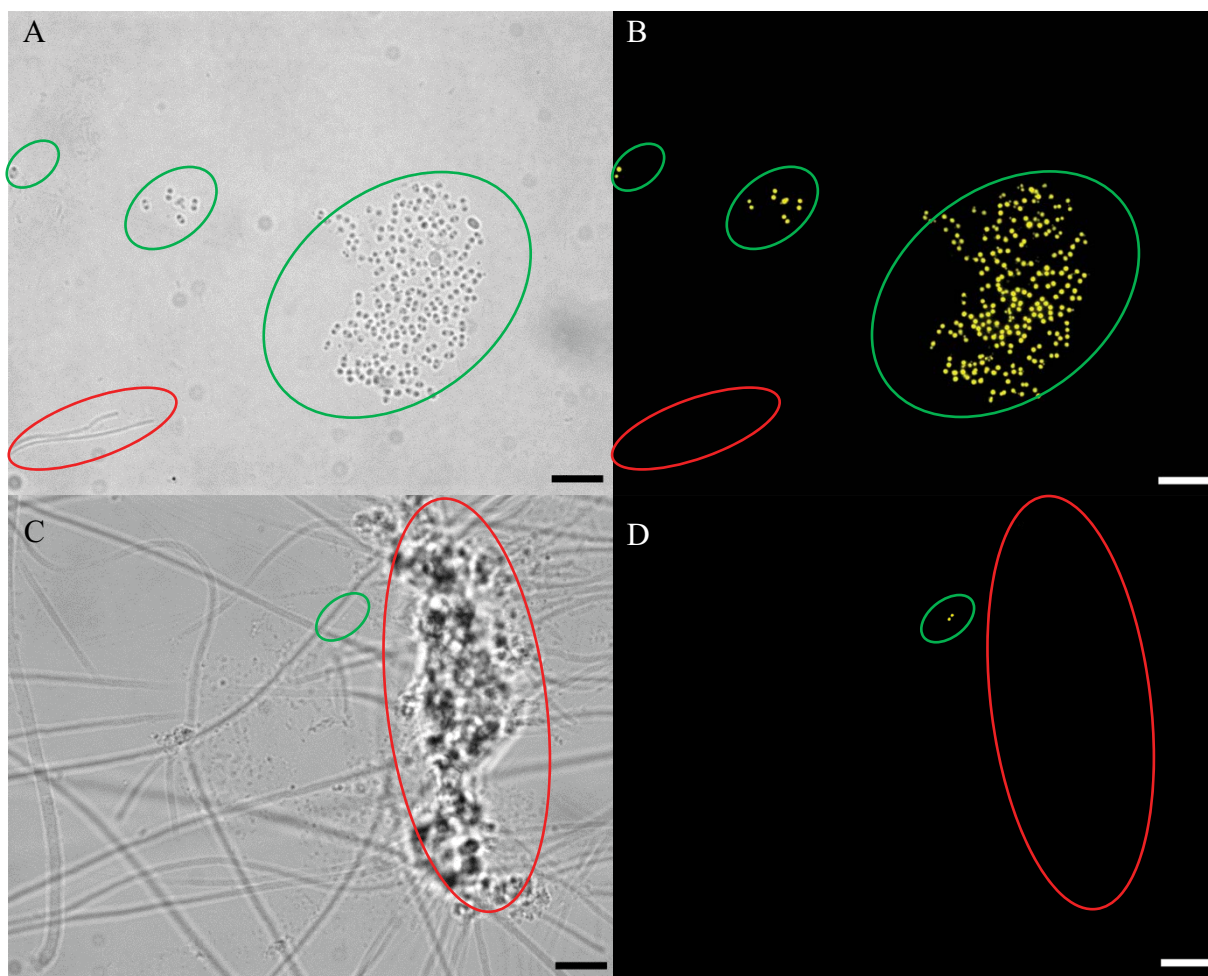
Fig. 8. Micrographs of biomass samples taken from anoxic-anaerobic-aerobic SBR1 (A and B) and truly aerobic SBR2 (C and D) after 97 d of the experiment. Same fields are presented in the left and right panels. (A) and (C) are phase contrast micrographs, (B) and (D) show fluorescent response from DAPI-polyphosphate complex. The green ellipses show bacteria cells with a positive bright yellow DAPI-polyphosphate signal which indicates the accumulation of polyphosphate. The red ellipses show bacteria cells without accumulated polyphosphate. Scale bars represent 10 μm.

dominated by *Accumulibacter* or other PAO that utilize acetate. However, the P_{rel}/DOC_{upt} ratio for SBR2 was calculated using the low values of ΔP and ΔDOC (Fig. 7) as it is described above, and the compatibility of both methods may be coincidental in this case.

4. Conclusions

Microbial community structure and PAO and GAO activity have been studied in anoxic-anaerobic-aerobic SBR and truly aerobic SBR. The obtained results show that:

- Synthetic wastewater with acetate as the only source of organic carbon equally influenced the abundance of bacterial phyla and classes in both reactors. In comparison to the seeding sludge from the municipal WWTP, Proteobacteria increased their abundance from 43% to 96% of all bacteria detected with EUBmix probe, and practically outcompeted Chloroflexi and Actinobacteria. Betaproteobacteria was the dominant class (increase from

17% to 73%–74%), abundance of Alphaproteobacteria dropped from 13% to 5%–7%, Gammaproteobacteria remained relatively stable (15%–18%). The percentage content of bacterial phyla and classes was rather unaffected by the way of bioreactors operation,
- Mode of operating bioreactors largely influenced the abundance of ecophysiological bacterial groups. Anoxic-anaerobic-aerobic conditions and the presence of low molecular weight soluble substrate (acetate) favoured the growth of PAO and GAO and promoted efficient biomass granulation. The truly aerobic conditions, along with the acetate, triggered an extensive growth of filamentous *Thiothrix*/021N group. The granulation process in anoxic-anaerobic-aerobic SBR was not attributed to the abundance of the main bacterial phyla and classes, but rather the PAO and GAO communities, known to produce exopolysaccharides, played a key role in the formation of matured aerobic granules,
- Despite the large differences between the PAO and GAO abundance in anoxic-anaerobic-aerobic SBR and truly

aerobic SBR, the PAO and GAO community structures did not differ significantly from one reactor to another. However, a marked change in the composition of these populations was observed in both reactors compared to the sludge seed from the full-scale plant, which indicates that the availability and type of organic substrate was a decisive factor shaping the PAO and GAO community structures. *Tetrasphaera*-related PAO, which were predominant in the seed, were completely outcompeted by *Accumulibacter* after just 35 d in the presence of acetate as the sole source of organic carbon. Clades IA and IIA, C, D constituted 78% and 73% of the *Accumulibacter* population in anoxic-anaerobic-aerobic and truly aerobic reactors, respectively, whereas 97% of *Accumulibacter* cells in the seed from the full-scale plant were not targeted by any of the two clade-specific probes. Compared to the seeding sludge, cluster 2 of *Defluviicoccus vanus* was partially replaced in the GAO community of both acetate-fed SBRs by the *Defluviicoccus* cluster 1 and *Competibacter*,

- Mode of operating bioreactors largely influenced the activity of ecophysiological bacterial groups. While the substantial PAO abundance in the anoxic-anaerobic-aerobic SBR (nearly 70% of the whole bacterial community) was reflected in the high EBPR activity (confirmed by anaerobic batch tests and positive poly-P staining in aerobic conditions), the biomass from the aerobic reactor did not show the activity typical of PAO cultures, despite a significant 7% of *Accumulibacter* abundance. The anaerobic EBPR activity expressed as P_{rel}/C_{upt} ratio can be successfully used to quantify the relative fractions of the PAO/GAO populations in activated sludge.

Authors' contributions

Conceptualization, A.M. and M.Z.-R.; methodology, A.M.; validation, M.Z.-R.; investigation, A.M.; resources, A.M. and M.Z.-R.; data curation, A.M.; writing—original draft preparation, A.M.; writing—review and editing, A.M. and M.Z.-R.; visualization, A.M..; supervision, M.Z.-R.; funding acquisition, M.Z.-R. Both authors have read and agreed to the published version of the manuscript.

References

[1] P.H. Nielsen, A.T. Mielczarek, C. Kragelund, J.L. Nielsen, A.M. Saunders, Y. Kong, A.A. Hansen, J. Vollertsen, A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants, Water Res., 44 (2010) 5070–5088.

[2] P.H. Nielsen, C. Kragelund, R.J. Seviour, J.L. Nielsen, Identity and ecophysiology of filamentous bacteria in activated sludge, FEMS Microbiol. Rev., 33 (2009) 969–998.

[3] R. Marques, J. Santos, H. Nguyen, G. Carvalho, J.P. Noronha, P.H. Nielsen, M.A.M. Reis, A. Oehmen, Metabolism and ecological niche of *Tetrasphaera* and *Ca. Accumulibacter* in enhanced biological phosphorus removal, Water Res., 122 (2017) 159–171.

[4] Z. Kondrotaite, L.C. Valk, F. Petriglieri, C. Singleton, M. Nierychlo, M.K.D. Dueholm, P.H. Nielsen, Diversity and ecophysiology of the genus OLB8 and other abundant uncultured Saprospiraceae genera in global wastewater treatment systems, Front. Microbiol., 13 (2022) 917553, doi: 10.3389/fmicb.2022.917553.

[5] P.H. Nielsen, S.J. McIlroy, M. Albertsen, M. Nierychlo, Re-evaluating the microbiology of the enhanced biological

phosphorus removal process, Curr. Opin. Biotechnol., 57 (2019) 111–118.

[6] S.J. McIlroy, A. Starnawska, P. Starnawski, A.M. Saunders, M. Nierychlo, P.H. Nielsen, J.L. Nielsen, Identification of active denitrifiers in full-scale nutrient removal wastewater treatment systems, Environ. Microbiol., 18 (2016) 50–64.

[7] H. Daims, E.V. Lebedeva, P. Pjevac, P. Han, C. Herbold, M. Albertsen, N. Jehmlich, M. Palatinszky, J. Vierheilig, A. Bulaev, R.H. Kirkegaard, M. von Bergen, T. Rattei, B. Bendinger, P.H. Nielsen, M. Wagner, Complete nitrification by Nitrospira bacteria, Nature, 528 (2015) 504–509.

[8] S. Ge, S. Wang, X. Yang, S. Qiu, B. Li, Y. Peng, Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: a review, Chemosphere, 140 (2015) 85–98.

[9] L. Wu, D. Ning, B. Zhang, Y. Li, P. Zhang, X. Shan, Q. Zhang, M.R. Brown, Z. Li, J.D. van Nostrand, F. Ling, N. Xiao, Y. Zhang, J. Vierheilig, G.F. Wells, Y. Yang, Y. Deng, Q. Tu, A. Wang, T. Zhang, Z. He, J. Keller, P.H. Nielsen, P.J.J. Alvarez, C.S. Criddle, M. Wagner, J.M. Tiedje, Q. He, T.P. Curtis, D.A. Stahl, L. Alvarez-Cohen, B.E. Rittmann, X. Wen, J. Zhou, Global diversity and biogeography of bacterial communities in wastewater treatment plants, Nat. Microbiol., 4 (2019) 1183–1195.

[10] M.K.D. Dueholm, M. Nierychlo, K.S. Andersen, V. Rudkjøbing, S. Knutsson, MiDAS Global Consortium, M. Albertsen, P.H. Nielsen, MiDAS 4: A global catalogue of full-length 16S rRNA gene sequences and taxonomy for studies of bacterial communities in wastewater treatment plants, Nat. Commun., 13 (2022) 1908, doi: 10.1038/s41467-022-29438-7.

[11] G. Dottorini, T.Y. Michaelsen, S. Kucheryavskiy, K.S. Andersen, J.M. Kristensen, M. Peces, D.S. Wagner, M. Nierychlo, P.H. Nielsen, Mass-immigration determines the assembly of activated sludge microbial communities, Proc. Natl. Acad. Sci. U.S.A., 118 (2021) e2021589118, doi: 10.1073/pnas.2021589118.

[12] Y.Y. Law, R.H. Kirkegaard, A.A. Cokro, X. Liu, K. Arumugam, C. Xie, M.S. Bjerregaard, D.I. Drautz-Moses, P.H. Nielsen, S. Wuertz, R.B.H. Williams, Integrative microbial community analysis reveals full-scale enhanced biological phosphorus removal under tropical conditions, Sci. Rep., 6 (2016) 25719, doi: 10.1038/srep25719.

[13] F. Petriglieri, C.M. Singleton, Z. Kondrotaite, M.K.D. Dueholm, E.A. McDaniel, K.D. McMahon, P.H. Nielsen, Reevaluation of the phylogenetic diversity and global distribution of the genus "*Candidatus Accumulibacter*", mSystems, 7 (2022) e0001622, doi: 10.1128/msystems.00016-22.

[14] A. Oehmen, P.C. Lemos, G. Carvalho, Z. Yuan, J. Keller, L.L. Blackall, M.A.M. Reis, Advances in enhanced biological phosphorus removal: from micro to macro scale, Water Res., 41 (2007) 2271–2300.

[15] H.T. Nguyen, V.Q. Le, A.A. Hansen, J.L. Nielsen, P.H. Nielsen, High diversity and abundance of putative polyphosphate-accumulating *Tetrasphaera*-related bacteria in activated sludge systems, FEMS Microbiol. Ecol., 76 (2011) 256–267.

[16] A. Muszyński, M. Załęska-Radziwiłł, Polyphosphate accumulating organisms in treatment plants with different wastewater composition, Archit. Civ. Eng. Environ., 8 (2015) 99–105.

[17] G. Qiu, R. Zuniga-Montanez, Y. Law, S.S. Thi, T.Q.N. Nguyen, K. Eganathan, X. Liu, P.H. Nielsen, R.B.H. Williams, S. Wuertz, Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources, Water Res., 149 (2019) 496–510.

[18] C.M. Singleton, F. Petriglieri, K. Wasmund, M. Nierychlo, Z. Kondrotaite, J.F. Petersen, M. Peces, M.S. Dueholm, M. Wagner, P.H. Nielsen, The novel genus, 'Candidatus Phosphoribacter', previously identified as *Tetrasphaera*, is the dominant polyphosphate accumulating lineage in EBPR wastewater treatment plants worldwide, ISME J., 16 (2022) 1605–1616.

[19] H.T. Nguyen, J.L. Nielsen, P.H. Nielsen, 'Candidatus Halomonas phosphatis', a novel polyphosphate-accumulating organism in full-scale enhanced biological phosphorus removal plants, Environ. Microbiol., 14 (2012) 2826–2837.

[20] F. Petriglieri, C. Singleton, M. Peces, J.F. Petersen, M. Nierychlo, P.H. Nielsen, "*Candidatus* Dechloromonas phosphoritropha" and "Ca. D. phosphorivorans", novel polyphosphate accumulating organisms abundant in wastewater treatment systems, ISME J., 15 (2021) 3605–3614.

[21] S.J. McIlroy, T. Nittami, E. Kanai, J. Fukuda, A.M. Saunders, P.H. Nielsen, Re-appraisal of the phylogeny and fluorescence *in situ* hybridization probes for the analysis of the Competibacteraceae in wastewater treatment systems, Environ. Microbiol. Rep., 7 (2015) 166–174.

[22] I. Bessarab, A.M. Maszenan, M.A.S. Haryono, K. Arumugam, N.M.M.T. Saw, R.J. Seviour, R.B.H. Williams, Comparative genomics of members of the genus Defluviicoccus with insights into their ecophysiological importance, Front. Microbiol., 13 (2022) 834906, doi: 10.3389/fmicb.2022.834906.

[23] A.M. Maszenan, I. Bessarab, R.B.H. Williams, S. Petrovski, R.J. Seviour, The phylogeny, ecology and ecophysiology of the glycogen accumulating organism (GAO) Defluviicoccus in wastewater treatment plants, Water Res., 221 (2022) 118729, doi: 10.1016/j.watres.2022.118729.

[24] T. Nittami, S. McIlroy, E.M. Seviour, S. Schroeder, R.J. Seviour, *Candidatus Monilibacter* spp., common bulking filaments in activated sludge, are members of cluster III Defluviicoccus, Syst. Appl. Microbiol., 32 (2009) 480–489.

[25] S.J. McIlroy, C.A. Onetto, B. McIlroy, F.-A. Herbst, M.S. Dueholm, R.H. Kirkegaard, E. Fernando, S.M. Karst, M. Nierychlo, J.M. Kristensen, K.L. Eales, P.R. Grbin, R. Wimmer, P.H. Nielsen, Genomic and *in situ* analyses reveal the *Micropruina* spp. as abundant fermentative glycogen accumulating organisms in enhanced biological phosphorus removal systems, Front. Microbiol., 9 (2018) 1004, doi: 10.3389/fmicb.2018.01004.

[26] M. Albertsen, S.J. McIlroy, M. Stokholm-Bjerregaard, S.M. Karst, P.H. Nielsen, "*Candidatus* Propionivibrio aalborgensis": a novel glycogen accumulating organism abundant in full-scale enhanced biological phosphorus removal plants, Front. Microbiol., 7 (2016) 1033, 10.3389/fmicb.2016.01033.

[27] R.L. Meyer, A.M. Saunders, R.J. Zeng, J. Keller, L.L. Blackall, Microscale structure and function of anaerobic-aerobic granules containing glycogen accumulating organisms, FEMS Microbiol. Ecol., 45 (2003) 253–261.

[28] R. Lemaire, Z. Yuan, L.L. Blackall, G.R. Crocetti, Microbial distribution of *Accumulibacter* spp. and *Competibacter* spp. in aerobic granules from a lab-scale biological nutrient removal system, Environ. Microbiol., 10 (2008) 354–363.

[29] T.W. Seviour, L.K. Lambert, M. Pijuan, Z. Yuan, Selectively inducing the synthesis of a key structural exopolysaccharide in aerobic granules by enriching for *Candidatus* "Competibacter phosphatis", Appl. Microbiol. Biotechnol., 92 (2011) 1297–1305.

[30] D.G. Weissbrodt, T.R. Neu, U. Kuhlicke, Y. Rappaz, C. Holliger, Assessment of bacterial and structural dynamics in aerobic granular biofilms, Front. Microbiol., 4 (2013) 175, doi: 10.3389/fmicb.2013.00175.

[31] L. Jahn, K. Svardal, J. Krampe, Comparison of aerobic granulation in SBR and continuous-flow plants, J. Environ. Manage., 231 (2019) 953–961.

[32] D. Xu, J. Li, J. Liu, X. Qu, H. Ma, Advances in continuous flow aerobic granular sludge: a review, Process Saf. Environ. Prot., 163 (2022) 27–35.

[33] D.J. Lee, Y.Y. Chen, K.Y. Show, C.G. Whiteley, J.H. Tay, Advances in aerobic granule formation and granule stability in the course of storage and reactor operation, Biotechnol. Adv., 28 (2010) 919–934.

[34] Q. Zhang, J. Hu, D-J. Lee, Aerobic granular processes: current research trends, Bioresour. Technol., 210 (2016) 74–80.

[35] S. Bengtsson, M. de Blois, B.-M. Wilén, D. Gustavsson, Treatment of municipal wastewater with aerobic granular sludge, Crit. Rev. Env. Sci. Technol., 48 (2018) 119–166.

[36] M.-K.H. Winkler, C. Meunier, O. Henriet, J. Mahillon, M.E. Suárez-Ojeda, G. Del Moro, M. De Sanctis, C. Di Iaconi, D.G. Weissbrodt, An integrative review of granular sludge for the biological removal of nutrients and recalcitrant organic matter from wastewater, Chem. Eng. J., 336 (2018) 489–502.

[37] F. Cai, L. Lei, Y. Li, Y. Chen, A review of aerobic granular sludge (AGS) treating recalcitrant wastewater: refractory organics removal mechanism, application and prospect, Sci. Total Environ., 782 (2021) 146852, doi: 10.1016/j.scitotenv.2021.146852.

[38] Y. Lv, C. Wan, D.-J. Lee, X. Liu, J.-H. Tay, Microbial communities of aerobic granules: granulation mechanisms, Bioresour. Technol., 169 (2014) 344–351.

[39] S.A. Zahra, N. Abdullah, K. Iwamoto, A. Yuzir, S.E. Mohamad, Alginate-like exopolysaccharides in aerobic granular sludge: a review, Mater. Today Proc., 65 (2022) 3046–3053.

[40] S.L.S. Rollemberg, A.F. dos Santos, T.J.T. Ferreira, P.I.M. Firmino, A.B. dos Santos, Evaluation of the production of alginate-like exopolysaccharides (ALE) and tryptophan in aerobic granular sludge systems, Bioprocess Biosyst. Eng., 44 (2021) 259–70.

[41] C.M. Schambeck, B.S. Magnus, L.C.R. de Souza, W.R.M. Leite, N. Derlon, L.B. Guimarães, R.H.R. da Costa, Biopolymers recovery: dynamics and characterization of alginate-like exopolymers in an aerobic granular sludge system treating municipal wastewater without sludge inoculum, J. Environ. Manage., 263 (2020) 110394, doi: 10.1016/j.jenvman.2020.110394.

[42] K. Affek, A. Muszyński, M. Załęska-Radziwiłł, N. Doskocz, A. Ziętkowska, M. Widomski, Evaluation of ecotoxicity and inactivation of bacteria during ozonation of treated wastewater, Desal. Water Treat., 192 (2020) 176–184.

[43] A. Muszyński, M. Załęska-Radziwiłł, N. Doskocz, Factors affecting *Accumulibacter* population structure in full- and laboratory-scale biological reactors with nutrients removal, Water Sci. Technol., 77 (2018) 2794–2802.

[44] M.C. van Loosdrecht, P.H. Nielsen, C.M. López-Vázquez, D. Brdjanovic (eds.), Experimental Methods in Wastewater Treatment, IWA Publishing, London, 2016.

[45] C.M. López-Vázquez, C.M. Hooijmans, D. Brdjanovic, H.J. Gijzen, M.C. van Loosdrecht, A practical method for quantification of phosphorus- and glycogen-accumulating organism populations in activated sludge systems, Water Environ. Res., 79 (2007) 2487–2498.

[46] D. Greuter, A. Loy, M. Horn, T. Rattei, ProbeBase: an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016, Nucleic Acids Res., 44 (2016) D586–D589.

[47] J.J. Flowers, S. He, S. Yilmaz, D.R. Noguera, K.D. McMahon, Denitrification capabilities of two biological phosphorus removal sludges dominated by different 'Candidatus Accumulibacter' clades, Environ. Microbiol. Rep., 1 (2009) 583–588.

[48] A. Muszyński, P. Marcinowski, J. Maksymiec, K. Beskowska, E. Kalwarczyk, J. Bogacki, Cosmetic wastewater treatment with combined light/Fe⁰/H₂O₂ process coupled with activated sludge, J. Hazard. Mater., 378 (2019) 120732, doi: 10.1016/j.jhazmat.2019.06.009.

[49] T.J. Collins, ImageJ for microscopy, Biotechniques, 43 (2007) 25–30.

[50] J.L. Zilles, C.H. Hung, D.R. Noguera, Presence of *Rhodocyclus* in a full-scale wastewater treatment plant and their participation in enhanced biological phosphorus removal, Water Sci. Technol., 46 (2002) 123–128.

[51] A. Muszyński, A. Miłobędzka, The effects of carbon/phosphorus ratio on polyphosphate and glycogen-accumulating organisms in aerobic granular sludge, Int. J. Environ. Sci. Technol., 12 (2015) 3053–3060.

[52] L.S. Clescerl, A.E. Greenberg, A.D. Eaton, Eds., Standard Methods for the Examination of Water and Wastewater, 20th ed. APHA/AWWA/WEF, Washington D.C., USA, 1999.

[53] M.K. de Kreuk, M.C.M. van Loosdrecht, Selection of slow growing organisms as a means for improving aerobic granular sludge stability, Water Sci. Technol., 49 (2004) 9–17.

[54] Z. Li, Q. Meng, C. Wan, C. Zhang, X. Tan, X. Liu, Aggregation performance and adhesion behavior of microbes in response to feast/famine condition: rapid granulation of aerobic granular sludge, Environ. Res., 208 (2022) 112780, doi: 10.1016/j.envres.2022.112780.

[55] T. Rocktäschel, C. Klarmann, B. Helmreich, J. Ochoa, P. Boisson, K.H. Sorensen, H. Horn, Comparison of two different anaerobic feeding strategies to establish a stable aerobic granulated sludge bed, Water Res., 47 (2013) 6423–6431.

[56] S. Kosar, O Isik., Y. Akdag, H. Gulhan, I. Koyuncu, H. Ozgun, M.E. Ersahin, Impact of seed sludge characteristics on granulation and performance of aerobic granular sludge process, J. Cleaner Prod., 363 (2022) 132424, doi: 10.1016/j. jclepro.2022.132424.

[57] T.P. Curtis, I.M. Head, D.W. Graham, Theoretical ecology for engineering biology, Environ. Sci. Technol., 37 (2003) 64A–70A.

[58] A. Muszyński, M. Łebkowska, A. Tabernacka, A. Miłobędzka, From macro to lab-scale: changes in bacterial community led to deterioration of EBPR in lab reactor, Cent. Eur. J. Biol., 8 (2013) 130–142.

[59] A. Muszyński, A. Tabernacka, A. Miłobędzka, Long-term dynamics of the microbial community in a full-scale wastewater treatment plant, Int. Biodeterior. Biodegrad., 100 (2015) 44–51.

[60] M. Schmid, A. Thill, U. Purkhold, M. Walcher, J.Y. Bottero, P. Ginestet, P.H. Nielsen, S. Wuertz, M. Wagner, Characterization of activated sludge flocs by confocal laser scanning microscopy and image analysis, Water Res., 37 (2003) 2043–2052.

[61] M. Beer, H.M. Stratton, P.C. Griffiths, R.J. Seviour, Which are the polyphosphate accumulating organisms in full-scale activated sludge enhanced biological phosphate removal systems in Australia?, J. Appl. Microbiol., 100 (2006) 233–243.

[62] M.-T. Wong, T. Mino, R.J. Seviour, M. Onuki, W.-T. Liu, *In situ* identification and characterization of the microbial community structure of full-scale enhanced biological phosphorus removal plants in Japan, Water Res., 39 (2005) 2901–2914.

[63] P.H. Nielsen, A.M. Saunders, A.A. Hansen, P. Larsen, J.L. Nielsen Microbial communities involved in enhanced biological phosphorus removal from wastewater – a model system in environmental biotechnology, Curr. Opin. Biotechnol., 23 (2012) 452–459.

[64] A.T. Mielczarek, C. Kragelund, P.S. Eriksen, P.H. Nielsen, Population dynamics of filamentous bacteria in Danish wastewater treatment plants with nutrient removal, Water Res., 46 (2012) 3781–3795.

[65] A. Miłobędzka, A. Muszyński, Population dynamics of filamentous bacteria identified in polish full-scale wastewater treatment plants with nutrients removal, Water Sci. Technol., 71 (2015) 675–684.

[66] M. Nierychlo, A. Milobedzka, F. Petriglieri, B. McIlroy, P.H. Nielsen, S.J. McIlroy, The morphology and metabolic potential of the Chloroflexi in full-scale activated sludge wastewater treatment plants, FEMS Microbiol. Ecol., 95 (2019) fiy228, doi: 10.1093/femsec/fiy228.

[67] D.S. Wágner, M. Peces, M. Nierychlo, A.T. Mielczarek, D. Thornberg, P.H. Nielsen, Seasonal microbial community dynamics complicates the evaluation of filamentous bulking mitigation strategies in full-scale WRRFs, Water Res., 216 (2022) 118340, doi: 10.1016/j.watres.2022.118340.

[68] L.B.M. Speirs, D.T.F. Rice, S. Petrovski, R.J. Seviour, The phylogeny, biodiversity, and ecology of the Chloroflexi in activated sludge, Front. Microbiol., 10 (2019) 2015, doi: 10.3389/fmicb.2019.02015.

[69] R.J. Seviour, C. Kragelund, Y. Kong, K.L. Eales, J.L. Nielsen, P.H. Nielsen, Ecophysiology of the Actinobacteria in activated sludge systems, Antonie van Leeuwenhoek, 94 (2008) 21–33.

[70] J. Cao, T. Zhang, Y. Wu, Y. Sun, Y. Zhang, B. Huang, B. Fu, E. Yang, Q. Zhang, J. Luo, Correlations of nitrogen removal and core functional genera in full-scale wastewater treatment plants: influences of different treatment processes and influent characteristics, Bioresour. Technol., 297 (2020) 122455, doi: 10.1016/j.biortech.2019.122455.

[71] A.J. Li, S.F. Yang, X.Y. Li, J.D. Gu, Microbial population dynamics during aerobic sludge granulation at different organic loading rates, Water Res., 42 (2008) 3552–3560.

[72] Y.Q. Liu, B. Moy, Y.H Kong., J.H. Tay, Formation, physical characteristics and microbial community structure of aerobic granules in a pilot-scale sequencing batch reactor for real wastewater treatment, Enzyme Microb. Technol., 46 (2010) 520–525.

[73] Z. Liang, W. Li, S Yang., P. Du, Extraction and structural characteristics of extracellular polymeric substances (EPS), pellets in autotrophic nitrifying biofilm and activated sludge, Chemosphere, 81 (2010) 626–632.

[74] Y.G. Zhao, J. Huang, H. Zhao, H. Yang, Microbial community and N removal of aerobic granular sludge at high COD and N loading rates, Bioresour. Technol., 143 (2013) 439–446.

[75] D.G. Weissbrodt, S. Lochmatter, S. Ebrahimi, P. Rossi, J. Maillard, C. Holliger, Bacterial selection during the formation of early-stage aerobic granules in wastewater treatment systems operated under wash-out dynamics, Front Microbiol., 3 (2012) 332, doi: 10.3389/fmicb.2012.00332.

[76] W.D. Tian, C. Ma, Y.M. Lin, L. Welles, C. López-Vázquez, M.C.M. van Loosdrecht, Enrichment and characterization of a psychrophilic 'Candidatus Accumulibacter phosphatis' culture, Int. Biodeterior. Biodegrad., 124 (2017) 267–275.

[77] J.T. Zou, F.F. Yu, J.Y. Pan, B.J. Pan, S.Y. Wu., M.J. Qian, J. Li, Rapid start-up of an aerobic granular sludge system for nitrogen and phosphorus removal through seeding chitosan-based sludge aggregates, Sci. Total Environ., 762 (2021) 144171, doi: 10.1016/j.scitotenv.2020.144171.

[78] P. Asvapathanagul, B.H. Olson, P.B. Gedalanga, A. Hashemi, Z. Huang, J. La, Identification and quantification of *Thiothrix eikelboomii* using qPCR for early detection of bulking incidents in a full-scale water reclamation plant, Appl. Microbiol. Biotechnol., 99 (2015) 4045–4057.

[79] T. Nittami, T. Shoji, Y. Koshiba, M. Noguchi, M. Oshiki, M. Kuroda, T. Kindaichi, J. Fukuda, F. Kurisu, Investigation of prospective factors that control *Kouleothrix* (Type 1851) filamentous bacterial abundance and their correlation with sludge settleability in full-scale wastewater treatment plants, Process Saf. Environ. Prot., 124 (2019) 137–142.