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PII: S0960-8524(16)31623-6
DOI: http://dx.doi.org/10.1016/j.biortech.2016.11.102
Reference: BITE 17348

To appear in: *Bioresource Technology*

Received Date: 23 September 2016
Revised Date: 24 November 2016
Accepted Date: 25 November 2016

Please cite this article as: Iqbal Hossain, M., Paparini, A., Cord-Ruwisch, R., Rapid Adaptation of Activated Sludge Bacteria into a Glycogen Accumulating Biofilm enabling Anaerobic BOD Uptake, *Bioresource Technology* (2016), doi: http://dx.doi.org/10.1016/j.biortech.2016.11.102

Rapid Adaptation of Activated Sludge Bacteria into a Glycogen Accumulating Biofilm enabling Anaerobic BOD Uptake

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Abstract

Glycogen accumulating organisms (GAO) are known to allow anaerobic uptake of biological oxygen demand (BOD) in activated sludge wastewater treatment systems. In this study, we report a rapid transition of suspended activated sludge biomass to a GAO dominated biofilm by selective enrichment using sequences of anaerobic loading followed by aerobic exposure of the biofilm to air. The study showed that within eight weeks, a fully operational, GAO dominated biofilm had developed, enabling complete anaerobic BOD uptake at a rate of 256 mg/L/h. The oxygen uptake by the biofilm directly from the atmosphere had been calculated to provide significant energy savings. This study suggests that wastewater treatment plant operators can convert activated sludge systems readily into a "passive aeration" biofilm that avoids costly oxygen transfer to bulk wastewater solution. The described energy efficient BOD removal system provides an opportunity to be coupled with novel nitrogen removal processes such as anammox.

Keywords: Glycogen accumulating organism (GAO), Activated sludge, Biofilm, Energy-efficient BOD removal, Wastewater.

1. Introduction

Conventional suspended growth activated sludge process, the core part of sewage treatment technology, have been extensively used for wastewater treatment since its introduction 100 years ago and have contributed greatly to our society in terms of environment protection and public health benefits (van Loosdrecht & Brdjanovic, 2014). Activated sludge is a mixture of inactive sewage solids combined with microbial populations, which facilitates the degradation and conversion of pollutants in wastewater treatment plants. The current activated sludge paradigm for wastewater treatment is characterized by relatively high-energy consumption and waste biomass production, which leads to high operational cost (Foley et al., 2010; Tchobanoglous et al., 2003). To overcome these problems, extensive research was undertaken that led to the development of new technologies, which have shown promise to treat wastewater more efficiently.

The sequencing batch reactor (SBR), a modification of the activated sludge process, where all nutrients (nitrogen, phosphorus, and organic carbon) are removed in a single reactor, have gained a great deal of attention due to their improved nutrient removal capacity. This process uses discrete phases regarding nutrient availability (e.g., feast/famine regime with respect to carbon source). Microbial populations normally exposed to this feast/famine condition results in the accumulation of large fraction of the soluble substrate, when available, as internal storage polymers such as poly-β-hydroxyalkanoates (PHAs) (Ciggin et al., 2013; Van Loosdrecht et al., 1997). The storage polymers act as an electron donor for respiration if electron acceptors (such as oxygen or nitrite) become available. This principle is used in "storage driven denitrification" process such as simultaneous nitrification and denitrification (SND) where heterotrophic bacteria rapidly store soluble substrate as storage

polymer (PHAs) that degrades slowly to provide the reducing power for the process to remove nitrogen from wastewater (Krasnits et al., 2013; Third et al., 2003).

In much the same way as PHA build-up by the bacterial biomass is advantageous for nitrogen removal, it is also critically involved in biological phosphorus removal as in enhanced biological phosphorus removal (EBPR) by poly-phosphate accumulating organisms (PAOs) (Oehmen et al., 2007). Polyphosphate accumulating organisms can take up organic BOD (e.g. in the form of acetate) and intracellularly store them as PHAs under anaerobic (feast) conditions (Mino et al., 1998). Energy for this biotransformation is generated by the cleavage of intracellular polyphosphate (poly-P) which they previously accumulated during the famine (aerobic) period (Mino et al., 1998), thus removing phosphorus from wastewater.

The dynamic feast-famine (anaerobic-aerobic) regime used in EBPR is also known to favour development of a different phenotypic group of bacteria called glycogen accumulating organisms (GAOs) (Liu et al., 1996; Satoh et al., 1992). Like PAO, these organisms are also able to store volatile fatty acids (VFA) as PHA anaerobically which they use in the subsequent aerobic phase as carbon and energy source. The energy and reducing power required for the anaerobic storage of PHA is provided by the hydrolysis of intracellularly stored glycogen. In aerobic conditions, PHA is oxidized for glycogen replenishment, biomass growth, and aerobic maintenance purposes. Since GAO competes with PAO for anaerobic uptake of VFA without contributing to the phosphorus removal process, they are considered undesirable and a major cause of EBPR failure (Kong et al., 2006; Zhou et al., 2008b). However, conventional EBPR processes generate excess sludge which increases the sludge disposal cost. To mitigate this problem, new technology such as biofilm based processes have been researched and developed.

In recent years, different forms of biofilm-based technology have been used around the world to remove nutrients and pollutants from wastewater. However, biofilm reactors have not been reported much in literature to be capable of developing bacteria that store biological oxygen demand (BOD) as PHA. Hughes et al. (2006) reported that storage driven biofilm reactor could be used to remove nitrogen from waste streams with high nitrogen relative to carbon effectively and efficiently. Moreover, in a recent report, Flavigny and Cord-Ruwisch (2015) described a biofilm reactor enriched in glycogen accumulating organisms that had been operated at very high biomass densities (50 g/L) for several years under alternating anaerobic/aerobic conditions. The biofilm was able to take up BOD anaerobically. After the biomass had removed the BOD and the treated, largely BOD (acetate) free synthetic wastewater was drained; the biomass could regenerate its biological storage capacity by oxidizing the stored PHA using oxygen directly from the atmosphere. However, it is not known how long it would take to develop such a biofilm from standard activated sludge and how effectively anaerobic biofilm BOD uptake will work. This is an impediment for plant operators intending to make use this technology.

The aim of the current paper is to describe the transition from activated sludge to GAO dominated biofilm by using selective conditions. The significance of the study is that the results give operators of wastewater treatment plants and design engineers a time estimate for the conversion of a traditional activated sludge biomass to a GAO dominated biofilm reactor that enables low-cost BOD removal via passive aeration.

2. Materials and Methods

2.1. Experimental setup and operation

Two reactors were constructed and operated in parallel; a sequencing batch biofilm reactor (test reactor) and a trickling filter reactor (control reactor) (Figure 1). The sequencing

batch biofilm reactor (SBBR) (4 cm diameter and 23 cm height) with a working volume of 0.255 L was equipped with dissolved oxygen (DO), pH and oxidation-reduction potential (ORP) probes. The reactor was completely automated; with all pumps, airflow valves and phase lengths controlled by National Instruments Instrumentation Control Software LabVIEW^{TM} (version 9.1). The trickling filter reactor (TFR) (dimension and working volume as of the sequencing batch biofilm reactor) was set up with a recycle vessel. Both reactors were filled with packing material (AMB^{TM} Biomedia Bioballs), whose specific surface area for biofilm growth and support is 500 m^2/m^3. These carrier materials have a cylindrical shape with 7 mm height and 11 mm diameter. The volume occupied by the empty carrier material was 20% ($V_{carrier}$/ $V_{reactor}$).

Prior to operation, described reactors were inoculated with activated sludge from local wastewater treatment plant (Subiaco, Western Australia). After seeding, the sequencing batch biofilm reactor was operated automatically by specifically timed phases. The reactor was filled with synthetic wastewater (within 5 min through a peristaltic pump), then maintained under anaerobic condition for about 2 hours, followed by gravity drainage (10 min) and exposure of the biofilm directly to air, which was recirculated within the reactor for 1 hour. In contrast, the control reactor was operated in trickling reactor mode at all time where feed (synthetic wastewater) was trickled by recycling over the carrier material.

2.2. Synthetic wastewater

Synthetic wastewater was used throughout the experimental period. The standard composition of the synthetic wastewater was (mg L^{-1}): CH_3COONa 660, NH_4Cl 160, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4$. $7H_2O$ 25, $CaCl_2$. $2H_2O$ 300, $FeSO_4$. $7H_2O$ 6.25, yeast extract 50, and 1.25 ml L^{-1} of trace element solution, which contained (g L^{-1}): EDTA 15,

$ZnSO_4$. $5H_2O$ 0.43, $CoCl_2$. $6H_2O$ 0.24, $MnCl_2$. $4H_2O$ 0.99, $CuSO_4$. $5H_2O$ 0.25, $NaMoO_4$. $2H_2O$ 0.22, $NiCl_2$. $6H_2O$ 0.19, $NaSeO_4$. $10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4$. $2H_2O$ 0.050.

2.3. Histochemical staining

The ability of biofilm material of both reactors to accumulate PHA was determined using chemical staining with Sudan Black B (Jenkins et al., 2004). Smears of biofilm materials deposited on a glass slide were stained with a 0.3% (w/v in 60% ethanol) Sudan Black B solution for 10 minutes and rinsed with water for 1 second. Slides were then counter-stained for 10 seconds with 0.5% safranin (w/v in deionized water), rinsed well with water and blotted dry. An Olympus BX51 microscope equipped with a charge-couple device (CCD) camera (PanasonicWV-CL830) was used for the observation of the biomass.

2.4. Analytical procedures

2.4.1. Chemical analysis

Total suspended solids (TSS), volatile suspended solids (VSS) and orthophosphate analysis were carried out according to standard methods (APHA, 2012). Five representative plastic carriers were taken from each reactor, and the biomass was detached from them for determination of the TSS and VSS. The total amount of TSS and VSS was calculated on the basis of total number of plastic carriers in the bioreactors.

2.4.2. Acetate analysis

Acetate was analysed using an Agilant 7820A gas chromatography (GC) with auto-sampler. Samples were acidified with formic acid (10% v/v) before 0.4 μL samples were injected onto an Altech Econo-Cap™ EC™-1000 column (30 m length × 0.250 mm internal diameter × 0.25 μm film thickness). The carrier gas (N_2) was set at a flow rate of 3 mL/min and the sample was split 10:1 at the inlet. The oven temperature was programmed as follows: initial temperature 70°C, increased at 5°C/min to 100°C, held for 2.0 min, increased at

70°C/min to 250°C, held for 2.0 min. Injector and detector were set at 250 and 300°C respectively. The peak area of the Flame Ionisation Detector (FID) output signal was computed via integration using the EzChrome Elite Compact Software© (V.3.3.2 SP2). The detection limit determined as 0.5 µmol/L of acetate.

2.4.3. Poly-β-hydroxyalkanoate (PHA) analysis

Poly-β-hydroxyalkanoate (PHA) including poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), was measured according to a method adapted from Smolders et al. (1994b). Briefly, approximately 20 mg freeze-dried samples of biomass were put into screw-topped glass tubes, and 1.45 mL of a mixture of 1-propanol and concentrated HCl (4:1), 1.5 mL dichloromethane and 50 µL benzoic acid solution as internal standard (2 g benzoic acid dissolved in 100 mL of 1-propanol) were subsequently added. The tubes were sealed with Teflon lids to prevent loss of volatile solvents. The samples were then digested for 4 hours at 100°C. After cooling, the organic phase was extracted with 3 mL distilled water; 1 mL of the organic phase was dried over Na_2SO_4 and transferred to the GC vials for analysis. 4.5µL of the sample was injected into an Agilant 7820A gas chromatograph (Agilant, USA) equipped with a FID detector and an Altech Econo-Cap™ EC™-1000 column (30 m length × 0.250 mm internal diameter × 0.25 µm film thickness). Nitrogen was used as a carrier gas (3 mL/min), and the sample was split 1:5 at the inlet. The temperature of injection was 250°C, the temperature of Flame Ionisation Detector (FID) was 300°C, and the temperature ramp of the column started at 80°C, then increased at a rate of 70°C/min until 152°C, further increased at a rate of 4°C/min until 160°C, and finally increased again at 70°C/min until 230°C and held for 2 min, to ensure a cleaning of the column after each injection.

2.4.4. Glycogen analysis

Biomass glycogen was analysed as glucose after acidic hydrolysis, according to the method used by Wang et al. (2015). Approximately 1-2 mg freeze-dried biomass was weighed into air-tight Pyrex tubes, to which 5 mL of 0.6 M HCl was added and heated at 100°C for 3 h. After cooling to room temperature, samples were sheared by a vortex mixer for 1 min, and transferred to 10-mL tubes, followed by centrifugation at 2600g for 10 min. About 1 mL supernatant was added to 4 mL of anthrone-H_2SO_4 reagent (0.2% anthrone (w/v) in 80% (v/v) H_2SO_4) in 10-mL colorimetric tubes. All tubes were placed in a water bath at 100 °C for 10 min. After cooling at 4°C for 5 min in cold water, samples were measured by a UV/VIS spectrophotometer (UVmini-1240, Shimadzu, Japan) at 625 nm. Glucose was used as the standard.

Three individual replicates of all experiments were performed. All the data were subjected to analysis of variance (ANOVA) using PAST software (Version 3.14). Statistical significance was tested using the least significant difference (LSD) at the $p<0.05$ level.

2.5. Microbial community structure analysis

Biofilm was scraped off the carriers, and genomic DNA was extracted using the Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction and using DNA-free reagents and consumables. A mock-extraction was also carried out, in parallel, using the same reagents and consumables, but no biofilm (extraction blank). The V_4 hypervariable region of the 16S rRNA gene was amplified with the modified version (Apprill et al., 2015) of 515F – 806R primers (Caporaso et al., 2012). Briefly, for each sample, polymerase chain reaction was carried out in a 25 µL total volume including 2.5 µL of normalized total genomic DNA (5 ng/µL), 0.2 µM of each primer and 12.5 µL of 2x KAPA HiFi HotStart Ready Mix (Kappa Biosystems, USA). The PCR cycling protocol

consisted of an initial denaturation step of 95°C for 3 min, followed by 35 cycles of DNA denaturation at 95°C for 30s, primer annealing at 55°C for 30s, strand elongation at 72°C for 30s, and a final elongation step at 72°C for 5 min. Extraction blanks and no-template control were always included in all PCR amplifications. For each sample or control, the PCR products from the three replicates were then pooled, checked by gel electrophoresis and purified using AMPure XP beads (Beckman Coulter, USA). After quantification with the Qubit dsDNA Assay Kit (Thermo Fisher Scientific, USA), amplicons were pooled at equimolar ratios, prior to index PCR using Nextera XT Index Kit V_2-V_5 indexes (Illumina, USA). Products were purified again using AMPure XP beads (Beckman Coulter, USA), quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) and pooled at the approximately equimolar ratio. The pool was then further concentrated and purified by a QIAquick PCR Purification Kit (Qiagen, USA) and quantified by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA), prior to dilution to 4 nM and paired-end sequencing (2 x 250 bp; 500 cycle V2) on the MiSeq platform (Illumina, USA).

Sequences were first processed in Geneious 8.0.4 (Kearse et al., 2012). Sequences were then quality filtered using USERARCH (Edgar, 2010), allowing only reads with a <1% error rate to remain and singletons were removed. To identify bacterial genera present in samples, operational taxonomic units (OTUs) were selected by clustering sequencing at 97% similarity with the UPARSE algorithm (Edgar, 2013) and filtered by UCHIME to ensure OTUs were not the result of chimeric reads. Genus level taxonomy was assigned to OTUs against the Greengenes 16S database (August 2013 release) (DeSantis et al., 2006) in QIIME 1.8.0 (Caporaso et al., 2010) using the UCLUST algorithm (Edgar, 2010) with default parameters. Bacterial genera that were identified in extraction reagent blanks and no-template control were removed from the dataset to eliminate background bacterial sequences. Some sequences

were manually cross-checked using the National Centre for Biotechnological Information nr collection of databases with the Basic Local Alignment Search Tool (BLAST) for nucleotides, including some sequences that were not identified at the genus level by QIIME.

3. Results and Discussion

The sequencing batch biofilm reactor (SBBR) and trickling filter reactor (TFR) were operated continuously under standard conditions by feeding synthetic wastewater for eight weeks after inoculation with activated sludge biomass. As expected, biofilms developed in both reactors over time. In order to quantify to what extent the alternating exposure of biofilm to anaerobic submerged conditions and to air (after draining) develops specialised storage bacteria such as the GAO in sequencing batch biofilm reactor, a number of parameters were studied that are indicative of the existence and predominance of GAO bacteria in the biomass. Amongst these parameters are anaerobic acetate storage capacity, microscopic observation, intracellular glycogen and PHA levels and microbial community structure analysis.

3.1. Development of anaerobic acetate storage capacity

A key indicator for a PHA accumulating organism-rich biofilm is its ability to take up acetate in the absence of oxygen or other electron acceptors such as nitrate. Such acetate uptake can only be explained by storage if methanogenesis is excluded. To test for such anaerobic acetate uptake and storage, both biofilms were submerged with synthetic wastewater and the decrease in acetate monitored over time.

The sequencing batch biofilm reactor (SBBR) that was operated under sequential anaerobic storage and subsequent biomass exposure to air conditions demonstrated the increasing capacity of anaerobic acetate storage over time (Figure 2A). Already after two weeks of continuous operation, the SBBR showed a clear tendency of acetate storage at a rate

of about 9 Cmmol/L/h (compared to 4.5 Cmmol/L in the trickling filter reactor), suggesting the selection of storage bacteria. The maximum speed of anaerobic acetate storage (initial 30 min) was 24 Cmmol/L/h (768 mg/L/h BOD) after eight weeks of operation. By comparison, the trickling filter reactor reached a maximum of 5.3 Cmmol/L at the end of the experimental period (Figure 2B). The measured acetate removal rate in sequencing batch biofilm reactor was significantly higher than the rates reported in the literature. Flavigny and Cord-Ruwisch (2015) reported an acetate removal rate of 10 Cmmol/L/h (320 mg/L/h BOD) in a similar SBBR operated with substantially higher biomass levels.

Interestingly, despite the fact that the SBBR underwent sequential anaerobic and aerobic phases, it developed a biofilm faster than the TFR. This is evident from microscopic observations and from the difference of aerobic acetate uptake rates after six weeks by both biofilms, which were 7.7 and 4.6 Cmmol/L/h for the SBBR and the TFR, respectively. Also, normal time curves of acetate uptake showed that anaerobic acetate uptake by the SBBR was faster than aerobic acetate uptake by the TFR (Figure S1), which is likely due to the higher biomass level in the SBBR. This result suggests that sequencing batch mode operation enhances biomass accumulation in a carrier material which is in accordance with the observation reported by Bassin et al. (2012).

3.2. Removal of carbon source without release of phosphorus

Two groups of microorganisms can store carbon source anaerobically: the polyphosphate accumulating organisms (PAO) and the glycogen accumulating organisms (GAO). To determine which types of microorganism enriched in this experiment, a batch experiment was done to study the anaerobic acetate (carbon source) uptake and P release profiles of both reactors (Figure 3). In sequencing batch biofilm reactor, biomass removed 17 Cmmol/L acetate while the phosphorus content remained almost equivalent in synthetic

236 wastewater throughout the anaerobic phase (Figure 3A). As expected, the biofilm in the
237 control reactor, in which PHA storage bacteria were not enriched, also did not release
238 phosphate (Figure 3B).

239 The sequential anaerobic and aerobic condition is known to be favourable for acetate
240 uptake and storage as PHA by phosphate accumulating organisms (PAO). These organisms
241 take up phosphate aerobically as an energy source, followed by hydrolysis and release of
242 phosphate in the anaerobic phase, which provides energy for anaerobic acetate uptake and its
243 polymerization as PHA. However, in this experiment, there is little chance for PAOs to
244 develop in the sequencing batch biofilm reactor because aerobic phosphate accumulation
245 cannot occur as phosphate containing synthetic wastewater has been drained just before the
246 aerobic phase. As a consequence, it is expected that an alternative mechanism of acetate
247 storage as PHA is used which is the mechanism of glycogen-accumulating organisms
248 (GAOs). These organisms use aerobically stored glycogen to enable anaerobic acetate uptake,
249 which is subsequently accumulated as PHAs (Liu et al., 1996). Hence, reactor operation in
250 SBBR would be likely to select for GAO rather than PAO.

251 From the established understanding of the physiology of GAO bacteria, the reasons
252 why the described operating conditions (anaerobic loading followed by aerobic exposure of
253 the biofilm to air) leads to the selective enrichment of GAO bacteria are as follows: During
254 the intitial establishment on the carrier material of an aerobic, acetate fed biofilm from
255 activated sludge, various types of bacteria may attach to the carrier (e.g. via producing expo-
256 polymers). However, after anaerobic loading of the reactor with synthetic wastewater, only
257 those bacteria that can store acetate as storage material (i.e. as PHA) will be able to profit
258 from the subsequent aerobic phase to produce ATP via aerobic respiration (electron transport
259 phosphorylation), hence allowing them to proliferate as a biofilm.

Other bacteria, including PAO, cannot profit from the oxygen. To PHA storing GAO bacteria, the aerobic phase not only provides energy in the form of ATP but also generates glycogen which, in the next anaerobic phase serves as the energy source (ATP from substrate level phosphorylation) for continued anaerobic storage of acetate. Again, only GAO bacteria are expected to absorb significant acetate in the next anaerobic phase as it requires a suitable anaerobic ATP source such as stored glycogen. A chance to store phosphate was not provided and hence the sequential anaerobic and aerobic operation specifically encourages GAO bacteria over PAO to develop the biofilm in SBBR which is in accordance with literature (Crocetti et al., 2002; Dai et al., 2007; Zeng et al., 2003). Moreover, the high acetate content available at the beginning of the anaerobic phase, favours GAO development via their ability to uptake acetate by diffusion (López-Vázquez et al., 2008).

3.3. Microscopic observation

Microscopic investigation of the biomass of SBBR showed that it was dominated by one morphological cell type, large coccobacilli (Figure S2). These cells positively stained with Sudan Black B, showing intracellular lipid granules, suggesting the accumulation of PHA. In samples taken after the aerobic period, this cell type did not show the characteristic stain. This finding is similar to the observation reported by Crocetti et al. (2002) who described the abundance of PHA storing spherical cells in an anaerobic and aerobically operated sludge. In contrast, only few lipid containing cells and a majority of rod shaped bacteria was observed in the trickling filter reactor.

3.4. Intracellular glycogen and PHA transformation

After 6 weeks of operation, the sequencing batch biofilm reactor reached a steady state as indicated by nearly identical cycle profiles. The results of one of these cycle studies are depicted in Figure 4. Under anaerobic condition, acetate was taken up, with concomitant

284 consumption of glycogen and accumulation of PHA (PHB + PHV) and without the release of
285 phosphorus. In the subsequent aerobic condition, anaerobically accumulated PHA was
286 oxidized to provide energy for glycogen replenishment and biomass growth. This observation
287 clearly shows that the enriched culture in the SBBR demonstrated the GAO behaviour,
288 confirming the selective enrichment of this functional group of microorganisms under the
289 used operating conditions.

290 The anaerobic and aerobic stoichiometric data was compared with other reports carried
291 out with GAO and PAO enriched cultures (Table 1). The anaerobic $Gly_{degraded}/Ac_{uptake}$ ratio of
292 SBBR was comparatively high with 1.58 (Cmol/Cmol). As glycogen accumulating organisms
293 rely on glycogen as their sole energy source, their $Gly_{degraded}/Ac_{uptake}$ ratio is known to be as
294 high as 1.68 (Cmol/Cmol) (Lopez-Vazquez et al., 2009a). Since the reactor was fed with
295 acetate based synthetic wastewater which was phosphorous limited, the high
296 $Gly_{degraded}/Ac_{uptake}$ ratio in SBBR indicates that the energy required for acetate uptake was
297 mainly derived from glycogen metabolism.

298 Also, the $PHA_{synthesized}/Ac_{uptake}$ ratio in SBBR was high with 2.14 (Cmol/Cmol) which is
299 close to the value (2.33 Cmol/Cmol) reported by Lopez-Vazquez et al. (2009a) for glycogen
300 accumulating organisms (GAOs). Further, the aerobic $Gly_{synthesized}/PHA_{degraded}$ ratio for SBBR
301 was 0.75 (Cmol/Cmol), which is similar to the ratio obtained by Filipe et al. (2001) for GAO
302 enriched cultures. These results agree with the glycogen accumulating metabolisms and
303 suggest that the enriched culture in SBBR was dominated by GAO.

304 ### *3.5. Microbial community structure analysis*

305 To investigate further the key constituents of the described biofilms, 16S rRNA
306 amplicon sequencing analysis was carried out at week 8. The initial bioinformatic analysis
307 (denoising, filtering out chimeras) yielded 11747 and 12283 high quality reads for SBBR and

TFR respectively, which were assigned to different taxonomic levels (from genus to family). A portion of the effective bacterial sequences could not be assigned to any taxon, suggesting that some bacteria were novel which was present in both reactors.

The relative abundances of different phyla and classes in *Proteobacteria* for both reactors are shown in Figure S3. The most abundant phylum in sequencing batch biofilm reactor was *Proteobacteria* (Figure S3 A), which accounted for 64.7% of the total bacterial 16S rRNA gene sequences. In contrast, *Bacteroidetes* (42.4%) was the largest component of the total OTUs in trickling filter reactor, followed by *Proteobacteria* (37.6%). The predominance of *Proteobacteria* is in line with previous studies of activated sludge (AS) communities (Zhang et al., 2012). This group is considered important for wastewater treatment because of their role in carbon, phosphorous and nitrogen removal (Yang et al., 2014). On the other hand, *Bacteroidetes* the dominant phylum in the TFR is responsible for sludge foaming and bulking which leads to increased operational cost of wastewater treatment plants (Yang et al., 2014).

Regarding relative abundances of different classes within *Proteobacteria*, there was a significant difference between SBBR and TFR reactor (Figure S3 B). While *Gamma-proteobactetia* (50.6%) was the most abundant class in the SBBR, the TFR reactor was dominated by *Beta-proteobacteria* (16.3%). This observation suggests that the sequential anaerobic and aerobic phase promotes the proliferation of *Gamma-proteobacteria* which is considered as the chief competitors of PAOs for anaerobic substrate uptake and has shown to be capable of PHA accumulation but lacks the ability to remove phosphorus. On the other hand, *Beta-proteobacteria* normally exists in aerobic bio-systems (Esplugas et al., 2013).

Bacterial community composition at the genus level (>1% relative abundance) is represented in Figure 5 and it can be shown that the sequencing batch biofilm reactor and

trickling filter reactor had different predominant bacterial groups. The SBBR is dominated by *Candidatus competibacter* (48.7%) belonging to *Gamma-proteobacteria*, followed by *Bacteroides* (11.17%). This observation is in line with the findings reported in the literature which has shown that *Candidatus competibacter* can be enriched in an anaerobic/aerobic system using acetate as the sole carbon source (Crocetti et al., 2002; Dai et al., 2007; Zeng et al., 2003). Moreover, other conditions such as increased temperature (about 25°C), low P/C (≤0.02 Pmol/Cmol) ratio used in this study may have contributed to GAOs enrichment and predominance in the SBBR (López-Vázquez et al., 2008; Lopez-Vazquez et al., 2009b).

On the other hand, the most abundant genus in trickling filter reactor is *Sphingobacterium* (8.1%), followed by *Saprospira* (7.7%) and *Bacteroides* (4.7%). *Sphingobacterium can* rapidly break down organic compounds (Yang et al., 2014) which might be responsible for the predominance of *Bacteroidetes* phylum in trickling filter reactor as they can utilize these available substances instantly (Acosta-Martínez et al., 2008). The low level of acetate storage capacity observed in the TFR could be attributed to the combined actions of *Candidatus competibacter* (4.04%), *Bacteroides* (4.74%), *Hydrogenophaga* (1.37%) present in the reactor.

3.6. Practical implications of this study

The current study shows activated sludge suspended biomass can be readily converted to a biofilm reactor that rapidly stores BOD as PHA under anaerobic conditions and subsequently oxidises PHA to glycogen and CO_2 when exposed to oxygen by mere drainage of the bioreactor. The selective enrichment of the responsible GAO bacteria can be accomplished within a few weeks. This observation suggests that wastewater treatment plant operators can readily implement a fixed bed reactor system that can remove a major proportion of BOD of wastewater. Moreover, the present study demonstrated that GAO

dominated biofilm reactor completely removes BOD at a rate of 8 Cmmol/L/h (256 mg BOD/L/h) which is 20 times faster than that of traditional wastewater treatment system such as trickle reactor (Table 2). The efficient and high BOD removal is due to the configuration (anaerobic/aerobic) of the reactor, high surface area (500 m^2/m^3) of carrier material and high biomass content (45 g dry biomass/L of the reactor) of the biofilm (Ahammad et al., 2013; Flavigny & Cord-Ruwisch, 2015). In addition to superior carbon removal performance, the proposed biofilm reactor is cost effective because it avoids the energy-expensive transfer of oxygen to the bulk wastewater as observed in typical activated sludge based processes. The energy requirement of the proposed biofilm reactor (with 3 m height and 3.25 h treatment time) is 2.5 W/m^3 which is about 60 - 75% less than that of trickling reactors (6 - 10 W/m^3) (Tchobanoglous et al., 2003). The energy efficiency phenomena could be attributed largely to the passive aeration of the biofilm and partly to the anaerobic-aerobic operation of the biofilm reactor (Ahammad et al., 2013; Flavigny & Cord-Ruwisch, 2015).

The described energy-efficient biofilm reactor lends itself to treat wastewaters rich in organic material in combination with a separate nitrification step to remove nitrogen as published for nutrients (carbon, nitrogen) removal using two reactors and three (anaerobic/anoxic/aerobic) stages (Cord-Ruwisch & Hughes, 2012; Zhou et al., 2008a). Alternatively, the proposed biofilm system could also be integrated upstream of low-energy required anammox based process to remove nitrogen.

Anammox is a biological process capable of anaerobic transformation of NH_4^+ to dinitrogen (N_2) gas using NO_2^- as an electron acceptor (Kartal et al., 2013) and has been successfully implemented in sidestream wastewater treatment system. Recently research focus has moved to the possible application of anammox based processes to mainstream wastewater treatment. However, one of the main challenges for applying anammox process to

the main wastewater stream is high C/N ratio. Anammox bacteria cannot compete with heterotrophic denitrifying bacteria at high organic content, which results in low levels of anammox bacteria in the population. Moreover, some organic compounds which are added to the wastewater to improve the nitrogen removal efficiency such as methanol, have been found to cause partial/complete inactivation of anammox activity (Ali & Okabe, 2015). In contrast, low concentration of organic matter does not affect anammox activity significantly but improves total nitrogen removal via heterotrophic denitrification. Since the currently described biofilm reactor is capable of removing soluble organic substances from wastewater rapidly and cost-effectively, it could represent an ideal partner process for subsequent anammox processing, resulting overall in one of the least energy consuming wastewater treatment options.

4. Conclusions

The following conclusions could be drawn:

- Within eight weeks, suspended activated sludge biomass was converted to a glycogen accumulating organism (GAO) dominated biofilm reactor.
- The biofilm removed all BOD in the form of storage energy, which enables direct oxygen uptake from the atmosphere (passive aeration) and associated energy savings.
- The biofilm reactor could be integrated with nitrogen removal systems such as parallel nitrification-denitrification (PND) or other anammox based methods which could facilitate the application of this technology to the mainstream wastewater treatment processes.

Acknowledgements

The authors would like to acknowledge Murdoch University for the financial support of this project (scholarship to M. I. Hossain).

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Figure Captions

Figure 1. Schematic diagram of the experimental setup. The biofilm in the SBBR was alternatively exposed to synthetic wastewater to facilitate BOD uptake (under anaerobic conditions) and to atmospheric air to regenerate biofilm's storage capacity. In TFR, synthetic wastewater was trickled by recycling over the carrier material containing the biofilm (SBBR = sequencing batch biofilm reactor, TFR = trickling filter reactor).

Figure 2. Anaerobic acetate storage of the sequencing batch biofilm reactor (A) and trickling filter reactor (B) after 2 (●), 4 (○), 6 (■) and 8 (□) weeks of operation. All values are represented as mean ± standard deviation of three replicates.

Figure 3. Anaerobic acetate and phosphate profiles of batch tests of the SBBR (A) and the TFR (B) biomass after 6 weeks of enrichment: acetate (●) and P-PO_4 (■). All values are represented as mean ± standard deviation of three replicates.

Figure 4. Typical carbon transformation during an anaerobic-aerobic cycle of the sequencing batch biofilm reactor: concentrations of acetate (▲), glycogen (■) and PHA (●). All values are represented as mean ± standard deviation of three replicates.

Figure 5. Relative abundance of OTUs at genus-level taxonomy. Only genuses comprising at least 1% of the total OTUs are listed; others are included in the "other" category, which also includes some OTUs that could not be classified at the genus level.

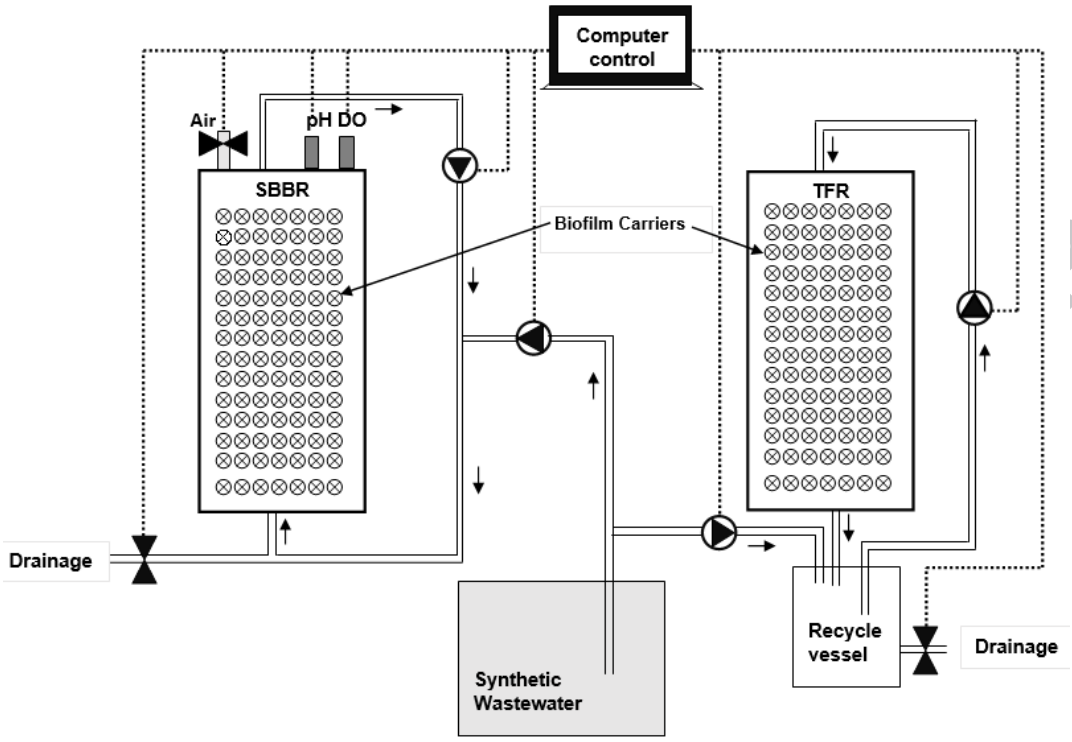
Table 1 - Stoichiometric parameters observed in this study in comparison with literature values, for processes that based on anaerobic acetate storage

	Anaerobic			Aerobic
	$P_{released}$ / Ac_{uptake}	$Gly_{degraded}$ / Ac_{uptake}	$PHA_{synthesized}$ / Ac_{uptake}	$Gly_{synthesized}$ / $PHA_{degraded}$
This study	0	1.58	2.14	0.75
Enriched GAO cultures				
Lopez-Vazquez et al. (2009a)	-	1.68	2.33	0.96
Oehmen et al. (2005)	0	1.17	1.85	-
Zeng et al. (2003)	0	1.12	1.86	0.65
Filipe et al. (2001)	0.020	0.92	1.53	0.80
Jeon et al. (2001)	0.015	1.21	2.04	-
Enriched PAO cultures				
Welles et al. (2015)	0.22	0.96	1.47	0.51
Acevedo et al. (2012)	0.73	0.35	1.36	0.39
Zhou et al. (2008b)	0.58	0.45	1.22	-
Smolders et al. (1994a)	0.50	0.50	1.33	0.42

All units expressed in Cmol/Cmol, apart from $P_{released}/Ac_{uptake}$, which is expressed in Pmol/Cmol

Table 2 - Comparison of the BOD removal rate of different systems

System	HRT (h)	BOD inflow (mg/L)	BOD removal rate (mg/L/h)	References
Trickle reactor	51.2	599.5	11.7	Doan et al. (2008)
Trickle reactor	50	250	5	Forster (2003)
Sequencing batch reactor	6.0	500	85.22	Zhao et al. (2016)
GAO biofilm	3.25	512	256	Present study



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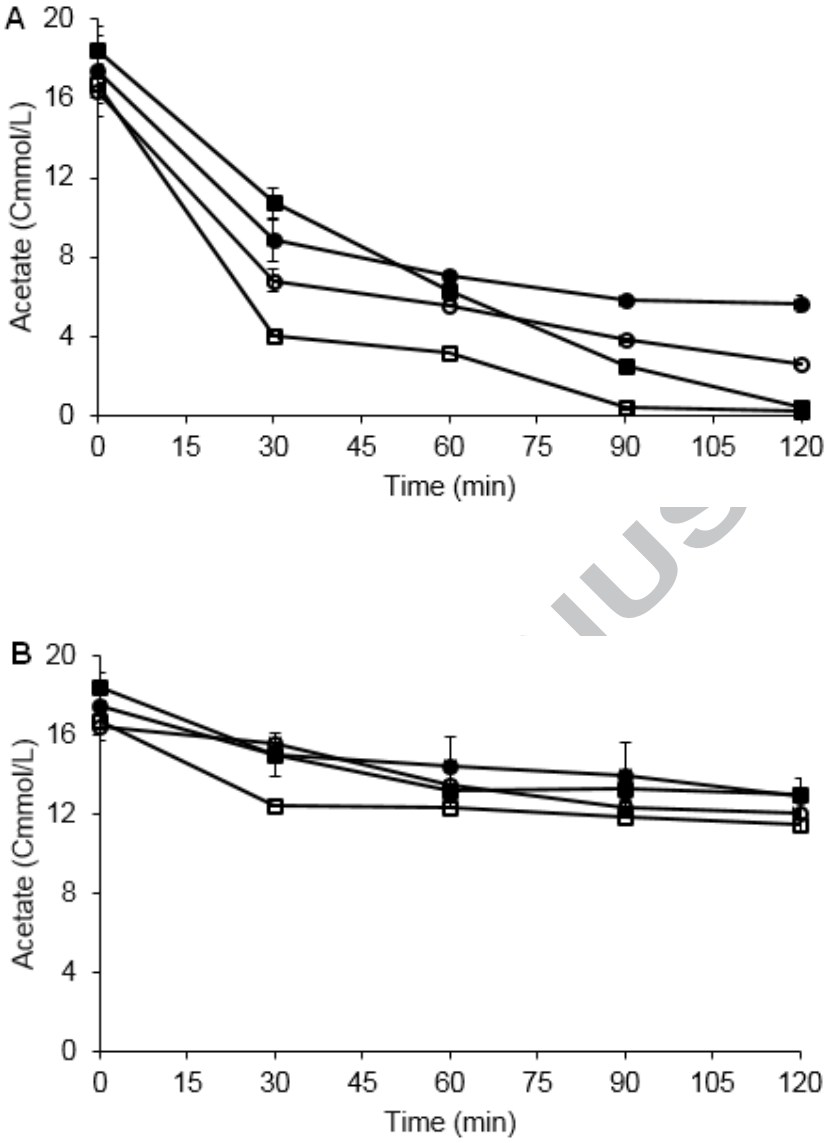

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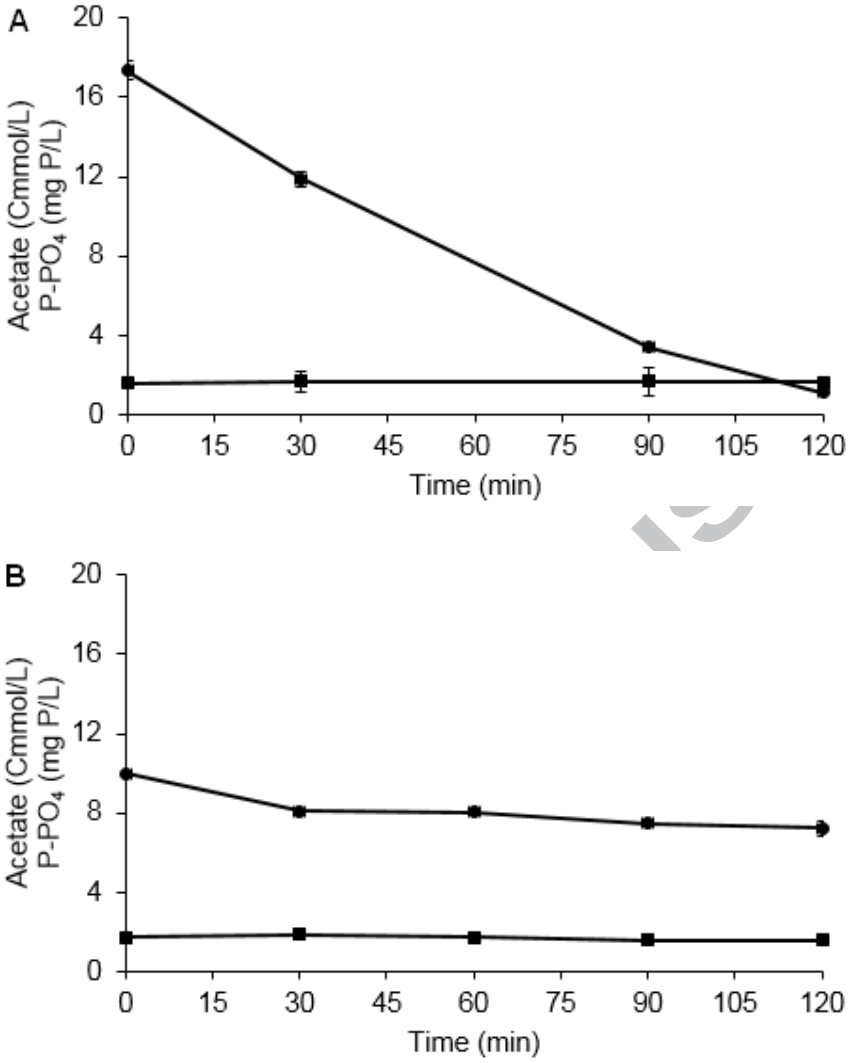

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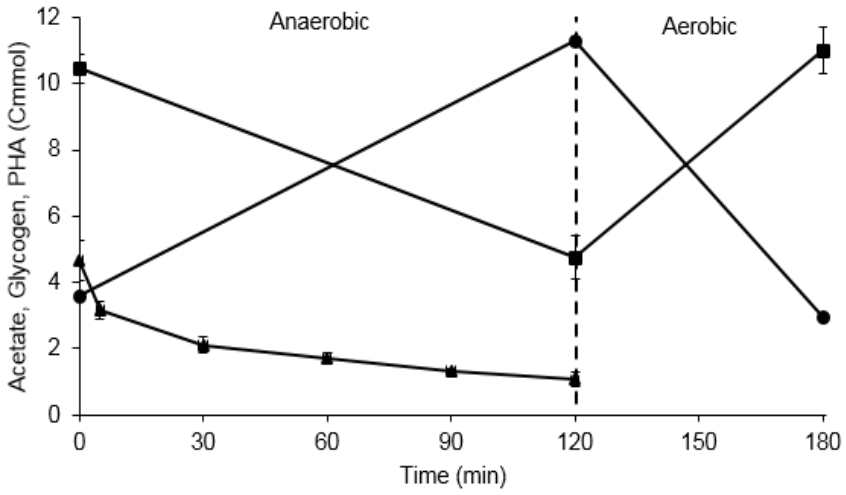

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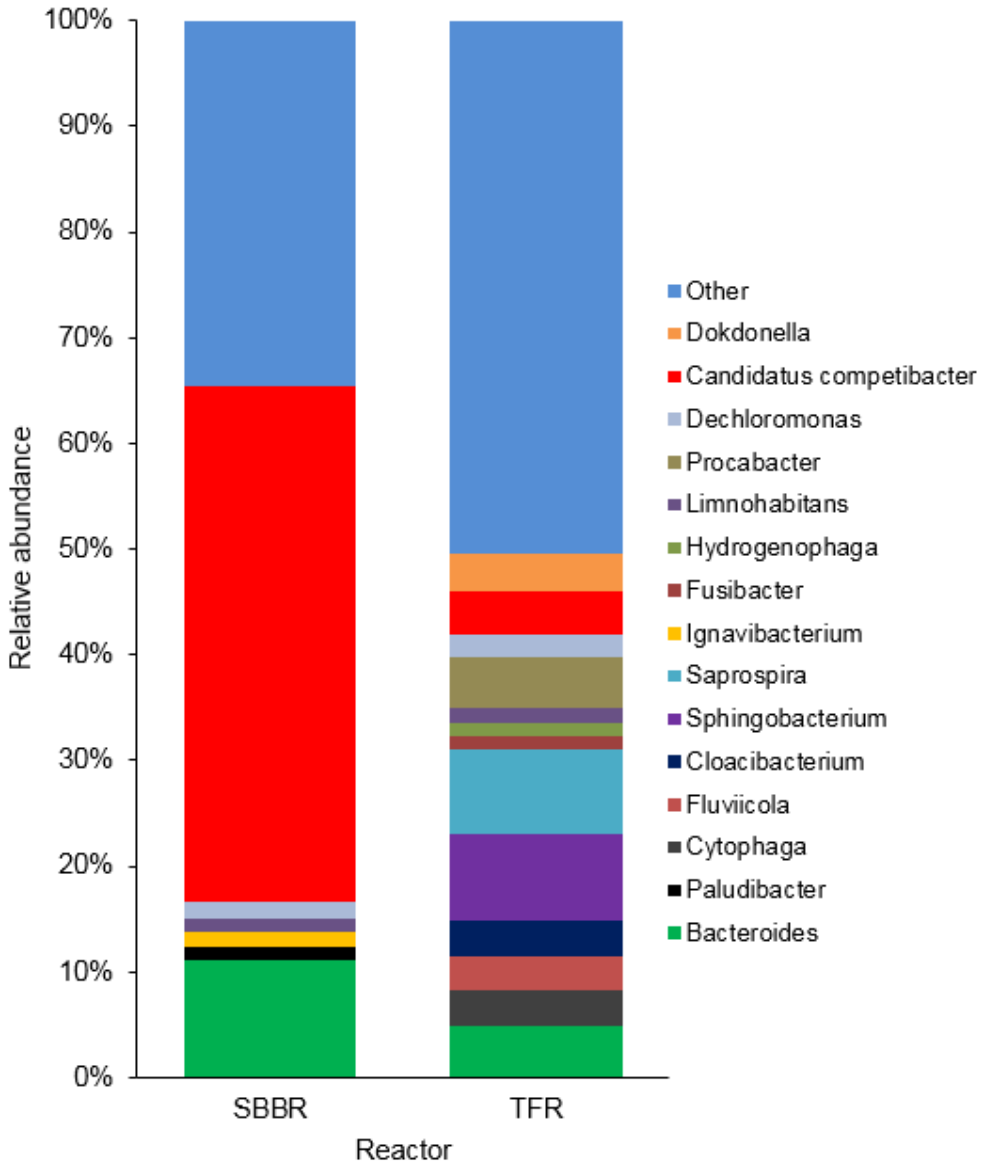

Figure 5. Relative abundance of OTUs at genus-level taxonomy. Only genuses comprising at least 1% of the total OTUs are listed; others are included in the "other" category, which also includes some OTUs that could not be classified at the genus level.

Highlights

- Activated sludge biomass was converted to GAO dominated biofilm within eight weeks
- Biofilm removed organic carbon anaerobically and stored as PHA
- Storage ability of biofilm was revived after exposure to atmospheric oxygen
- Biofilm operation required less energy as it avoids costly O_2 transfer into wastewater
- The reactor could be integrated with nitrogen removal (e.g., anammox) technologies