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Filamentous and non-filamentous bulking of activated sludge encountered under nutrients limitation or deficiency conditions

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Abstract: Although the limitation or deficiency of nutrients, such as nitrogen (N) and phosphorus (P), has been one of the frequently reported factors causing filamentous or non-filamentous bulking of activated sludge, the mechanisms are still unclear. In this work, the long-term effects of N and P limitation or deficiency on sludge settleability and bioflocculation characteristics were investigated in six sequencing batch reactors (SBRs) fed with wastewater with different nutrient availability. The sludge volume index (SVI), microbial community structures, intracellular poly-β-hydroxyalkanoates (PHAs) and extracellular polymeric substances (EPS) were characterised over time. Bulking was not observed in SBRs with N limitation or deficiency, in which SVI remained below 150 mL/g. In contrast, bulking was encountered in those reactors with P deficiency. The occurrence of non-filamentous bulking was associated with a higher carbohydrates fraction and a lower proteins fraction in EPS. In the case of filamentous bulking, SVI correlated negatively with the amount of PHAs. Our experimental data support the hypothesis that the occurrence and/or the type of bulking in activated sludge could be affected by the combination of kinetic selection, microbial storage, as well as the EPS composition.

Keywords: sludge settleability; filamentous bulking; non-filamentous bulking; substrate storage; extracellular polymeric substances (EPS); filamentous bacteria

1. Introduction

The performance of an activated sludge system for biological wastewater treatment is often deteriorated due to sludge separation problems caused by sludge bulking.

Bulking consists of filamentous bulking due to excess proliferation of filamentous bacteria [1] and non-filamentous bulking (also known as *Zoogloeal* bulking or viscous bulking) [2], resulting from certain microbes that produce large amounts of biopolymers on their surface [3].

The causes for inducing filamentous bulking are complicated [4] and include factors such as low dissolved oxygen (DO) concentrations [5-7], low organic loading rates [8], low substrate concentration gradients [9], low pH [10], and low temperatures [11]. Nutrient limitation [12] has also been identified as a factor for the proliferation of filamentous bacteria in activated sludge. In order to obtain well-settling sludge, the ratio of biological oxygen demand (BOD) to nitrogen (N) to phosphorus (P) in influent should generally satisfy 100:5:1 [1]. Peng et al. [13] showed that filamentous bulking was stimulated by the lack of either N or P in the feed. However, the simultaneous absence of both N and P did not induce filamentous bulking [13]. Low nutrient supplies have also been suggested to cause non-filamentous bulking. It was reported that activated sludge treatment of nutrient-deficient wastewater such as some types of industrial wastewaters led to severe slime formation and consequently biomass separation difficulties due to non-filamentous bulking [2, 14, 15]. Non-filamentous bulking at a full-scale wastewater treatment plant (WWTP), which was hypothetically due to low concentrations of soluble phosphate (0.2 mg/L), was solved by supplying additional soluble phosphate [16].

However, the mechanisms involved in both filamentous and non-filamentous bulking induced by nutrient limitation/deficiency are not fully understood at present.

There is still controversy about which bulking type would be caused under nutrient limitation. On one hand, it is hypothesised that nutrient deficiency has an effect on the competition between floc-forming and filamentous bacteria, causing filamentous bulking when filamentous bacteria proliferate due to their enhanced ability to uptake substrates under stress conditions [13, 17]. On the other hand, nutrient limitation has also been hypothesised to induce the production of extracellular polymeric substances (EPS) on the surface of microorganisms [18]. The EPS are important for the physicochemical properties of activated sludge flocs and have been implicated to affect sludge settling properties [19], inducing non-filamentous bulking. In addition, when sludge is subject to nutrients limitation or deficiency, more carbon substrate can be used for accumulation of poly-hydroxyalkanoates (PHA) and glycogen [20], which would affect the competition between filaments and floc-formers, as well as sludge settleability.

The objective of this study was to shed light on the mechanism of filamentous and non-filamentous bulking of activated sludge induced by nutrients limitation or deficiency, through a comprehensive experimental study. Six lab-scale sequencing batch reactors (SBR) were operated for 130-230 days with various nutrients-supplying conditions. The sludge volume index (SVI), PHA storage and EPS composition, Gram and Neisser staining, fluorescent *in situ* hybridization (FISH) and microscopic observations were used to monitor sludge properties and to track the changes of microbial morphology and community structure. These experimental data led to the connections between bulking type and the associated sludge properties, including

sludge settleability, microbial structure, intracellular storage and extracellular polymeric substrates under the stress condition of nutrients limitation or deficiency.

2. Materials and Methods

2.1 Lab-scale SBR reactors

The experiments were performed in six identical SBRs each with a 12-L working volume. Each reactor was equipped with an air compressor for aeration and a stirrer for mixing. Operation of the SBRs was based on 6 h cycles consisting of a feed phase (10 min) in which 6 L fresh medium was supplied giving rise to a hydraulic retention time of 12 h, an anoxic phase (110 min), an aerobic phase (180 min), a settling phase (50 min) and an effluent withdrawal phase (10 min) in which 5.85 L of reactor supernatant were withdrawn. The bulk liquid DO concentration in aerobic periods was controlled at 2.0±0.2 mg/L under aerobic periods. Temperatures in all reactors were controlled at 25±2 °C. pH was recorded but not controlled, and fluctuated between 7.0 and 7.5. The biomass concentrations in all reactors were kept in the range of 1800~2400 mg/L with sludge wasting that ensured an operation at a sludge age of 20 days of each reactor. The surfaces of tube, pumps and reactors were cleaned manually weekly in order to prevent biomass attachment.

The feed conditions for the SBRs are summarised in Table 1. SBRs 1-4 were operated for 233 days, to investigate the effects of nutrient deficiency on sludge settleability and microbial community structure. With COD/N/P ratios being 300:30:10 in the feed, SBR1 was operated as a control. SBR2 (COD/N/P set at

300:0:10), SBR3 (COD/N/P set at 300:30:0) and SBR4 (COD/N/P set at 300:0:0) were operated to investigate the effects of N, P and simultaneous N&P deficiency respectively. In order to investigate the combined effects of sludge cultivation history and influent nutrient ratios, which may affect the types of bulking and the dominant filaments, two additional reactors (SBRs 5-6) were operated for 130 days in three phases. Phase I (days 1-24) was used to collect base line data with normal feed (COD/N/P set at 300:30:15). The effects of N limitation (COD/N/P set at 300:5:15) and P limitation (COD/N/P set at 300:30:1) on sludge proprieties were investigated during Phase II (days 25-66) in SBR5 and SBR6, respectively. The effects of N deficiency and P deficiency on sludge proprieties were further investigated during Phase III (days 67-130).

2.2 Synthetic wastewater and seeding sludge

The medium for the SBRs consisted of a carbon source, a nutrient solution and a trace element solution. The normal synthetic wastewater contained CH_3COONa of 4.69 mM (300 mg COD/L), NH_4Cl of 2.14 mM (30 mg N/L), KH_2PO_4 of 0.32 mM (10 mg P/L in SBRs 1-4) or 0.48 mM (15 mg P/L in SBRs 5-6), $MgSO_4 \cdot 7H_2O$ of 0.37 mM, KCl of 0.48 mM, $CaCl_2 \cdot 2H_2O$ of 0.10 mM and 1 mL/L of the following trace element solution: EDTA 10 g/L, $ZnSO_4 \cdot 7H_2O$ 0.12 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.06 g/L, $MnCl_2 \cdot 4H_2O$ 0.12 g/L, KI 0.18 g/L, $CuSO_4.5H_2O$ 0.03 g/L, H_3BO_3 0.15 g/L, $FeCl_3 \cdot 6H_2O$ 1.5 g/L. Under the conditions of nutrients limitation and deficiency, N and/or P concentrations in the synthetic wastewater were modified according to the specific values describe in Table 1.

Each SBR was inoculated with 2 L seed sludge from the secondary clarifier of the GaoBeiDian WWTP (Beijing, China). The seed sludge had a good settling property (SVI< 100 mL/g), in which only limited filamentous bacteria (Type 0092 as the dominant filament) were present as a floc backbone.

2.3 Analytical methods

The temperature, pH and DO were monitored on line using WTW pH/DO meters (WTW Multi 340i, Germany). Supernatant samples in all reactors were collected 2-3 times every week to monitor effluent quality. Cycle studies were performed every 3 weeks for all SBRs. Samples were analyzed after filtration through 0.45 μm filter. COD, NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}-P, mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured according to Standard Methods [21]. When conducting cycle studies, PHA and acetate analysis was carried out as described by Oehmen et al. [22].

2.4 Microscopic observation and fluorescence in situ hybridization (FISH)

Microscopic observation of mixed liquor samples was performed every week using an OLYMPUS-BX61 (Japan). Filamentous index (FI), a method of subjective scoring of filamentous bacteria abundance suggested by Eikelboom [1], was used to evaluate the abundance of filamentous bacteria present in the samples. The dominant filamentous bacteria were identified based on morphology observation, Gram and Neisser staining, and a sulfur deposit test according to Eikelboom [1] and Jenkins [2]. FISH was also conducted for further identifying the dominant filamentous bacteria as previously described [23-25]. Table S1 (Supporting Information) shows a list of oligonucleotide

probes used in this study. The images of FISH samples were captured using an OLYMPUS-BX61 fluorescence microscope.

2.5 EPS extraction

Sludge samples were collected from all reactors every 1-2 weeks for characterisation of the EPS composition over time. The EPS extraction methods were previously described in Guo et al. [26]. Total extractable EPS was defined as the sum of proteins, carbohydrates and DNA. Carbohydrates were determined using the anthrone method with the glucose standard (Aldrich). Proteins were measured with the Lowry procedure using BSA (bovine serum albumin) as standard. DNA was measured in the extracted EPS samples according to the method described in Frolund et al. [27].

3. Results

3.1 Reactor performance

The COD removal efficiency of SBRs 1, 2 and 5 were consistently above 85%, with the average COD concentration in effluent lower than 50 mg/L. In comparison, COD removal efficiencies were consistently lower for SBRs 3, 4 and 6 (50~65%). The effluent COD concentration was frequently higher than 100 mg/L, particularly when bulking occurred. For the control reactor (SBR1), the average NH_4^+-N, NO_2^--N, NO_3^--N and PO_4^{3-}-P concentrations were 0.2, 0.8, 10.3 and 4.3 mg/L, respectively. SBRs 5 and 6 in Phase I (with normal feed) showed good nitrification, phosphorus release and uptake performance. However, the deterioration of the P removal was caused by N limitation or deficiency, and deterioration of the N removal was caused by P

limitation or deficiency in Phases II and III. Similar phenomenon was observed in other reactors (SBRs 2, 3 and 4) with limited nutrient influent. No nitrification and denitrification were observed in SBRs 2 and 4. No clear phenotype of enhanced biological phosphorus removal was found in SBRs 3 and 4. The phenomenon of phosphorous release and uptake was observed in SBRs 1, 2 and 5, despite with poor P removal efficiency. Phosphorous concentrations in effluent of SBRs 1 and 5 were lower than 5 mg P/L, while the average effluent phosphorous concentration in SBR 2 was higher than 9.1 mg P/L. The detailed effluent concentrations can be found in Table S2 (Supporting Information).

3.2 Sludge settling properties

Various influent nutrient ratios led to different sludge settleability, as evidenced by different SVI profiles (Fig. 1). SVIs in SBRs 1-4 slightly increased in the initial 30 days but gradually decreased to below 150 mL/g in the following 60 days, which might be associated with the adaptation of microorganisms to laboratory conditions including the synthetic feed. From day 90, sludge settleability expressed in SVI showed different trends. The settling property of the SBR1 sludge with a normal influent nutrient ratio was generally very good with a SVI in the range of 40–130 mL/g. Good sludge settleability with SVI of 40–125 mL/g was also obtained in SBR2, to which an influent without N was fed (C: N: P as to be 300/0/10). In SBR3 fed with influent without P, SVI was significantly higher than that in SBR1 and was in the range of 90–150 mL/g. However, the simultaneous N and P deficiency applied to SBR4 resulted in poor sludge settleability with SVI of 122-355 mL/g from day 90 to

day 230, which was higher than that in SBR3 during the corresponding period. The deficiency of P seemed to lead to poorer settleability while the deficiency of N did not.

SVI levels of the SBRs 5 and 6 sludges slightly increased to 150 mL/g in the initial 10 days and then became below 100 mL/g at the end of Phase I (Fig. 1b). In Phase II, good sludge settleability was maintained in both reactors, with the average SVI being 62 and 80 mL/g, respectively, despite of the imposition of N and P limitation, respectively. Subsequently, SBR5 and SBR6 were operated with wastewater without N or P, respectively (Phase III). Distinctly, the SVI of SBR6 (no P in the feed) rose rapidly after Day 100 (up to 500-600 mL/g) due to serious sludge bulking. In comparison, the settleability SBR5 (no N in the feed) remained stable.

3.3 Growth of filamentous bacteria

Microscopic observation was conducted to monitor the change of sludge morphology (as shown in Fig. 2) and the growth of filamentous bacteria. The growth of filamentous bacteria (FI of 2-3 in a scale of 0-5, as shown in Table 2) was observed in SBR3 with the deficiency of P. Many filamentous bacteria were present in the sludge (FI of 3-4 in a scale of 0-5) in SBR4 (Fig. 2d), in contrast to other reactors SBRs 1, 2 and 5, where much fewer filamentous bacteria were present (FI lower than level 2). Interestingly, the microorganisms in the SBR6 sludge were primarily floc-forming bacteria, and few filamentous bacteria (FI below 1) extended from the flocs, although high SVI was observed in this reactor (Fig. 2f). Thus, filamentous bacteria were not responsible for the increased SVI and non-filamentous bulking (viscous bulking) occurred in SBR6. In fact, microscopic observation showed a change from normal

compact flocs (average diameter ca. 100 μm) to much more open, loose and irregularly shaped flocs, and with many free-swimming bacteria. In comparison, the well-settling sludge (average SVI of 50 mL/g) in SBR5 contained dense and compact flocs (Fig. 2e), in spite of some limited filaments growing out of flocs.

Gram and Neisser staining and FISH analysis were employed for identification of the dominant filamentous bacteria in systems with filamentous bulking. Various influent nutrient ratios resulted in different dominant filamentous bacteria (Table 2, Fig. 3). In SBR1 with normal influent nutrient ratios, no distinct filaments extended from the flocs, despite that some Type 0092 and Type 0041 filamentous were found inside the flocs. Although no filamentous bulking occurred in SBR2 fed with influent without N, short and slightly bowed Type 0092 (filament length usually smaller than 200 μm) extended out from the flocs. Compared to SBR2 with lower filament diversity, more types of filaments, including Type 021N, Type 0092 and *M. parvicella* with very small numbers were detected in SBR5. The dominate filamentous bacteria in SBR3 have a morphology similar to that of the *Nostocoida limicola*-like filaments as described in [1, 2], but did not bind to the probes of NLIMI91 or NLIMIII301 [28]. Moreover, FISH analysis indicated that *T. nivea* were present in very small numbers in SBR3. There seemed to be two types of *N. limicola* possibly occurred in SBR3. One type produced relatively long filaments (> 200 μm). Cells were oval shaped with cell septa clearly observable. This type of filament was not only found with the floc structure but also in the bulk solution. The filament staining is Gram positive and Neisser negative (yellow). On the other hand, another type of *N. limicola*-like was

mostly observed in the bulk solution, and sheathed filaments composed of rectangular, also with distinct septa. Gram staining is positive. Specially, Neisser staining is positive (purple). In SBR4, the most dominant filaments, in an excessive abundance level (FI of 3-4), were *N. limicola*-like. A relatively smaller number of Type 0092 (FI of 1-2) was also found. However, the characteristics of *N. limicola*-like in SBR4 showed some differences from those in SBR3. They are Gram positive and Neisser positive (purple). As reported, *N. limicola*-like are often found in industrial wastewater with low nutrients [2]. With decreased the influent N concentration in SBR5, a limited number of Type 021N and Type 0092 extended out from the flocs.

3.4 PHA storage

Although it is widely reported that nutrient limitation is favourable for PHA synthesis in microbial cells (Third et al., 2003), the effect of storage phenomena on sludge settleability has not been thoroughly studied [29], in particular under the nutrients limitation or deficiency condition. In this study, sludge samples were collected every 3 weeks for the measurement of PHA, including poly-β-hydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV) and poly-β-hydroxy-2-methylvalerate (PH2MV). Table 2 compares the average PHA concentration in all reactors and Fig. 4 plots SVI against the stored intracellular PHA, measured in all reactors during the course of the study. PHA storage amounts in N limitation or deficiency systems (SBRs 2 and 5) were clearly higher than in all other reactors including those with P limitation or deficiency (SBRs 3 and 6). Our results are consistent with the previous observation that PHA accumulation is favoured under low ammonium concentrations [20]. Surprisingly,

however, the limitation or deficiency of P, which is also key element for cell growth, did not stimulate PHA accumulation. SBR4 with simultaneous N and P deficiency had the lowest PHA concentration. SBRs 3 and 6 (Phase III) fed with P-deficient influent, also had low PHA contents. The higher accumulation of PHA in SBR2 and SBR5 coincided with the excellent sludge settleability. In comparison, poorer settleability was obtained in SBRs 3, 4 and 6, where lower PHA contents were accumulated in sludge samples. Storage phenomenon in the form of PHA has previously been found to have an important role on sludge settleability [29, 30]. Compared to floc-formers, most of filaments are supposed to have no or lower ability to store substrates [30, 31]. In this case, most of substrates were used for storage under the feast phase and the growth of filaments would be restricted.

3.5 Formation of EPS

Sludge settleability of activated sludge is greatly related to its EPS properties [32, 33]. Therefore, the main components of EPS including carbohydrates, proteins and DNA were quantified in order to identify any correlation between SVI and the amount and composition of EPS (Figure 5 and Table S3). There is no significant correlation between the total amount of EPS and sludge settleability (Fig. 5a), which is consistent with the results reported by Liao et al. [34]. However, the carbohydrates fraction of EPS is positively correlated with SVI (R^2=0.5990), while the proteins fraction is negatively correlated with SVI (R^2=0.5073) (Fig. 5b and 5c, respectively). For SBRs 2 and 5, both with N limitation/deficiency and good sludge settleability, the composition of EPS is very similar, despite. In both reactors, 17-19% of the extracellular substances is attributed to carbohydrates and 75-80% to proteins. Increases in the carbohydrates contents were observed in all SBRs (3, 4 and 6) with P

deficiency. For SBRs 3 and 4 with filamentous bulking sludge, low proteins (62-76%) and high carbohydrates content (20-30%) were observed in EPS. In contrast, a higher carbohydrates content (27±10%) and a lower proteins content (69±9%), compared to other reactors, were observed in Phase III in SBR6, where viscous bulking occurred. When serious non-filamentous bulking was encountered at the end of Phase III, the carbohydrates fraction reached 42% and the proteins fraction dropped to a level of 55%.

When SVI was plotted against the carbohydrates or proteins fractions for each reactor (Fig. S1, SI), no significant correlation between SVI and carbohydrates or proteins fractions is observed for SBRs 1, 2 and 5 with sludge of good settleability. However, for reactors with bulking (SBRs 3, 4 and 6), the carbohydrates fraction of EPS has a more positively correlation with SVI (R^2=0.7850, 0.7821 and 0.9486 for SBR3, 4 and 6, respectively), while the proteins fraction is negatively correlated with SVI (R^2=0.7418, 0.7446 and 0.9277 for SBR3, 4 and 6, respectively).. The correlations are much stronger for SBR6 with viscous bulking than for other reactors. This suggests that the observed non-filamentous bulking was possibly caused by the overproduction of extracellular carbohydrates in the biomass. A similar phenomenon was found by Jobbagy et al. [15] in which an exponential correlation between the extracellular carbohydrates contents and SVI values was observed.

4. Discussion

Many hypotheses explaining filamentous bulking such as kinetics selection, diffusion selection and storage selection have been put forward to date [17, 29, 35]. Kinetic selection theory, formulated by Chudoba et al. [36], assumed that floc-formers and filaments have different kinetic parameters K_S (half-saturation constant) and μ_{max}

(maximum growth rate) for the substrate. The floc-formers usually dominate over filaments at high substrate concentrations, since they have high μ_{max} and K_S for soluble substrates. In contrast, the filamentous bacteria would be more favoured at low substrate concentrations, since filaments are thought to have lower *K*s values. However, Martins et al. [37] proposed that substrate diffusion limitation inside the flocs might be a critical cause for filamentous bulking than kinetics selection. They assumed that floc-formers grow in three dimensions and form the floc matrix, while filaments grow in only one or two dimensions [38]. Thus, the floc-formers would be more affected by diffusion resistance of substrates at low substrate concentrations. The storage selection theory is based on the assumption that floc-formers have higher substrate uptake rates and capacities to store substrates, while most of the filamentous microorganisms are supposed to have no or lower ability to store substrates [29]. Therefore, floc-formers are favoured at high substrate concentrations, compared to filamentous bacteria.

Two types of bulking, i.e. filamentous bulking and non-filamentous bulking were encountered in this study. In SBRs 2 and 5 with N limitation or deficiency, filamentous bulking did not occur. In comparison, filamentous bulking occurred in SBR3 and SBR4 with P deficiency. The correlation between SVI and PHA supports the storage selection hypothesis. In SBRs 2 and 5, part of the substrates were stored as intracellular PHA in the feast phase. In the famine phase, growth would have occurred based on the storage product. Such a growth mechanism is known to favour floc-formers over filaments as most of filamentous bacteria are known to have a lower

ability to produce carbon storage products [30, 31], while several types of filaments (like *Microthrix parvicella* and *Thiothrix nivea*) have a similar capacity to store substrates [37, 39-41]. In this case, well-settling sludge was obtained due to the limited proliferation of filaments. Compared to SBRs 1, 2 and 5, PHA formation was not stimulated but attenuated in SBRs 3 and 4. Without PHA formation giving advantage to floc-formers, filamentous bacteria probably gained advantage through their competitiveness for nutrients at low concentrations due to their kinetics advantages. Consequently, filamentous bulking was caused in these reactors (i.e. SBRs 3 and 4).

In comparison to SBRs 2 and 5 with N limitation/deficiency, SBRs 3, 4 and 6 produced more carbohydrates (and less proteins) in EPS as compounds without N or P. It is unclear, why PHA formation was stimulated by N limitation/deficiency while carbohydrates formation was stimulated by P limitation/deficiency. The strong positive correlation between SVI and the carbohydrates fraction in EPS suggests the excessive production of carbohydrates in EPS is detrimental to sludge settleability. This supports the observation in [18] that a higher carbohydrates fraction and a lower proteins fraction in EPS would deteriorate sludge settleability. Different from SBR3 and SBR4, non-filamentous bulking rather than filamentous bulking occurred in SBR6. With P limitation/deficiency, all these reactors had similar levels of PHA, carbohydrates and proteins. It is not clear why filamentous bacteria did not develop in SBR6. This could be related to the relatively short operational time of SBR6 after bulking occurred (Day 110 – Day 130). Indeed, the SBR6 sludge, prior to bulking,

contained filaments at very low levels (FI was lower than 1). Given the high SVI observed in SBR6 in the absence of filaments, a possible contribution of viscous bulking to the high SVIs in SBR3 and SBR4 should not be ruled out.

The dominant filamentous bacteria under nutrient deficiency were seldom documented in the previous studies [17]. Through microscopic observation, staining reactions and FISH analysis, the dominant filaments grew in reactors with filamentous bulking were identified in this study. In SBRs 3 and 4, the proliferation of two typical bacteria, *N. limicola*-like and *T. nivea* were identified. These filaments' affinities for nutrient are critical parameters in the competition for the limited nutrients. It is assumed that *N. limicola*-like and *T. nivea* have high affinities for nutrients, including N and P. *N. limicola*-like are usually found in systems where there is low DO or septicity, and often found in industrial WWTPs with low nutrients [2]. Similarly, *T. nivea* are also often detected in industrial WWTPs [33]. The filaments have a high affinity for substrate (e.g. acetate) and nutrients, but a relatively low substrate uptake rate [42].

N and P are the basic elements for the growth of microorganisms. The activated sludge still kept a stable growth phenomenon in the systems fed without N (SBRs 2 and 5 in Phase III). The MLSS concentrations in these reactors were kept around 2000 mg/L, although the growth rate of activated sludge in SBR5 became slower during Phase III, compared to that during Phases I and II. It is assumed that some unknown fixation microorganisms might be capable of supplying the entire N requirements of the system. It is reported that genomic analysis of some polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) indicates an ability

to fix nitrogen [43, 44], which might be relevant in these nitrogen-limiting systems, given the FISH analysis suggested the presence of PAOs and GAOs (data not shown). Although P was not supplied in SBR6 during Phase III, microorganisms in the reactors might still gain them for their growth, which might be from the sludge decay products. In addition, it is assumed that P was taken up in excess and stored to be re-utilized during P limitation or deficiency. It is still not clear what is the mechanism of the microorganism growth in SBRs 3 and 4 without any phosphorus. It is necessary to detect N and P levels in the internal of sludge to clarify why the cells still keep growing under no nutrients feeding.

Moreover, from the PHA and EPS data it can be inferred, N limitation or deficiency increased the intracellular PHA and extracellular proteins levels and was associated with good settling in SBRs 2 and 5. However, P limitation or deficiency distinctly did not stimulate the PHA storage, while increased the carbohydrates content of EPS in SBRs 3, 4 and 6, which was associated with poor settleability. Non-filamentous bulking occurred when the carbohydrates fraction was distinctly high in the EPS matrix. It is assumed that the intracellular PHA and the extracellular EPS matrix would change if encountering nutrient unavailability. The occurrence and/or the type of bulking in activated sludge could be affected by the combination of kinetic selection, microbial storage, as well as the EPS composition.

This study shows that P limitation seems to stimulate the production of extracellular carbohydrates rather than intracellular carbohydrates (PHA), which deteriorates sludge settleability through filamentous or non-filamentous bulking. P limitation should be avoided in activated sludge systems, especially when treating wastewater with low levels of P. Considering that the requirement of P for the growth of microorganisms is relatively lower compared to N, adding P in influent would not

distinctly increase operation cost, which can be used in practice. But the dosage warrants further batch tests.

Conclusions

This study investigated the long-term effects of nutrients (N and P) limitation or deficiency on sludge settleability, EPS, substrate storage and microbial community structure. N limitation or deficiency does not necessarily lead to sludge bulking, likely attributed to its stimulating effect on the formation of intracellular storage products, which gives competitive advantages to floc-formers over filamentous bacteria. In comparison, P deficiency caused sludge bulking. Both filamentous bulking and non-filamentous bulking could be induced by P limitation/deficiency, with reasons to be further identified. Filamentous bulking was strongly correlated with the excessive growth of *N. limicola*-like and *T. nivea*, while non-filamentous bulking is accompanied by a higher carbohydrates fraction and a lower than proteins fraction of EPS.

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Figure Captions

Table 1. Summary of feed conditions in six reactors with different influent nutrient ratios

Table 2. The properties of sludge under different nutrient-supplying conditions

Figure 1. SVI profiles in six SBRs (a: SVIs of the sludge in SBRs 1-4; b: SVIs of the sludge in SBRs 5-6)

Figure 2. Microscopic observations of activated sludge in all SBRs at the end of the study

Figure 3. Typical FISH, Gram and Neisser staining images of dominant filaments from various reactors (Bar=20 μm.)

Figure 4. SVI vs. PHA in all reactors. Higher amounts of PHA accumulated in SBRs 1, 2 and 5 with good settleability, as shown in the right oval; Lower amounts of PHA accumulated in SBRs 3 and 4 with filamentous bulking, as shown in the left oval; SBR6 with non-filamentous bulking has a moderate amount of PHA, as shown in the middle oval.

Figure 5. Correlation between SVI and EPS, extracellular carbohydrates and proteins contents of total EPS in the biomass

Table 1. Summary of feed conditions in six reactors with different influent nutrient ratios

Reactor	Phase	Time (days)	C/N/P (mgCOD:mgN:mgP)	Remarks
1	—	1-233	300/30/10	Control
2	—	1-233	300/0/10	N deficiency
3	—	1-233	300/30/0	P deficiency
4	—	1-233	300/0/0	N&P deficiency
5	I	1-24	300/30/15	Control
	II	25-66	300/5/15	N limitation
	III	67-130	300/0/15	N deficiency
6	I	1-24	300/30/15	Control
	II	25-66	300/30/1	P limitation
	III	67-130	300/30/0	P deficiency

Table 2. The properties of sludge under different nutrient-supplying conditions

Reactor	Phase	C/N/P ratio	SVI* (mL/g)	PHA* (mmol C/L)	FI (0-5)	Detected filaments
SBR1	—	300/30/15	108±67	7.2±0.7	0-1	Type 0092, Type 0041
SBR2	—	300/0/10	108±63	13.5±0.1	0-1	Type 0092, Type 0041,
SBR3	—	300/30/0	153±65	4.6±0.5	2-3	*N. limicola*-like, *T. nivea*
SBR4	—	300/0/0	186±60	2.5±0.5	3-4	*N. limicola*-like, *T. nivea*, Type 0092
SBR5	I	300/30/15	111±15	7.0±0.9	0	No filaments extended from flocs
	II	300/5/15	62±15	14.6±1.3	0-1	Type 021N, Type 0092
	III	300/0/15	49±17	8.9±0.6	1-2	Type 021N, Type 0092
SBR6	I	300/30/15	111±17	6.8±0.5	0	No filaments extended from flocs
	II	300/30/1	80±9	4.6±0.4	0	No filaments extended from flocs
	III	300/30/0	150±150	5.6±0.4	0	No filaments extended from flocs

*: values showed as mean ± standard deviation (n=58 for SVI calculation; n=8 for PHA calculation)

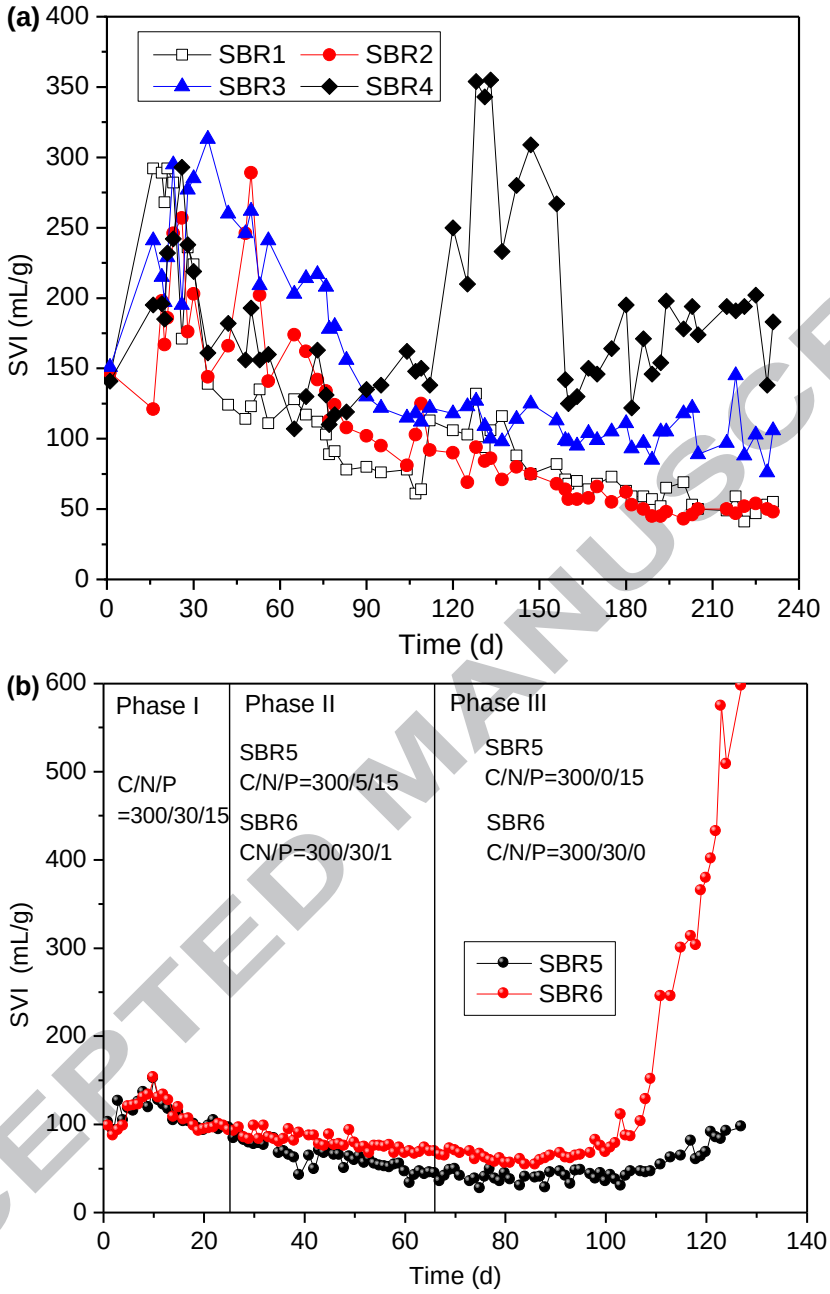
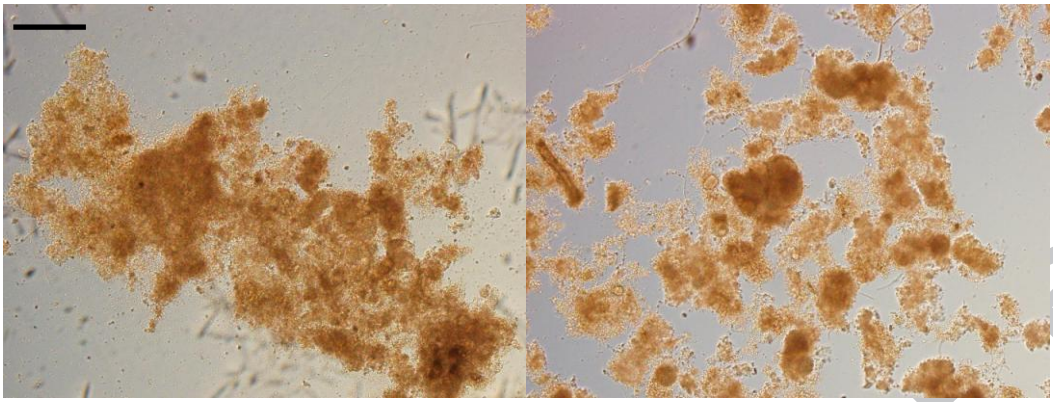
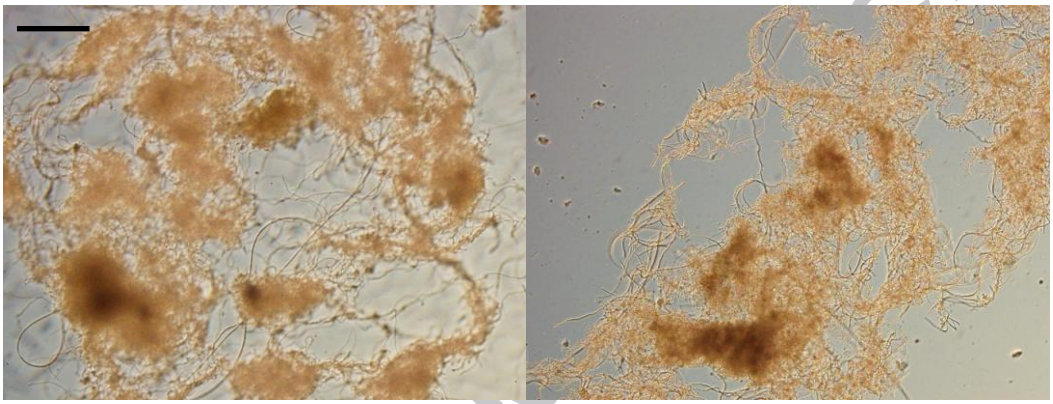


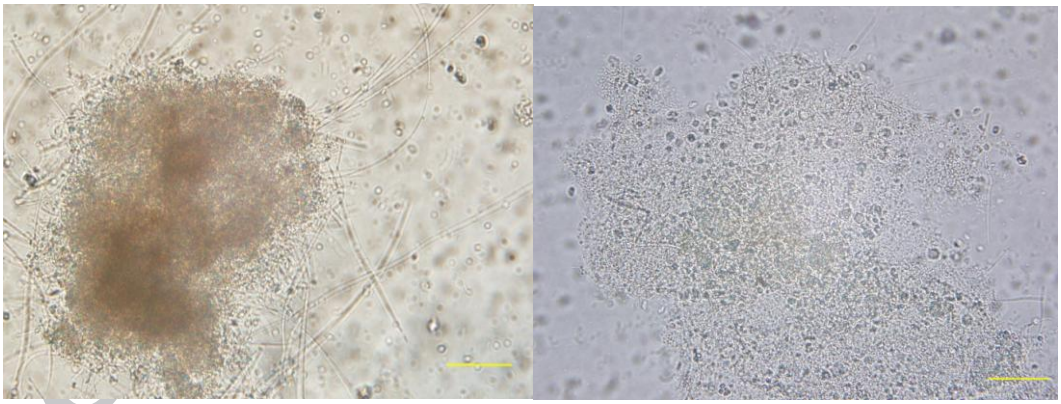
Figure 1. SVI profiles in six SBRs (a: SVIs of the sludge in SBRs 1-4; b: SVIs of the sludge in SBRs 5-6)



(a) SBR1 (100×, bar =200 μm) (b) SBR2

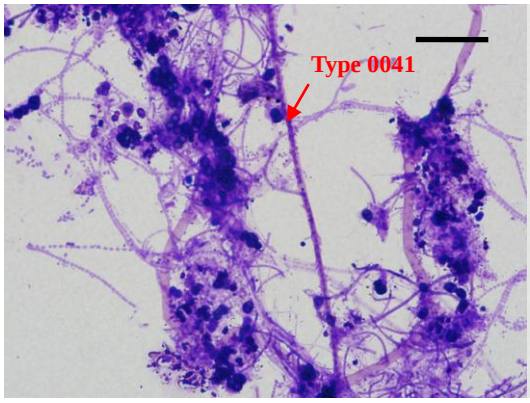


(c) SBR3 (100×, bar =200 μm) (d) SBR4

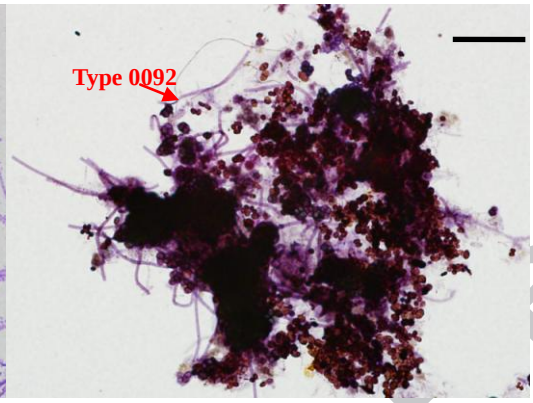


(e) SBR 5 (400×, bar =200 μm) (f) SBR 6

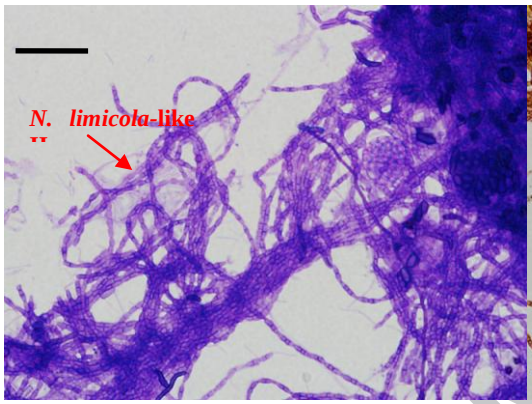
Figure 2. Microscopic observations of activated sludge in all SBRs at the end of the study



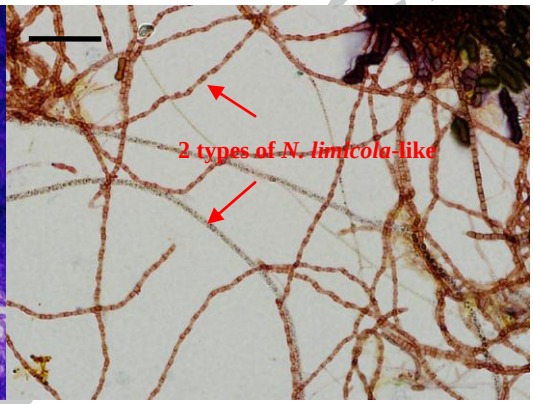
(a) SBR2, Gram staining image



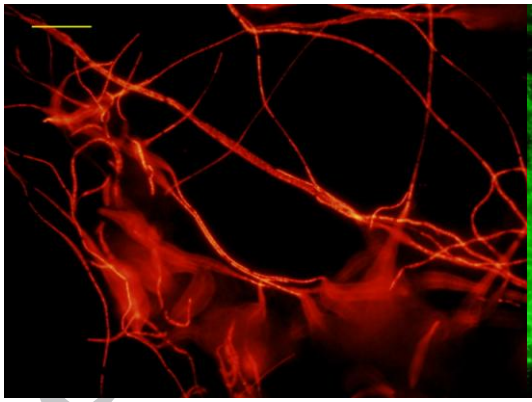
(b) SBR2, Neisser staining image



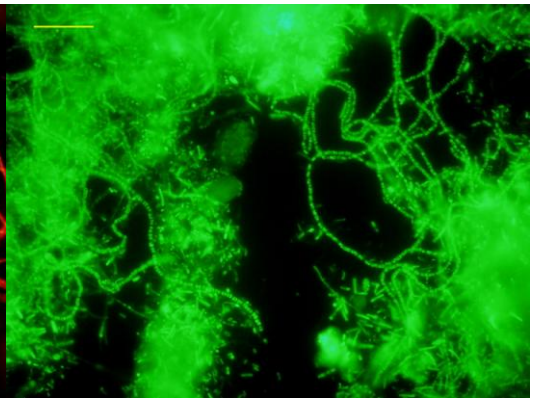
(c) SBR3, Gram staining image



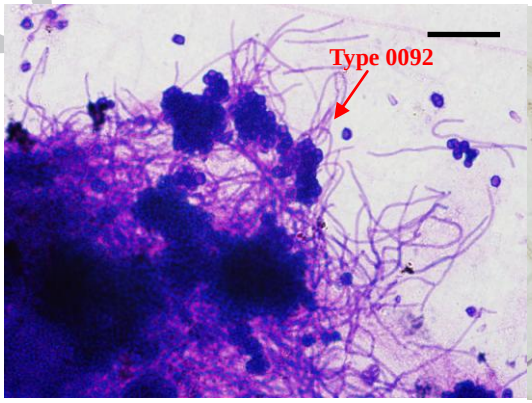
(d) SBR3, Neisser staining image



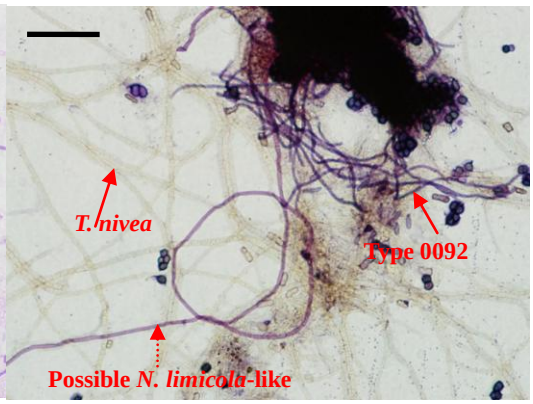
(e) SBR3, FISH image of *T. nivea*



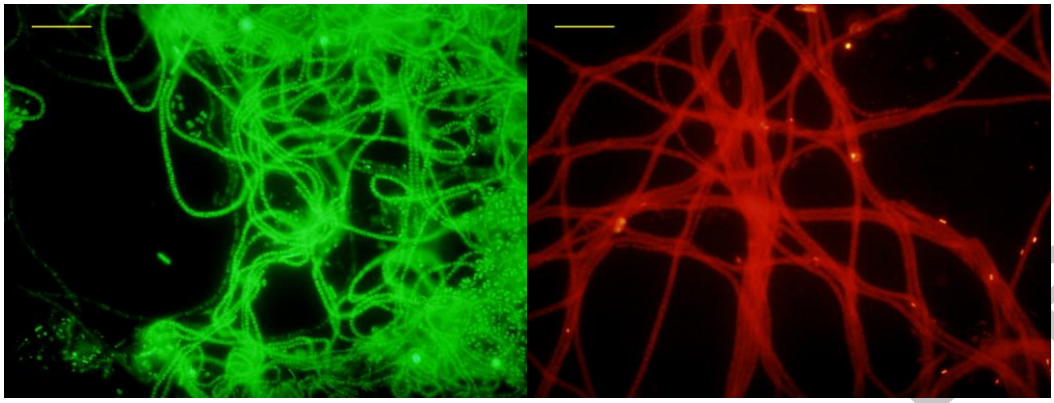
(f) SBR3, FISH image of Eubmix probe



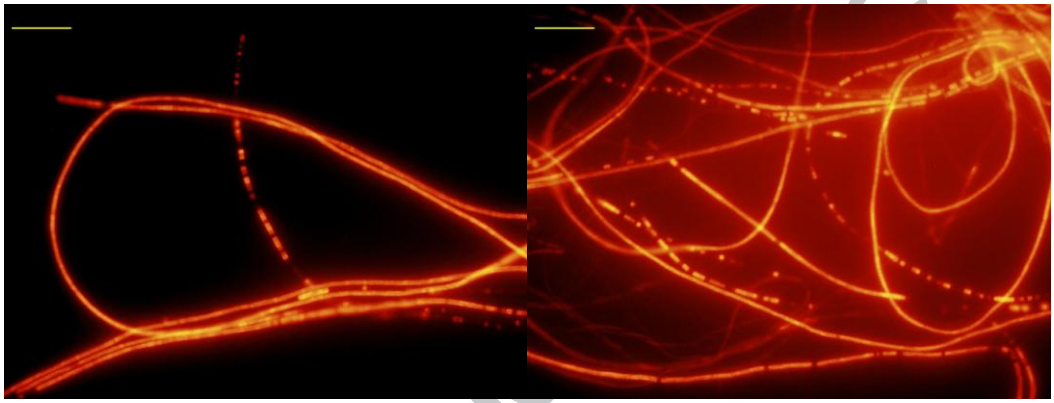
(g) SBR4, Gram staining image



(h) SBR4, Neisser staining image

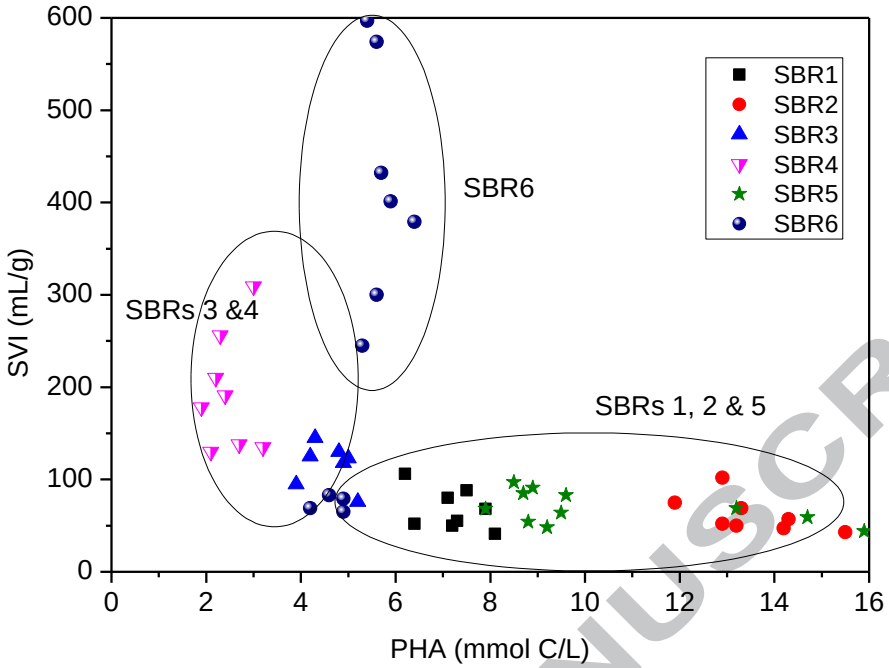


(i) SBR4, FISH image of Eubmix probe (j) SBR4, FISH image of *T. nivea*



(k) SBR5, FISH image of Type 021N in Phase II (m) SBR5, FISH image of Type 021N in Phase III

Figure 3. Typical FISH, Gram and Neisser staining images of dominant filaments from various reactors (Bar=20 μm.)



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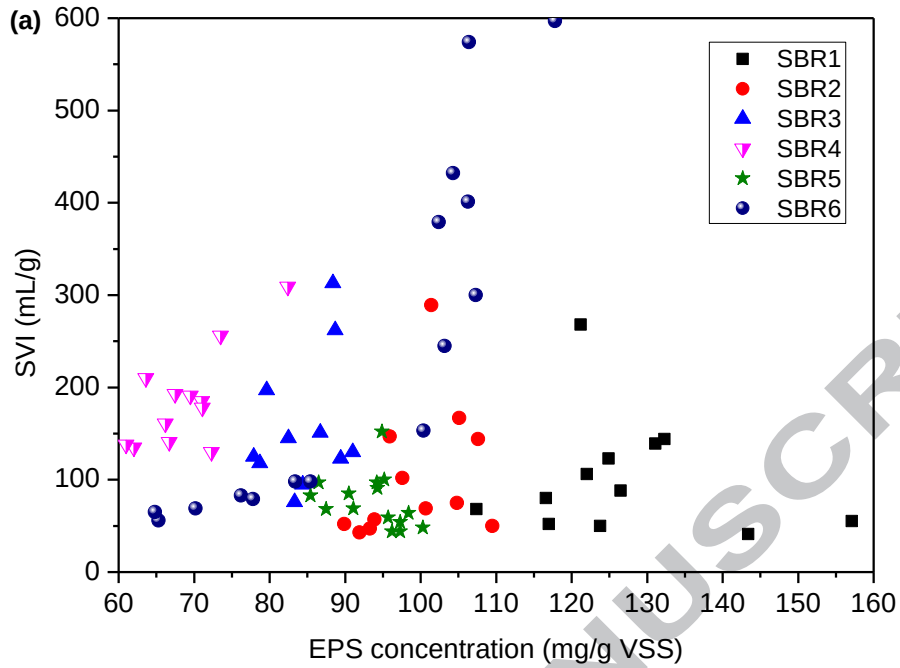

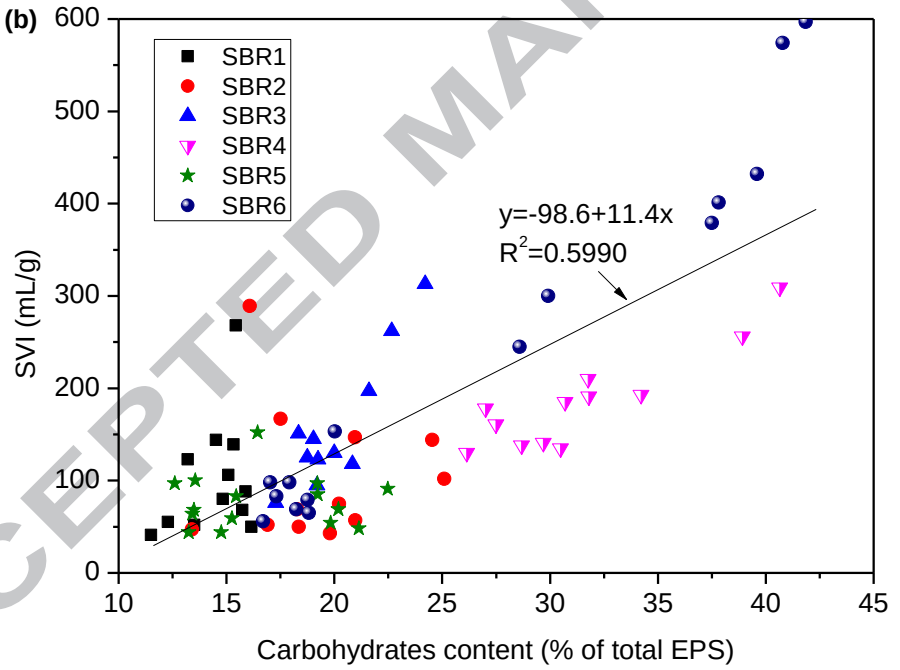

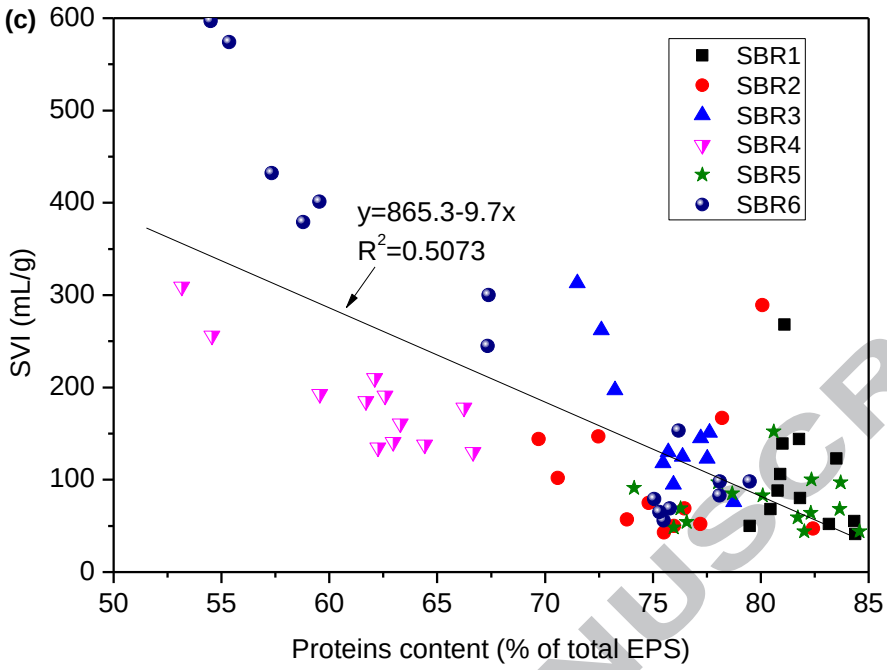

Figure 5. Correlation between SVI and EPS, extracellular carbohydrates and proteins contents of total EPS in the biomass

Research Highlights

- Effects of N/P deficiency on SVI, EPS, PHA and microbial community structure were examined.
- Bulking was not encountered in reactors with nitrogen limitation or deficiency.
- Bulking was encountered in those reactors fed with wastewater deficient in phosphorus.
- N limitation/deficiency stimulates formation of intracellular storage products (PHA).
- P limitation stimulates formation of carbohydrates.

Graphical abstract