Linking floc structure and settling properties to activated sludge population dynamics in an SBR

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Abstract Over a period of 227 days properties of activated sludge grown in an aequencing batch reactor (SBR) operated under stable conditions were analyzed. Settling properties (sludge volume index (SVI)) of the activated sludge were compared with on-line measurements of floc size and size distribution obtained by using a laser light scattering technique (Malvern Mastersizer/S, Malvern, UK), and with measurements of microbial community dynamics analyzed by denaturing gradient gel electrophoresis (DGGE) patterns of 16S rRNA genes. In addition, microscopical observations were used to confirm the results. Three distinct stages in the SBR evolution were observed. In the first stage the structural floc properties showed predominant presence of floc-forming bacteria in the activated sludge. A good correlation between floc size, settling properties and microbial community evolution was observed. The second stage showed a good balance between floc-forming and filamentous bacteria, with good settling properties and a highly dynamic community in the SBR. In the third stage, an increase in the filamentous bacteria, which became predominant in the system was observed. Again, a good correlation between settling properties and floc size distribution was obtained and a new dominant species was observed in the DGGE patterns, which can be assumed to be a filamentous organism.

Keywords DGGE; floc size distribution; population dynamics; sequencing batch reactor; sludge volume index

Introduction

The effectiveness of the activated sludge process is subject to a good solid–liquid separation, which is strongly determined by the activated sludge settling properties. In turn, the settling properties depend mainly on the flocs' structural properties and on the activated sludge microbial population.

Monitoring of the activated sludge settling properties as well as the structural properties and the dynamics of microbial populations can provide insights into the relation between the microbial community changes and process performance. Several attempts have already been made to evaluate the settling and floc structural properties of activated sludge (Andreadakis, 1993; Wilén and Balmer, 1999; Dagot *et al*., 2000; Seka *et al*., 2001) or to monitor the population dynamics under stable environmental conditions (Fernandez *et al*., 1999; Kaewpipat and Grady, 2002). However, due to the high sensitivity of the microorganisms to environmental conditions, many characteristics of the biological treatment system remain poorly understood and therefore, difficult to control.

In this study, the changes in activated sludge settling properties (SVI), structural properties (FSD) and bacterial community composition (DGGE) were monitored in an SBR during a period of 227 days. The aim of this approach was to investigate possible links between these properties.

Evaluating the structural properties, floc size and size distribution measurements open

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opportunities for detecting changes in the floc properties during various treatment steps and provide valuable information about how well the separation processes work. Various methods can be found in the literature to measure the size of the activated sludge flocs, such as direct microscopic observation (Barbusinski and Koscielniak, 1995; Li and Ganczarczyk, 1987), image analysis (Li and Ganczarczyk, 1991; Grijspeerdt and Verstraete, 1997) and Coulter counter (Andreadakis, 1993). In this study, the laser light scattering technique was used to obtain floc size measurements. This technique allows on-line determination of the changes in floc structure such as fractal dimension (Guan *et al.*, 1998) or direct size distribution (Biggs and Lant, 2000).

A DGGE analysis of 16S rRNA genes was used to examine temporal differences within the activated sludge bacterial community. The procedure is based on the electrophoresis of polymerase chain reaction (PCR)-amplified 16S rDNA fragments, obtained from total sludge DNA, in polyacrylamide gels with a linearly increasing gradient of denaturants parallel to the direction of electrophoresis (Muyzer *et al.*, 1993). It generates a community fingerprint pattern, in which a band generally corresponds to one bacterial ribotype, i.e., bacteria with the same 16S ribosomal RNA fragment, often belonging to one species. This fingerprint pattern allows one to detect changes in the presence and abundance of the numerically dominant bacterial populations (ribotypes) in the community.

Materials and methods

SBR

An 80 L pilot-scale SBR for nitrogen and phosphorus removal was inoculated with 40 L activated sludge from the Ossemeersen WWTP (Ghent, Belgium). A schematic drawing of the SBR unit is shown in Figure 1. All devices controlling the SBR set-up and performing the on-line measurements of pH and DO are connected to a PC-based data acquisition (DAQ).

The SBR runs with 4 cycles per day. Each 6-hour cycle contains 6 phases. The purpose of these phases and their duration have been reported elsewhere (Demuynck *et al.*, 1993). From each cycle, 2 L of mixed liquor are wasted, in order to have a sludge age of 10 days. In the last phase, 38 L of clarified effluent is wasted from the SBR.

Every day during one cycle of the SBR sludge and effluent samples are collected from the reactor and stored at 4°C before being analysed. Effluent analysis consisted in daily monitoring of COD, COD_{sol}, N_{tot}, NO_3-N, PO_4-P (Dr. Lange, Germany) and BOD (OxiTOP, WTW, Germany). Activated sludge characteristics are studied by daily measurements of the SVI, mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MVLSS) according to *Standard Methods* (APHA, 1992).

A synthetic sewage is used as SBR influent, which aims at mimicking real pre-settled

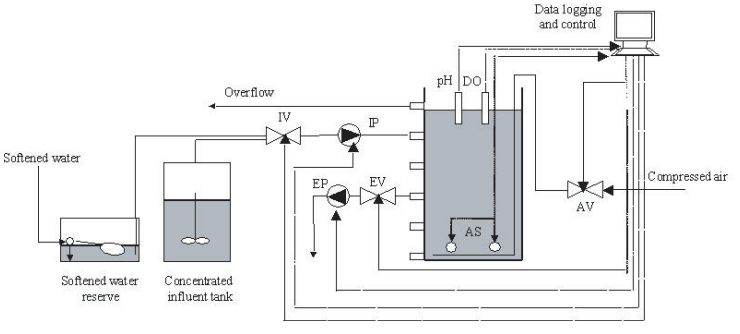


Figure 1 Schematic overview of the SBR pilot-plant (IV-influent valve; IP-influent pump; EP-effluent pump; EV-effluent valve; AS-aeration system; pH-pH probe; DO-DO probe; AV-air valve)

domestic wastewater (Boeije *et al*., 1998). The calculated COD:N:P ratio of the synthetic sewage is 100:13.7:2.14. The concentrated feed and the dilution water are dosed separately. This procedure enables a rather small volume (75 L) of synthetic feed stock to be used during one week and minimizes the influent preparation effort. The concentrated influent is stored at pH = 3 to maintain the influent characteristics as constant as possible.

Floc size measurement

Floc sizing was done on-line using a Malvern Mastersizer/S (Malvern, UK) that is based on static laser light scattering (Fraunhofer diffraction theory). The Mastersizer software (version 2.15, Malvern) generates a volume-weighted floc size distribution (FSD). In order to describe the mean particle size, the volume-weighted average diameter, which is also known as the mass mean diameter, D[4,3] was used. This parameter is calculated as:

$$D[4,3] = \frac{\sum_{i=1}^{n} \Delta F_N(x)_i \, x_i^4}{\sum_{i=1}^{n} \Delta F_N(x)_i \, x_i^3} \tag{1}$$

where: x_i is the diameter of size class i and $\Delta F_N(x)_i = N_i/\Sigma N_i$ is the number fraction in size class i.

A 300RF lens was used for all experiments corresponding to a size range of 0.05–900 μm. Dilution of the activated sludge sample prior to analysis was required, since too concentrated samples lead to multiple scattering, which is not accounted for in the determination of the final result of the FSD. Fresh activated sludge samples collected directly from the SBR were analysed weekly. In order to obtain identical initial conditions 1 L undiluted sludge was mixed 30 min by using a SW6 flocculator (Stuart Scientific, USA) at 35 rpm. For analysis, a small volume of sludge (20–30 mL) was diluted into 1 L of filtered effluent (0.45 μm). The sludge concentration was controlled by fixing the obscuration level at 15% in the Mastersizer software. Obscuration helps to set the concentration of the sample when it is added to the dispersant. An ideal range is between 10 and 30%. Experiments have been conducted by using the MSX17 automated wet sample dispersion unit (Malvern, UK). Preliminary experiments demonstrated that a flow rate of 3 mL/s and a mixing speed of 210 rpm were the optimal conditions for maintaining the flocs in suspension without creating floc break-up. For deflocculation experiments the ultrasonic bath of an MSX17 automated wet sample dispersion unit was used at a power of 50 W.

Microscopy

The microscopical observations were performed by using an optical microscope, Olympus CX40 (Olympus, Japan) equipped with a Ikegami ICD-46E video camera (Ikegami Electronics Inc., USA). A drop of mixed liquor was carefully deposited on a glass slide and covered with a cover slip before being observed through the microscope.

DNA extraction, PCR and DGGE

Total DNA was extracted from the sludge sample based on the extraction protocol presented by Boon *et al*. (2000). A 100 μL aliquot of the crude extract was further purified using Wizard PCR preps (Promega, Medison,Wis.). The clean DNA was stored at –20°C. 1 μL of the extracted DNA was amplified by PCR with the bacteria specific 16S rRNA forward primer 338f (ACT CCT ACG GGA GOL AGC A) and the reverse primer 518r (ATT ACC GCGGCT GCT GG) based on a universally conserved region, as previously described

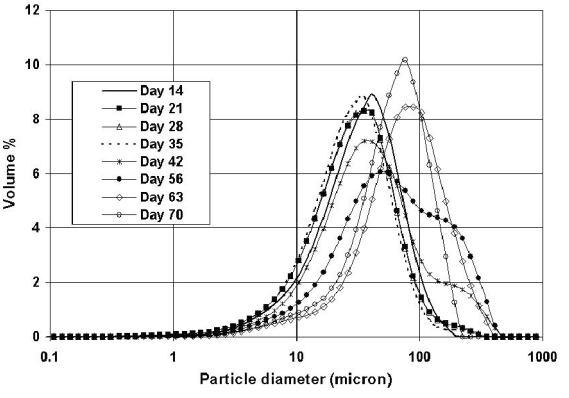
(Muyzer *et al.*, 1993). The PCR product contains a GC-clamp of 40 bases, added to the forward primer.

PCR products were subjected to DGGE as described previously (Boon *et al.*, 2000). In brief, PCR samples were run for 16 h at 45 V on 6% (wt/vol) polyacrylamide gel with a denaturing gradient ranging from 45–60% (where 100% denaturant contains 7 M urea and 40% formamide). After electrophoresis the gels were stained with SYBR Green I nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, Maine) and photographed. Cluster analysis (WARD algorithm) of the DGGE patterns was performed with the GelCompar 4.1 software package (Applied Maths, Belgium).

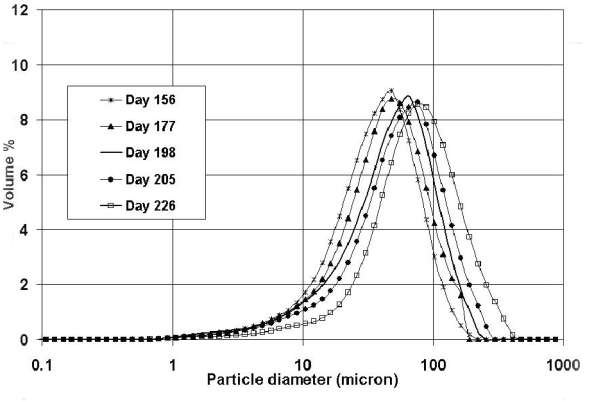
Results and discussion

Floc size and size distribution experiments were performed weekly for two time periods. The first period covered day 1 to 70 (Figure 2a), whereas the second period lasted from day 156 to 226 (Figure 2b).

In the first 28 days a monomodal distribution with a small decrease in floc size and a mass mean diameter decreasing from 35.12 µm to 32.04 µm was observed. A high level of similarity in the FSD appeared between day 21 and day 28. From the 21st day on a bimodal floc size distribution with a weak tendency to form larger flocs of approximately 400 µm was observed. This tendency became more pronounced in time (days 42 and 56) until again



a.

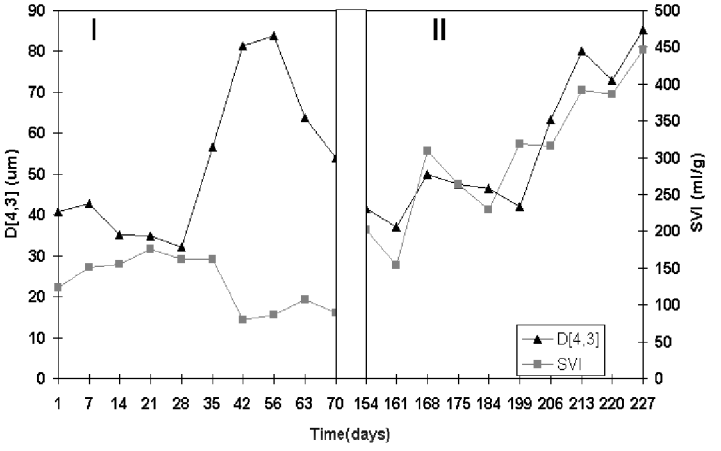


b.

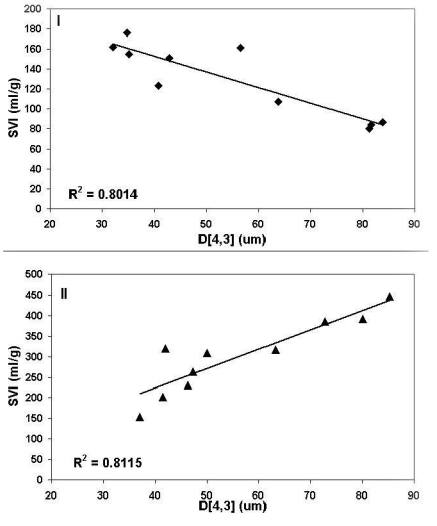
Figure 2 Particle size distribution evolution with time for: the (a) first and (b) the second experimental periods

a monomodal distribution (day 63), this time with predominant larger flocs was obtained. The first 35 days could be associated to a transient period of the microbial community that adapts to the new operating conditions. During the second period (Figure 2a) an increasing trend in the floc size was observed.

A comparison between SVI and floc size measurements shows two distinct periods in SBR evolution for the monitored period. During the first period (Figure 3a – I) the increase in mean floc size diameter correlated well with a decrease in the SVI (Figure 3b – I). In the second period (Figure 3a – II) filamentous bulking occurred and increasing SVI correlated with an increase in mean floc size (Figure 3b – II). This is similar to the observations of Wilén and Balmer (1999) where an increase of the floc size was shown to correspond to an increase of the SVI due to the presence of filamentous microorganisms. It also has to be mentioned that no correlation between the activated sludge settling properties and flocs size was found when the results for the whole monitored period of time were compared. These observations suggest that, in general, a correlation between floc size and settling properties is highly dependent of the type of microorganisms present in the floc structure.



a.



b.

Figure 3 D[4,3] and SVI evolution during: (a) the two experimental periods and (b) their correlation

Additional microscopic examination of the sludge during the experimental period showed that filamentous organisms were hardly present in the sludge in the first 91 days. Sezgin (1982) demonstrated that SVI is influenced by sludge characteristics such as floc size, sludge concentration and the filamentous organisms abundance and found that for a very low content of filaments compact, high density "floc-to-floc" aggregates were formed, which had no significant changing effect on the SVI. A similar finding was also observed for the first 91 days (Figure 4), where SVI revealed good settling properties accentuated during the period when the sludge adapted to the SBR operating conditions (days 42–91). Due to the fact that no significant variations in the sludge concentrations were observed it could be assumed that the SVI was especially influenced by floc size (Figures 3 and 4). The absence of filamentous organisms as observed microscopically, together with the floc size and SVI measurements for the first 91 days suggest that the flocs structure predominantly consists of floc-forming bacteria. Starting from day 91 the number of filamentous bacteria increased and even though a slight increase of the SVI was observed, settling experiments showed a good and fast sedimentation. Due to the lack of data on floc size measurements for this period a comparison between SVI and floc size could not be done. SVIs appeared again not to be influenced by the sludge concentration, which suggests that structural properties of the flocs primarily affect the SVI (Figure 4). On day 126, sludge bulking occurred and microscopical observations showed a large number of filaments. The decreasing trend observed for SVI between days 154–161 and days 184–191 respectively (Figure 4) is associated with a sharp increase in the sludge concentrations. However, no changes in the floc structure were observed. During this period continuous growth of filamentous organisms could be a cause of the increasing trend observed in floc size and SVI, which was demonstrated to be well correlated.

These observations allow us to conclude that three distinct stages exist in the evolution of floc structural properties. The first one corresponds to the predominant presence of floc-forming bacteria. The second stage is a short period (days 91–126) in which the filaments started to grow but a balance between floc-forming and filamentous bacteria resulted in good activated sludge settling properties. Finally, in the third stage, which is associated with the formation of a large number of filamentous organisms, bulking occurred.

The flocs' capability for break-up and aggregation was also investigated on-line (Figure 5). After 10 minutes testing of the floc size distributions' stability at continuous mixing (210 rpm) the sludge was subjected to a disrupting effect by using a sonication bath for 10 minutes after which the initial conditions were imposed again. (De)flocculation studies showed a clear difference in the activated sludge behaviour between those phases. It could be noticed that deflocculation by sonication produced an accentuated disrupting effect

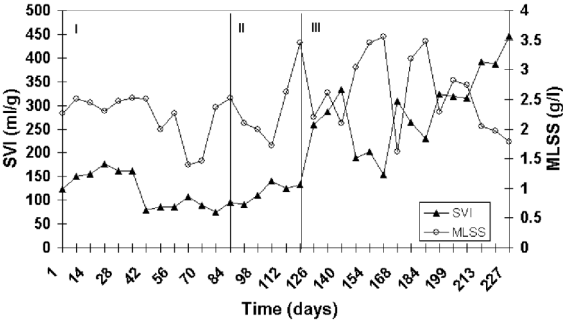


Figure 4 SVI and MLSS evolutions over 227 days. Three distinct stages were observed: I – predominantly floc-forming bacteria; II – equilibrium between floc-forming and filamentous bacteria; III – predominantly filamentous bacteria

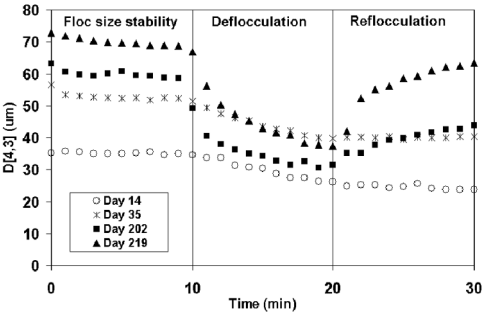


Figure 5 Mean diameter evolutions during deflocculation and reflocculation experiments

when filaments were present (Figure 5, days 202 and 219). A faster reflocculation occurred for the flocs containing filaments, whereas non-filamentous flocs reflocculated only slightly (Figure 5, days 14 and 35). These facts suggest that filaments reinforce the flocs structure and an equilibrium between the floc-forming and filamentous bacteria is necessary to obtain good settling performance. This confirms the filamentous backbone theory introduced by Parker *et al*. (1971), which suggests that filamentous bacteria form a backbone onto which extracellular polymeric substance (EPS) producing bacteria can attach.

Before microbial community analysis by DGGE was performed, the measurement variability was evaluated. To achieve this goal, nine duplicate samples taken over a one month period were collected during the sludge wasting phase and stored at –20°C before analysis. Results show that duplicates yielded the same DGGE-patterns (Figure 6). This suggested a homogenous community within the sample. This result also demonstrated that further analysis of duplicates would not be necessary.

The DGGE patterns of the sludge samples taken during the monitored period were compared and analysed by cluster analysis, revealing 3 major groups (Figure 7).

The first group (I) corresponded to the first 42 days. The inoculum is characterised by two dominant bands, which are already present in the first operating day of the SBR. During the first week (days 3–5–7) some new ribotypes appeared in the patterns and the two original bands became less dominant. The patterns of the next 35 days evolved into separate clusters with highly diversified ribotypes appearing on day 14. A high similarity was observed between days 21–28 and days 35–42, which clustered together. When comparing

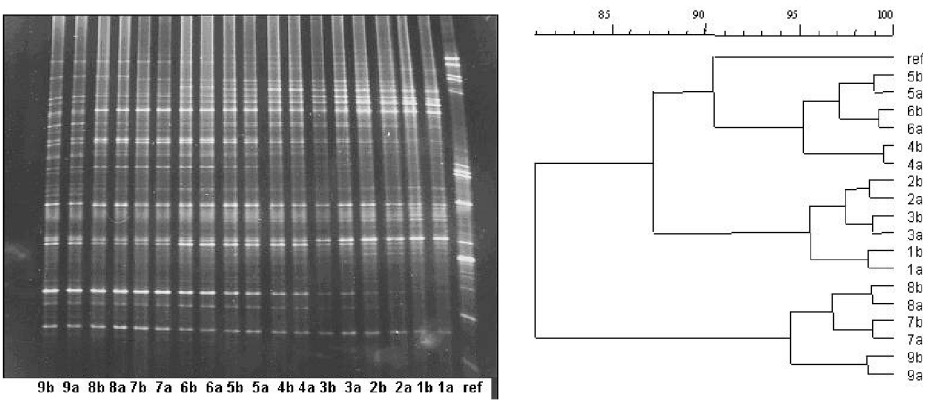


Figure 6 DGGE profile for replicate samples (left) and cluster analysis (right)

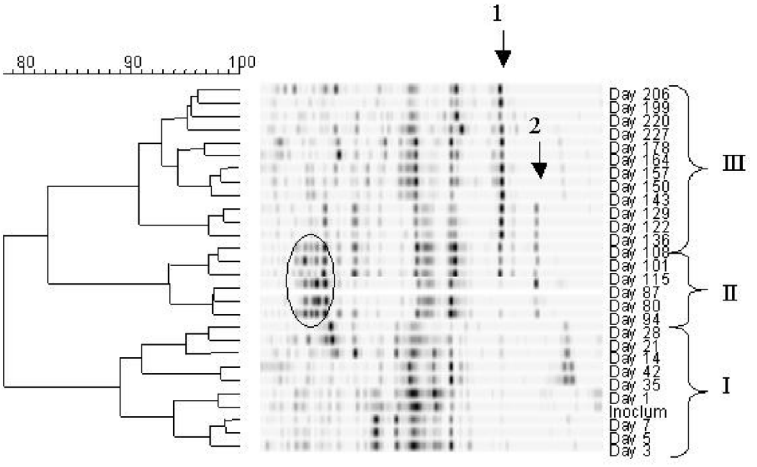


Figure 7 DGGE profiles (right) at different time periods and cluster analysis (left)

the floc size with these DGGE results, it was observed that the changes of the community structure were correlated with the FSD evolution. In Figure 2a the FSD of day 14 looks very different than those of days 21 and 28, which present a rather similar size distribution. Similarly, the FSD of day 14 is different from those of days 35 and 42, which correspond to the moment when large particles started to be formed. This first major group could be assigned to the period of adaptation of the community to the SBR conditions where new populations became dominant and others disappeared from the pattern. The structural properties also reveal dominant floc-forming bacteria during this period.

The microbial communities further evolved rather dynamically. In the second major period (Figure 7, II), the appearance of diverse populations was observed (oval mark in Figure 7). The large diversity observed in this period coincided well with the period where structural properties revealed a good balance between filamentous and floc-forming bacteria. During this period, two new ribotypes became visible as well (arrows 1 and 2 in Figure 7).

Another major cluster (III) started in the period when filamentous bacteria became abundant. During this period the bands on the top of the gel, as well as the band indicated by arrow 2 disappeared again (day 143). The band indicated by arrow 1 became brighter, indicating that the corresponding organism seemed to be adapted very well to the SBR conditions because its presence was rather stable until the end of the monitored period. This species could be one of the developing filamentous organisms, but this needs further validation by determining the sequence of the DNA fragments. This could also explain the slightly and constantly increasing floc size observed during the third stage.

The results suggest a dynamic bacterial community. The stable operating conditions of the SBR in terms of nutrient removal did not imply stability in floc structure and microbial community. A similar finding was suggested by Fernandez *et al.* (1999), they found that even when the environmental conditions were stabilized after 400 days, the microbial community was extremely dynamic for a period of 2 years.

Conclusions

The activated sludge floc structural properties evolved in three stages during the monitored period. The first one corresponded to the predominant presence of floc-forming bacteria. In the second stage a balance between floc-forming and filamentous bacteria was observed and, finally, in the third stage a large number of filamentous organisms developed. A good

correlation between the SVI and flocs size was obtained. (De)flocculation analysis showed less capacity for reflocculation after break-up for floc-forming bacteria and a higher break-up and reflocculation effect when the filamentous organisms were present in the floc structure. A dominant band, which could be due to a filamentous bacterium, was observed in the microbial community pattern at that stage. Moreover, the DGGE method was shown to be useful for monitoring the changes of the microbial community structure over time.

The method proposed in this study for monitoring the properties of the activated sludge in an SBR is a good procedure to find a link between changes in the activated sludge settling properties, floc structure and the microbial community dynamics. This should lead to a better understanding of the process performance.

Acknowledgements

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