MONITORING MICROBIAL COMMUNITY DEVELOPMENT IN A NEWLY-COMMISSIONED WWTP

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ABSTRACT

The commissioning of a WWTP provided a unique opportunity to link floc structure dynamics with the changes in the microbial community during start-up and stabilization.

An AS plant operating under an SBR regime was seeded with AS from a continuous process. The microbial community was monitored using microscopic examination and molecular methods. Floc characteristics were inferred from particle size distribution and EPS production.

Monitoring was carried out for eight weeks. During this time the AS maintained excellent settling properties (SVI < 100) while the microbial community and floc architecture adapted. The bacterial diversity increased 20% and half the seed population disappeared. Multi-dimensional scaling of microbial community profiles indicated a continuous shift away from the seed population. The amount of EPS doubled and then dropped after week two. Average floc size increased from 100 µm to 350 µm.

The last two samples indicated a change in microbial community coinciding with an increase in small particulates. Further analyses will determine whether such changes are a reliable diagnostic measure of changes in WWTP performance.

This study contributes towards understanding the relationship between AS microbial populations and performance indices and highlights new technology available for everyday use in microbiological monitoring of wastewater treatment.

KEYWORDS

Particle size distribution, activated sludge, EPS, 16S rRNA, DGGE, ARISA, SBR, microbial community dynamics, OTU, floc structure

1 INTRODUCTION

The formation and maintenance of flocs that settle well is a critical component in the proper functioning of AS treatment processes, for both good solid-liquid separation and for retention of a functional biomass. The optimal size, shape and density of flocs for rapid settling have been investigated (Li & Ganczarczyk, 1987; Sezgin et al., 1978; Wilen et al., 2006) and it is generally accepted that large (>100µm), compact flocs, with a rounded structure and distinct separation from the surrounding liquid are desirable characteristics (Eikelboom, 2000). Research suggests that critical determinants of floc structure include: a diverse and active microbial community (Klausen et al., 2004; Wilen et al., 2000); the matrix of extracellular polymeric substances (EPS) that aid cellular attachment (Jorand et al., 1995; Keiding & Nielsen, 1997); the number of filamentous bacteria (Parker et al., 1972; Sezgin et al., 1978), and the presence of higher grazing organisms (Gude, 1979; Luxmy et al., 2000). Establishing links between these components is essential to understanding floc formation and process stability.

Current models of floc formation and maintenance such as those reported by Jorand et al. (1995), Keiding & Nielsen (1997) and Sezgin et al. (1978) are based largely on the response of floc macrostructure to changes in

process parameters such as shear and oxygen stress. As microorganisms are acutely sensitive to changing environmental conditions, measuring the response of the microbial communities to perturbation may provide better predictive models of floc stability. It is now possible to monitor even small changes in microbial community structure using high-throughput culture-independent methods and recent advances in technology have provided the means to monitor the other components of AS flocs that are indicative of microbial gene expression, including EPS production and floc size. Fourier transform infrared spectroscopy (FTIR) is a rapid and simple tool for determining the amount of polysaccharide in bacterial communities (Marcotte et al., 2007) and can be used to provide an indication of both the total amount of EPS and EPS composition – a factor that has been correlated with AS flocculating ability (Sheng et al., 2006; Wilen et al., 2003). Automated particle size distribution provides a rapid means of determining floc size and variation and can be adapted to analyse floc density and porosity, properties that both determine the effectiveness of solid-liquid separation and reflect the aggregating properties of bacterial populations (Li & Ganczarczyk, 1987; Li & Ganczarczyk, 1991).

The microbial communities in AS wastewater treatment are critical in nutrient removal and flocculation. The development of these communities is often overlooked in the commissioning of new treatment plants as operators focus on the engineering aspects of reactors. Gaining an understanding of the baseline microbial populations in a wastewater treatment process provides a means for predicting the effects of changes on process stability.

2 METHODS

Commissioning of the new municipal WWTP in Pukekohe commenced on the 26th of April 2010. Over the first two days 8 loads of seed in the form of waste activated sludge at 7 m^3 each, comprising approximately 2240kg of biomass, were received from a large municipal AS treatment plant. The SBR reached operating capacity on day four of operation. On day eight the plant commenced automatic operation with 5 hour fill and draw cycles and an average flow rate of 3900 $m^{3/}$d. 500 ml grab samples of mixed liquor were taken daily for the first two weeks of operation and weekly for the following six weeks.

2.1 PARTICLE SIZE DISTRIBUTION

Particle sizing was performed within 24 hr of sample collection. Approximately 20 ml of sample was diluted with tap water until the obscuration was within range using laser diffraction in a Malvern Mastersizer 2000 (stirrer speed, 440 rpm; pump speed, 900 rpm). Five consecutive measurements, with a 60 s delay between measurements, were made of each sample to determine the stability of the flocs within the instrument.

2.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY

Samples were analyzed by Fourier-transform infrared (FTIR) spectroscopy to quantify EPS using a method adapted from Marcotte et al. (2007)(Marcotte, Kegelaer, Sandt, Barbeau, & LaXeur, 2007). This method determines the total polysaccharide content and is used as an indicator of EPS quantity. The amount of polysaccharide was estimated using the area under the peaks between 950 and 1201 cm^{-1} in the FTIR spectra, associated with C-O stretching modes of the alcohol and ether functional groups (Nivens et al., 1993). Normalisation of the polysaccharide amount (970-1182 cm^{-1}) to the quantity of the bacterial biomass was performed using the peak intensity of the amide II group (1530-1560 cm^{-1}) since this spectral region falls within an area where interference from other bacterial components is minimal.

FTIR spectra were recorded between 4,000 and 650 cm−1 with a Thermo Electron Nicolet 8700 FTIR spectrometer using attenuated total reflectance. 100 µl of each sample was spread in a thin layer onto a ZnSe crystal and dried under a stream of N_2 gas. The absorption spectrum of the crystal plate coated with cells was then recorded. A total of 64 scans were performed with a resolution of 2 cm^{-1} to obtain each spectrum. The angle of incidence of the IR beam was 45º. The spectra were ATR corrected and baseline corrected using Omnic spectroscopic software. The areas of all peaks were determined using the curve-fitting application in GRAMS 32 software v. 5.

2.3 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

DGGE analysis was used for microbial community characterization. This method generates a community fingerprint that reflects the bacterial diversity and abundance within the original sample. DNA was extracted

from samples of mixed liquor using a bead beater technique described by (Foght et al., 2004). The primers 341F-GC, incorporating a GC clamp, and 907R described by (Muyzer, Waal, & Uitterlinden, 1993) were used to amplify approximately 500 bp of the 16S rRNA gene. The resulting PCR products were separated on a 7.5% polyacrylamide gel containing urea as a denaturing agent in a gradient of 40-70%. Gels were run at 100V for 16 hrs then stained for 40 mins in 1 x Tris-acetate-EDTA containing a 1 x concentration of SYBR Gold (Invitrogen). Gel images were captured using a UV transilluminator. Each band was considered to represent a group of closely related bacteria, from this point referred to as an operational taxonomic unit (OTU).

2.4 AUTOMATED RIBOSOMAL INTERGENIC SPACER ANALYSIS (ARISA)

ARISA is an alternative method to DGGE for bacterial community profiling. The intergenic spacer region between the 16S and 23S was amplified with the primers SDBact and LDBact (Ranjard et al., 2001) in a PCR reaction described previously (Lear & Lewis, 2009). The fluorescently-labeled products were purified using a QIAquick® PCR purification Kit (Qiagen). Products were analysed with an internal LIZ1200 standard on a 3130XL Capillary Genetic Analyzer using a 50cm capillary (Applied Biosystems Ltd., NZ).

Results from ARISA were analyzed though Genemapper software v 3.7 to create bacterial community profiles for each sample. Multi-dimensional scaling (MDS) plots were constructed from community profile data using Primer 6 software. Bray-Curtis similarity was used to compare the profiles in terms of differences in abundance and length of fragments. The distance between samples on MDS plots was used to infer the similarity of the bacterial populations.

2.5 MICROSCOPIC INVESTIGATION

Protozoan and metazoan populations and floc morphology were assessed by direct microscopic observation using standard methods (Eikelboom, 2000). This involves ranking the number of each organism on a scale of 1 to 3 where 1 is none and three is numerous organisms per slide.

3 RESULTS

3.1 FLOC STRUCTURE

Numerous small flocs were observed during the first seven days of operation (Fig. 1). Over the following period the floc size increased and dense, rounded flocs were present by day 22. These appeared to form around large inorganic fibres present in the SBR system. The filament index (FI, ranked from 0-5 with 0 being very few) increased steadily and from day 30 to the end of monitoring the FI = 4. An increase in the diversity of filaments was also observed (data not shown). Floc structure remained dense and SVI remained <100 ml/g despite the high FI.

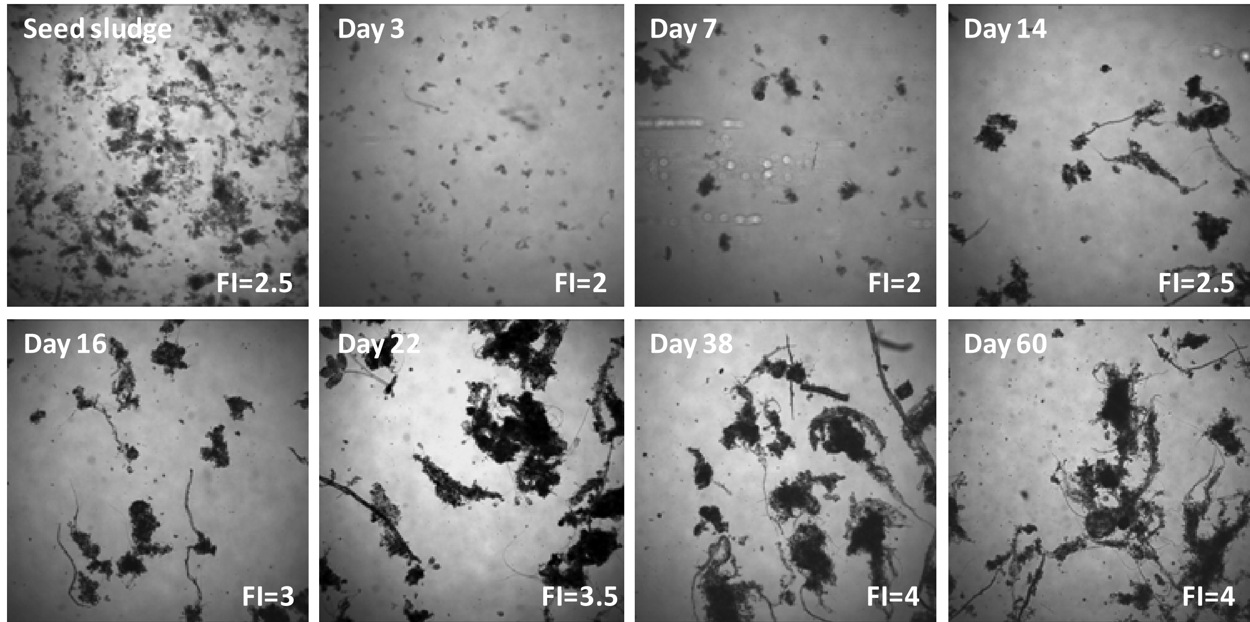

Figure 1: Bright field microscope images of SBR mixed liquor at 40X magnification. FI=filament index.

Average floc size increased over the sampling period, reaching a maximum average diameter of 371 µm on day 38 then decreasing slightly to 321 µm on day 57, the last day of sampling (Table 1). The average size of the largest 10% increased 3-fold, from 282 µm to 874 µm. The average size of the flocs in the smallest 10% of the particle size distribution showed more variation than the mean, maintaining an average of ~100 µm between day 23 and day 38 then declining to 76 µm on the final day of sampling.

Table 1. Average diameter of flocs in the 10th, 50th and 90th percentiles of the particle size distribution.

Sample	***d(0.1)***	***d(0.5)***	***d(0.9)***
Seed sludge	29.7	109.6	282.6
Day 1	28.9	101.5	274.9
Day 11	61.7	178.3	423.1
Day 15	84.4	258.2	614.3
Day 18	95.0	283.5	748.0
Day 21	88.8	287.3	676.7
Day 23	96.6	302.3	697.9
Day 25	100.5	309.3	705.2
Day 30	105.4	352.4	874.7
Day 38	99.4	371.1	830.7
Day 44	87.2	365.7	785.3
Day 53	75.6	335.7	866.1
Day 57	76.1	321.7	853.1

The distribution of particles in each sample is shown in Figure 2A. The samples from the seed sludge and during the first 20 days of operation followed a normal distribution. From day 25 the distribution became slightly skewed, with a long tail and an less pronounced decline in the distribution below the mean. This became more pronounced over the rest of the monitoring period. The last two samples, from day 53 and 57, show a distinct irregularity in particle size distribution (Fig. 2B).

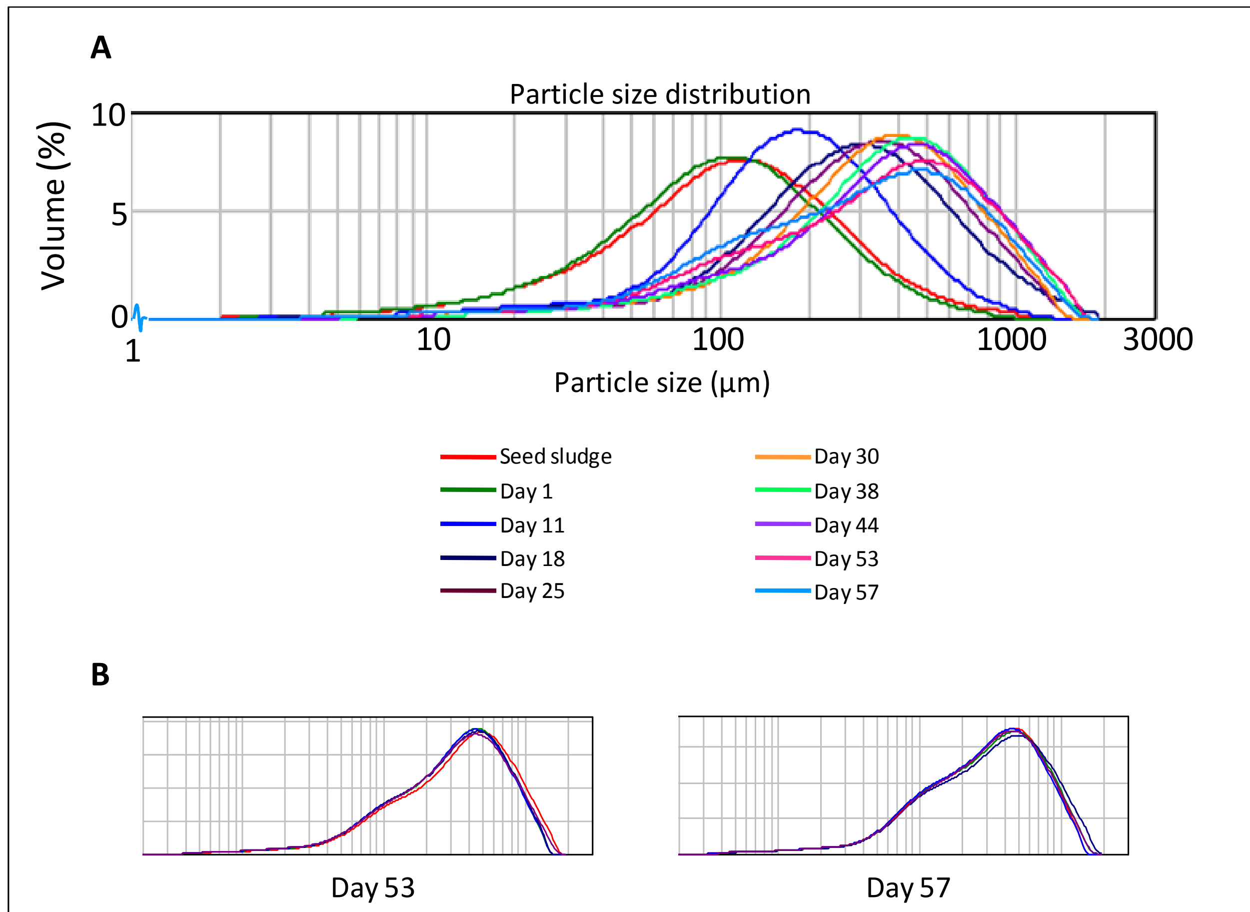

Figure 2: ***A.*** *Particle size distribution of all samples.* ***B.*** *Detail of samples from days 53 and 57, showing the skewed distribution that developed during the adaptation of the floc structure to SBR conditions.*

The ratio of absorbance in the polysaccharide spectral region, $A_{polysaccharide,}$ to the area of the amide II peak, $A_{amideII,}$ was calculated to quantify total polysaccharide in AS samples and gives an indication of EPS production by the microbial communities. The amount of EPS in samples peaked during the first two weeks of SBR operation and dropped again after day 14 (Fig. 3).

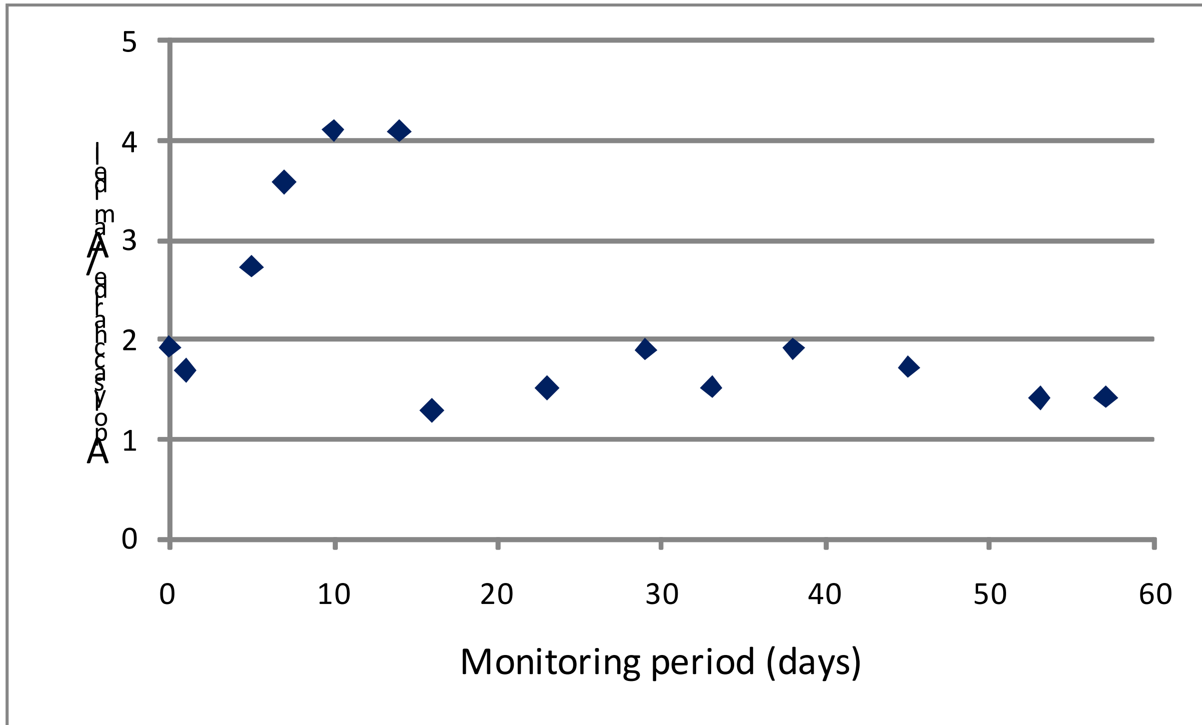

Figure 3: Amount of polysaccharide in SBR samples measured by FTIR. Day 0=seed sludge sample.

3.2 MICROBIAL COMMUNITY COMPOSITION

3.2.1 BACTERIAL COMMUNITIES

DGGE showed a change in diversity of the bacterial communities over the sampling period, estimated from the number of OTUs in each sample. The lowest diversity was shown in the seed sludge and day 1 of SBR operation, with 42 and 41 OTUs, respectively, in the denaturing gradient gel. By day 4 the diversity had increased to 53 OTUs and remained consistently between 52 and 56 OTUs for the remainder of the sampling period. Of the 42 OTUs in the seed population, 21 were detected in all samples (see Fig. 4).

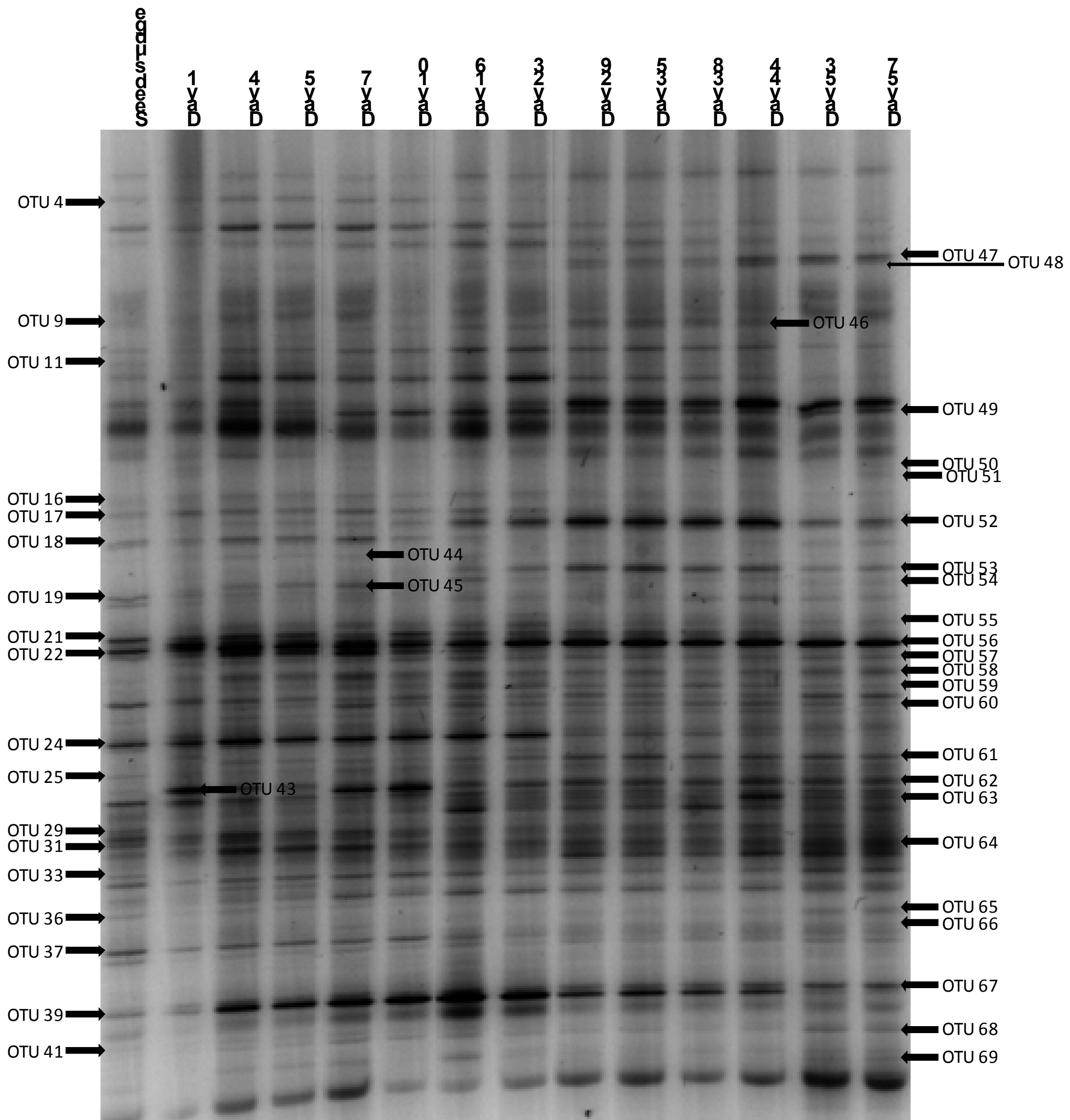

Figure 4: Denaturing gradient gel showing the bacterial community profiles of each sample. Each unique band was assigned an OTU number. The arrows on the left indicate OTUs that disappeared from profiles over the sampling period. Arrows on the right indicate OTUs that were not present in the seed population. Arrows in the middle of the gel indicate transitional OTUs.

At days 7, 29 and day 53and there are changes in the banding patterns and abundance (indicated by the intensity of the bands) of each OTU, suggesting that at these times there was a change in the bacterial populations. This is supported by the results from ARISA.

The bacterial populations showed a clear progression away from the starting community, with ARISA profiles from the seed sludge and the last two samples at opposite corners of the multi dimensional scale plot (Fig. 5). An overlying trajectory line in the MDS plot indicates a one-way and continuous change. Along this line, the remaining samples can be distinguished into three groupings. Day 1, 4 and 5 lie close together, indicating a close degree of relatedness and little change over this period. Day 7, 10 and 16 are distinctly separated, indicating a high rate of change during this period. This is followed by another closely grouped cluster of samples, Day 23, 29, 35 and 38.

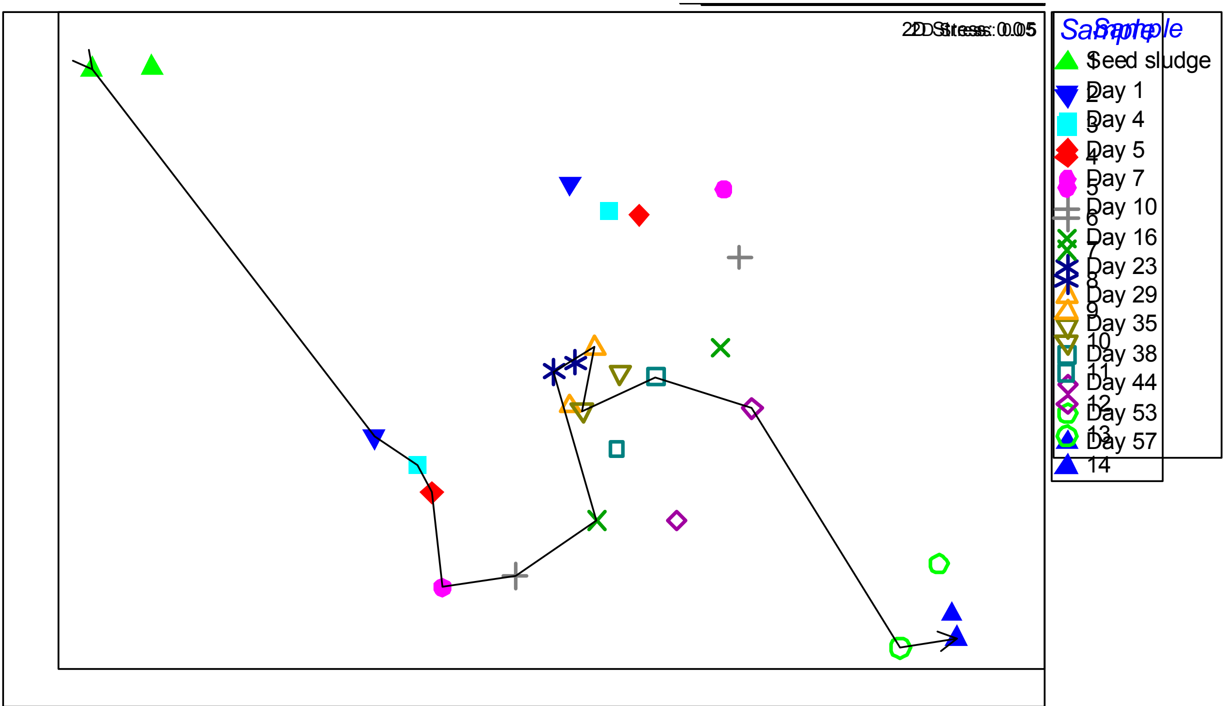

Figure 5: Multidimensional scale (MDS) plots of ARISA data representing bacterial community structures of samples. Each symbol within the graph represents a bacterial community for one given sample, and the distance between samples is an indicator of bacterial community relatedness. The arrow overlaying the data follows sampling time.

3.2.2 EUKARYOTIC COMMUNITIES

Numbers of protozoa and metazoa changed slightly over the monitoring period (Fig. 6). The seed community was dominated by ciliates and flagellates, with low numbers of testate amoeba, rotifers and worms. At the end of the monitoring period there were high numbers of testate amoeba and elevated numbers of rotifers.

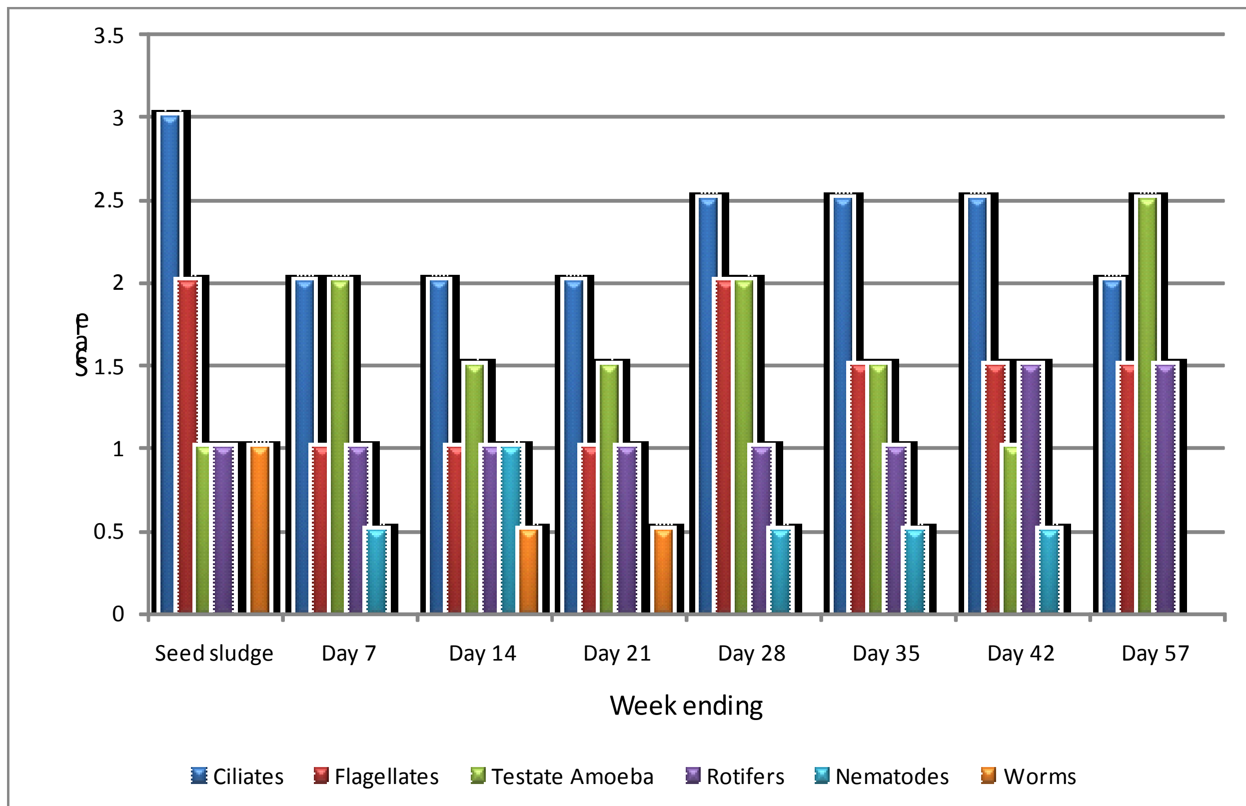

Figure 6: Populations of eukaryotic microbes over the monitoring period. Bars represent weekly averages.

4 CONCLUSIONS

- Over the monitoring period the bacterial community changed in both composition and diversity as it adapted to the SBR operating conditions. Despite this continuous change, good solid-liquid separation was maintained throughout the monitoring period, suggesting that microbial expression, rather than the abundance of any particular group, is a determinant of floc maintenance.
- The particle size distribution changed from a normal distribution in flocs from the continuous process to a skewed distribution in the SBR. The last two samples showed a distinctly skewed distribution and an increase in small particles. This coincided with changes in microbial community composition.
- The production of EPS was not directly related to floc size or microbial community composition, but rather appeared to be influenced by the change in operating regimes, seen by the sharp increase in polysaccharide between day 1 and day 14.

These results suggest that monitoring indicators of microbial expression in addition to the microbial communities themselves is the key to understanding links between microbial community dynamics and floc structure. Further analyses will determine whether particle size distribution and FTIR monitoring of AS flocs can be used as predictive indices of treatment plant performance.

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