Water and Environment Journal
Promoting Sustainable Solutions

CIWEM

Water and Environment Journal. Print ISSN 1747-6585

Comparison of microbial populations and foaming dynamics in conventional versus membrane enhanced biological phosphorous removal systems

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Keywords
EBPR; foaming; membrane; population dynamics.

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doi:10.1111/wej.12164

This paper is dedicated to the memory of Fred Koch.

Abstract

In this study, molecular community analysis and analytical measurements were combined to assess and compare the identity, functionality, structure, and temporal changes of membrane and conventional enhanced biological phosphorous removal systems (which ran stable over 300 days) and to identify the dynamics of the foam forming population. Magnesium was added to the system with the goal to enhance phosphate removal. Both systems behaved similarly with respect to phosphate and nitrogen removal efficiency and had similar community evenness, but they differed in community composition. A principle component analysis indicated that, the community of the membrane system changed permanently whereas the conventional system returned to approximately its initial composition. The relative intensity of DGGE bands were transformed in a numerical matrix and based on this matrix a model was constructed, which predicted some bands as foam forming bands, which were identified as foam-forming filamentous bacteria by sequencing.

Introduction

Understanding bacterial population dynamics in biological treatment systems is of high interest because the process relies on bacteria to achieve treatment goals (Crocetti *et al*. 2000; Okunuki *et al*. 2004). Structural and functional aspects of the bacterial community in individual wastewater treatment plants (WWTPs) are informative, (Head *et al*. 1998; Kong *et al*. 2002; Rossetti *et al*. 2005; Gillbride *et al*. 2006) and can give insights about changes in removal performance over time. Studies have already attempted to quantify community shifts occurring in mixed liquor over time (Pholchan *et al*. 2010; Cai-Yun *et al*. 2011; van Nostrand *et al*. 2011); however, means to predict foaming events on the basis of community changes in the mixed liquor and foam largely remain unknown. It is important to understand factors leading to malfunctions, such foam production caused by filamentous growth or nutrient availability, in order to determine and evaluate alternative remedies to control the growth of problem organisms and promote the presence of the functional relevant population (Cloete and Theron 2003).

All over the world simultaneous bulking and foaming problems in activated sludge plants have been reported (Tandoi *et al*. 2006), but only minor attempts have been made to prevent either (Seviour and Nielsen 2010). Over 30 different kinds of filamentous microorganisms have been found to cause problems with bulking and foaming (Pipes 1978; Eikelboom and van Buijsen 1981; Jenkins *et al*. 1986; De los Reyes 2010, Wanner *et al*. 2010). Microscopy and fluorescence in situ hybridisation (FISH) on activated sludge samples have been used to predict foam-formation and to identify foam-forming microorganisms. However, both methods have their drawbacks. Through microscopy it is often not possible to make a certain identification of foam-forming microorganisms up to species level and the available FISH probes do not cover all foam forming *Mycolata* (Kragelund *et al*. 2007). Furthermore, FISH probes rely on cell ribosome content and they will show no signal if the foaming bacteria have low activity and ribosome content (Oerther *et al*. 2001) or if they are not permeable to the FISH probes. Foaming bacteria such as Nocardia cells have a

hydrophobic cell surface and can hence float and cause foaming problems even if dead (Carr *et al*. 2005).

One essential process known to be inhibited by filamentous bacteria is enhanced biological phosphate removal (EBPR) (Vaiopoulou *et al*. 2007). Rational improvement of EBPR technology is developing in part from the understanding of the ecological rules structuring the microbial community in the system. Oxygen availability (Smolders *et al*. 1994) and the availability of trace metals, such as Mg^{2+} and K^{+} ions, are key factors driving the system. These trace metals are important cofactors for the EBPR organisms (Pattarkine & Clifford 1999), because these bivalent cations are needed for uptake of phosphate in from polyphosphate (polyp) (Rickard & McClintock 1992; Schöneborn *et al*. 2001). Other factors affecting the growth and performance of polyphosphate accumulating organisms (PAOs), such as temperature, carbon source and pH, have been extensively studied (Lopez-Vazquez *et al*. 2009), but the effects of added bivalent cations on the microbial population structure has not yet been tested on a pilot scale.

The goal of this study was to compare bacterial community composition and rates of change of the mixed liquors and foam communities of two parallel reactors designed for EBPR removal (one conventional and the other membrane) under conditions preserving functional stability. Both reactors were fed with municipal waste water and operated under the same conditions for 1.5 years. This study additionally aimed to produce numerical matrices from microbial data in order to describe the dynamics of foam-formers and determine effects of changes in bivalent cations (magnesium) on community dynamics.

Material and methods

Field site and operational conditions

The UBC pilot plant consisted of two parallel 25 m^3 activated sludge tanks, operated on the University of Cape Town principle (Ekama & Marais 1977). A secondary clarifier was used in the case of the conventional EBPR process, and a membrane module replaced the clarifier in the case of the membrane EBPR process (Supporting Information S1). The membrane unit was a Zenon ZeeWeed® hollow fibre membrane system (Zenon, Ontario, Canada) with a total membrane surface of 12 m^2 and a nominal pore size of 0.04 μm. The mixing in the aerobic zone relied on an aeration system at an air flow rate of 0.34 m^3/min, which was used to prevent cake formation, packing of solids between the membrane fibres, and biofilm formation. The municipal wastewater was pumped into two 15 m^3 equalization basins which were filled four times per day. The solid retention time of 20 days and the hydraulic retention time of 10 h were maintained throughout the study (400 days). The influent characteristics are listed in Table 1.

Table 1 Wastewater characteristics. (Srinivas, 2001)

Parameter	Mean	Min-Max
TSS (mg/L)	90.1	16–180
COD_{tot} (mg/L)	291	122–590
COD_{sol} (mg/L)	186	80–467
Acetate (mg/L)	18.2	0.0–43.4
Propionate (mg/L)	5.2	0.0–24.4
Tot VFA's (mg COD/L)	19	0.0–76.57
TKN (mg N/L)	35.9	25.8–47.3
NH_4-N (mg N/L)	26.7	9.1–39.2
NO_3-N (mg N/L)	Not detec.	
TP (mg/L)	4.2	2.1–7.9
PO^{2-}_4-P (mg/L)	3.0	1.1–6.7
Mg	1.2	
T °C	20.2	14.0–24.0
pH	7.2	6.4–7.8

As the wastewater entered the anaerobic zone, it was supplemented with 5000 mg/L acetate. The phosphate nitrogen and carbon removal efficiency of both reactors were monitored over 400 days and were measured spectrophotometrically (Supporting Information S2). Vancouver's water has a relatively low pH, which occasionally drops below the threshold value of 6.5 and it has a limited amount metal ions (16–18 mg Mg^{2+}/l; water hardness of A–B) (Höll 2002; Water-Quality-Report 2005). Therefore, Mg^{2+} was supplemented to the wastewater because it is known that it is an important cofactor for the EBPR organisms (Rickard & McClintock 1992; Pattarkine & Clifford 1999; Schöneborn *et al*. 2001) and it was therefore hypothesized that the addition of Mg^{2+} would increase the stability of phosphorous removal of the UBC-pilot plant.

Biomass collection

After Magnesium addition began, sample collection for the determination of microbial community composition started. The experiment was divided into two parts: (1) five weeks of Mg^{2+} addition, and (2) seven weeks without Mg^{2+} addition. The operational conditions remained the same throughout the study. The influent wastewater, the mixed liquor and the foam present in all compartments were sampled twice per week. The solids in the samples (influent: 100 ml, mixed liquor: 1.8 ml, and foam: 0.7ml) were harvested by centrifugation (5 min at 14 000 rpm).

DNA extraction

Nucleic acids were extracted from the harvested solids using the Q-Biogene BIO 101 FastDNA SPIN Kit for Soil (QBiogene, Mississauga, Ontario, Canada). The integrity of the extracted DNA was evaluated by electrophoresis on a 1% agarose gel

for 35 min at 90 V, then stained for 20 min with 1 μg/ml of ethidium bromide, and visualized by using a Bio-Rad Gel Doc 2000 (Bio-Rad Canada, Mississauga, Ontario, Canada). To determine the concentration of the extracted DNA, the adsorption was measured with a spectrophotometer at a wavelength of 260 nm. Samples with a 260/280 coefficient of at least 1.8 and a 260/230 coefficient of 2.0–2.2 were considered as sufficiently pure DNA. Samples below these ratios were not analysed.

Denaturing gradient gel electrophoresis analysis

The microbial community composition was assessed by denaturing gradient gel electrophoresis (DGGE) of the 16S-rRNA gene fragment spanning the variable regions V3–V5. The polymerase chain reaction (PCR) amplification of this fragment used the forward primer 357f-GC and the fluorescently-labelled reverse primer 907rCy5 (Schäfer & Muyzer 2001). The PCR mixture consisted of 30 pmol of each forward and reverse primers, a total of 0.35 mM of $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphates (dTTP, dATP, dGTP, dCTP) (Invitrogen Canada, Burlington, Ontario, Canada.) 1.25 U Taq DNA polymerase (Qiagen, Mississauga, Ontario, Canada.), and 1× Taq buffer. The amount of template added to the mixture was 20 ng. PCR amplifications were preformed using a Hybaid PCR Express thermocycler (Hybaid Ltd., Teddington Middlesex, UK) following the program: 4 min denaturing at 94°C for the first cycle then 1 min denaturation at the same temperature for following cycles including 45 s annealing at 65°C and a decrease of the annealing temperature by 1°C at each subsequent cycle, until the temperature of 55°C was reached, the extension was held at 72°C for 90 s. Then 9 cycles of denaturation at 94°C for 1 min, extension of 72°C and annealing of 55°C were performed, followed by a final extension for 7 min at 72°C. After PCR amplification, the quality and quantity of the products were evaluated by electrophoresis. The mixtures of PCR amplicons obtained from the DNA extracted from the solids samples were analysed using DGGE. The 6% polyacrylamide gel in 0.5× TAE buffer (45 mM Tris, 45 mM acetic acid, 1 mM EDTA, Beacham *et al*. 1992) contained a gradient from 45 to 65% of denaturant (100% denaturant solution corresponds to 7 M urea and 40% formamide). The gels were poured using the Bio-Rad DCode system (Bio-Rad Canada, Mississauga, Ontario, Canada) and run at 65°C for 14 h in 0.5× TAE buffer at 75 V. Each lane was loaded with 50 ng of the PCR product labelled with Cy5, and 50 ng of standard PCR-DGGE fragments labelled with FAM to provide an independent reference system in each lane. The DGGE gels were imaged using a Typhoon 9400 Imaging System (Amersham Biosciences) equipped with a Red (633) laser and a 670BP30Cy5 emission filter to detect the Cy5 fluorochrome, and a Blue2 (488) laser with a 520BP40Cy2BlueFAM emission filter to detect the FAM fluorochrome. The sensitivity was set to normal and a 480 photomultiplier tube was used. The gel images were analysed using the GelCompar Software (Applied Maths, Inc., Austin, TX, USA). The location of the DGGE bands within a lane was normalized using the standard reference system, which allowed a proper alignment of the co-migrating bands within a single gel. After alignment, the integrated intensity of each band was measured to obtain a final dataset of DGGE profiles.

Statistical analysis

The intensities of the bands in each DGGE profile (lanes) were normalized by dividing by the sum of the band intensity in the profile. Thus, the band intensities in the normalized profiles can be understood as the relative band intensities. For statistical analysis, the evenness measurement was calculated based on the entropy measurements of the Shannon–Weaver index $H_1 = -\sum_{i=1}^{q} p_i \ log_2 \ (p_i)$, where p_i is the probability of having species i present (Pielou 1981). The measurement of order one (H_1) is the logarithm of intensities (abundance). A closely related evenness measure is the Partic index $H_0 = log_2 q$ (Legendre & Legendre 1979), where q is the number of bands. The measurement of zero order (H_0) is the logarithm of the number of bands (richness) in each DGGE lane. The evenness indicates how heterogeneous the value of a measured variable in a sample series is. The Pielou evenness is defined as $J = H_1/H_0$ and is a concentration measurement which will take its maximal value (1) when all species are equally represented (Pielou 1981). These metrics were selected because they are widely accepted in ecological literature (Buzas & Heyek 2005). The dataset of normalized DGGE profiles was analysed by measuring the similarity between the different sampling points. Four similarity indices were used in this study because different indices will be sensitive to different properties of the data; they are summarized in Table 2. The indices used can be classified according to two criteria. The first criterion is binary or quantitative. Binary indices compare two samples on the basis of bands being present or absent. In contrast, the quantitative indices compare the relative abundance of the bands between two samples. The second criterion is the symmetrical and asymmetrical. In the case of a symmetrical coefficient, a band being absent in two samples will make the samples more similar (Legendre & Legendre 1998). In the case of asymmetrical coefficients, the bands that are absent in the two samples under comparison are excluded from the calculation of the coefficient. The coefficients used were *Simple Matching Similarity* (S_1), and *Sørensen Similarity* (S_8) *Bary-Curtis Similarity* (S_{17}) and the *Eucledian Distance* (S_{D1}) (Legendre & Legendre 1998) (Table 2). Similarities between mixed liquor samples collected over time were analysed using

Table 2 Shows the different indices used in this study

Coefficient	Symbol	Equation	Coefficient	Properties	Reference
Simple matching	S1	$\frac{(a+b)}{a+b+c+d}$	Binary	Symmetrical	(Sokal R.R. and Michener C.D., 1958)
Sørensen	S8	$\sqrt{1-\frac{2a}{(2a+b+c)}}$	Binary	Asymmetrical	(Dice L.R., 1945; Sørensen T., 1948)
Euclidean Distance	S_{D1}	$\sqrt{\sum_{j=1}^{p}(y_{1j}-y_{2j})^2}=1-\frac{S_{D1}}{S_{D1MAX}}$	Quantitative	Symmetrical	
Bray-Curtis	S_{17}	$\frac{\sum 2Min(y_{1j}-y_{2j})}{\sum(y_{1j}+y_{2j})}$	Quantitative	Asymmetrical	(Motyka J., 1947)

a-d define counts of bands in terms of presence and absence [a:0/0; b:0/1; c:1/0 d:1/1], y is used for relative intensities of bands.

chronological clustering. Thus, for this clustering algorithm, complete linkage agglomerative clustering was conducted. Chronological clustering was performed using the R package, a freeware produced by (Legendre & Legendre 1998) and available on the web at http://www.bio.umontreal.ca/legendre. The cluster formation was based on the broken stick distribution according to McArthur 1957 and the magnitude of changes (i.e. resolution) between the clusters of samples was set by the alpha level. The higher the alpha level are the easier significant differences between samples (or clusters) and the more groups are build. An alpha level of 0.3 was picked for all following analysis. Principal coordinate analysis of the distance matrix based on the S_8 similarity matrix (Distance $= \sqrt{(1-S_8)}$).

Statistical analysis of foam forming bands

According to the analyses of (Klein *et al*. 2007) the foam forming bands were analyzed with the student *t* distribution and population of interest in a sample were denoted as *p*. The enrichment factor was defined as pfoam/(1 − pfoam) × (1 − pmixed liquor)/pmixed liquor. The standard error (SE) was in the order of 1/nfoam(1/pfoam + 1/(1 − pfoam) + 1/nmixed liquor (1/pmixed liquor + 1/(1 − pmixed liquor). A band was identified as a putative foam-forming population if the relative intensity of a band was significantly ($p < 0.1$) higher in the foam layer than in the underlying mixed liquor.

Microscopic observations

The 4′,6-diamidino-2-phenylindole (DAPI) staining procedure followed the method of (Zillers *et al*. 2002b). Fluorescence from DAPI staining was visualized using the G365/FT395/LP420 filter set. Images were recorded with a Nikon Coolpix 8800 digital camera using a microscope adapter produced by Martin Microscope (Martin Microscope Co., Easley, SC, USA).

Band sequencing and phylogenetic analysis

Bands of interest were excised from the gel for sequencing using a sterile scalpel blade, and eluted overnight at 4°C in 50 μl of molecular grade water. The resulting solution was used as template and re-amplified by PCR as described above, except that the forward primer was primer 357f (Turner *et al*. 1999). The PCR products were purified using the Qiagen QIAquik PCR Purification Kit (Qiagen, Mississauga, Ontario, Canada), their quality was evaluated by agarose gel electrophoresis, and they were sequenced at the Nucleic Acids Protein Services Unit, University of British Columbia. The nomenclature adopted in presenting the sequence results is in accordance with Bergey's Manual of Systematic Bacteriology. All sequences were approximately 500 base pairs (bp). The initial phylogenetic affiliation of each sequenced band was determined using the Basic Local Alignment Search Tool (BLAST) search program (http://www.ncbi.nlm.nih.gov/blast/) to search the National Centre for Biotechnology (NBCI) Nucleotide database. Reference sequences most similar to the rRNA gene sequences originating from this study were obtained using the Ribosomal Database Project (RDP) database (Cole *et al*. 2003) in order to build phylogenetic trees. All sequences (obtained in this study and retrieved from the NCBI Nucleotide database) were aligned using Clustalw (Thompson *et al*. 1997). The Parsimony algorithm was used to construct phylogenetic trees. The modules dnapars, drawgram, and retree of the Phylip package (Felsenstein 1997) were used to construct phylogenetic trees. All entries of sequences in the algorithm were bootstrapped 50 times.

Result and discussion

Diversity

Complex substrates were shown to increase microbial diversity (Beer *et al*. 2005), and since the UBC pilot plant was fed with municipal wastewater containing different organic carbon sources, the results shown here increase validity and

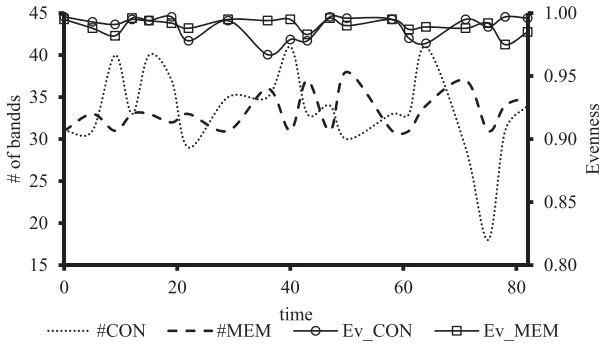

Fig. 1. •••.

applicability for full-scale municipal WWTPs. Diversity is thought to confer robustness to system functioning, because after a disturbance, less abundant functional groups within a diverse ecosystem can act as a buffer, herewith replacing a negatively affected group thus conserving functional stability (Yachi & Loreau 1999). DGGE analysis of the influent showed that the population in this study remained very constant over time (Fig. 1, Supporting Information S4). The similarity in community composition for the three compartments within each reactor (anaerobic, anoxic, and aerobic zone) was high (>90%) (Supporting Information S3). Thus, samples from one compartment were taken as representative of all chambers. Even though the reactors were operated under the same conditions, bacterial communities with different species composition developed within each reactor, which may have led to differences in the temporal dynamics of the communities. This is in line with the study of (Kaewpipat & Grady 2001), in which the bacterial community of two identically operated batch reactors were not identical. The similarity in community composition between the membrane and conventional system were low (<30% similarity). Despite this difference both systems had on average highly similar average richness and evenness (Fig. 1). This is consistent with other studies investigating functional stability of microbial communities (Winkler *et al*. 2013; Wittebolle *et al*. 2009). Among the studied systems, bacterial communities showed on average comparable OTU

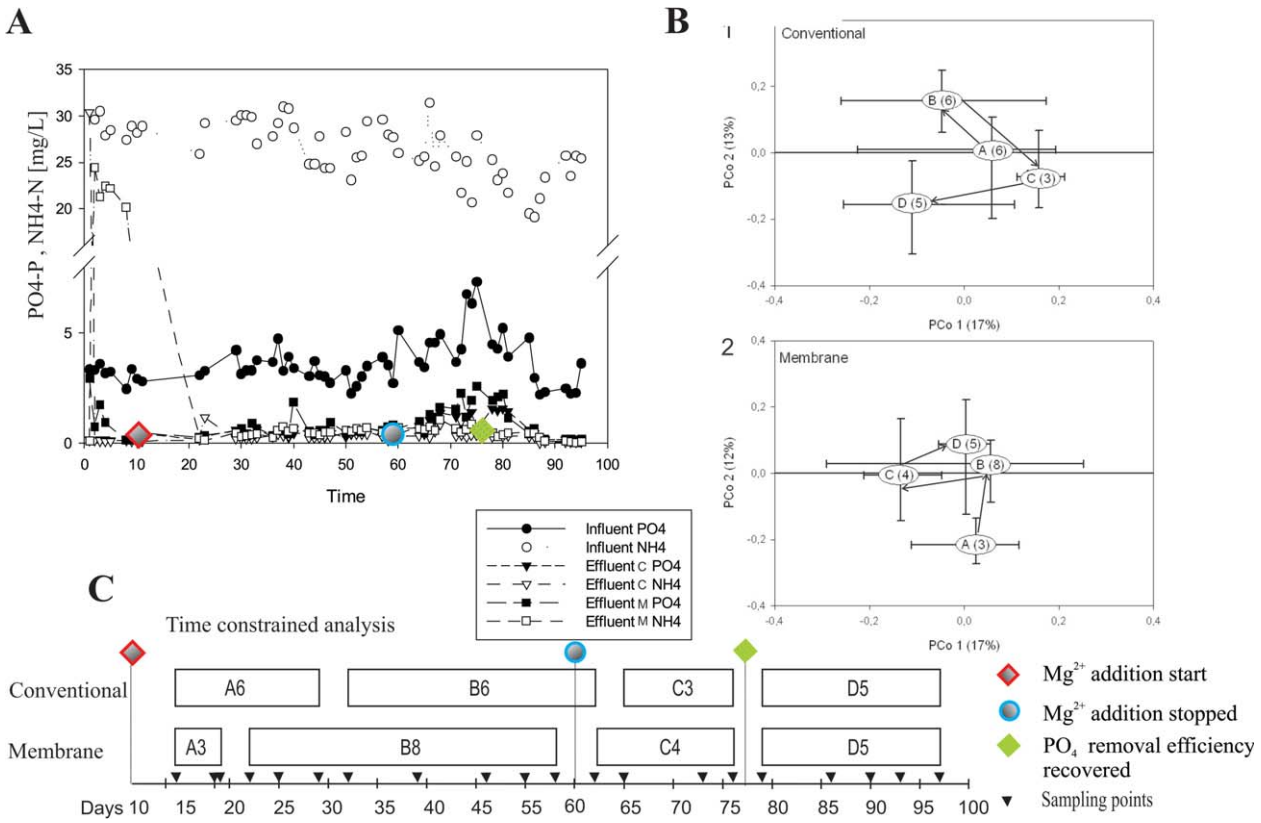

Fig. 2. (a) Total phosphorus in the influent and soluble phosphate in the effluent for the membrane (MEBPR) and conventional (CEBPR) EBPR-reactors. At day 0 reactor was operated stable for 300 days (Figure S2). Sample collection for community composition analysis started at Day 16. Mg2+ addition started Day 10 and lasted for 50 days. (b) Principal coordinate analysis of the distance matrix. The points (A, B, C, D) represent the centroid of the clusters determined by time-constrained cluster analysis and the error bars indicate the position of the extreme sampling points associated with each cluster. The number of observations associated with each cluster is indicated in parentheses. (c) Segmentation of the sample time series by time-constrained cluster analysis at an alpha level of 0.3 of the mixed liquor DGGE profiles from the conventional and membrane reactor.

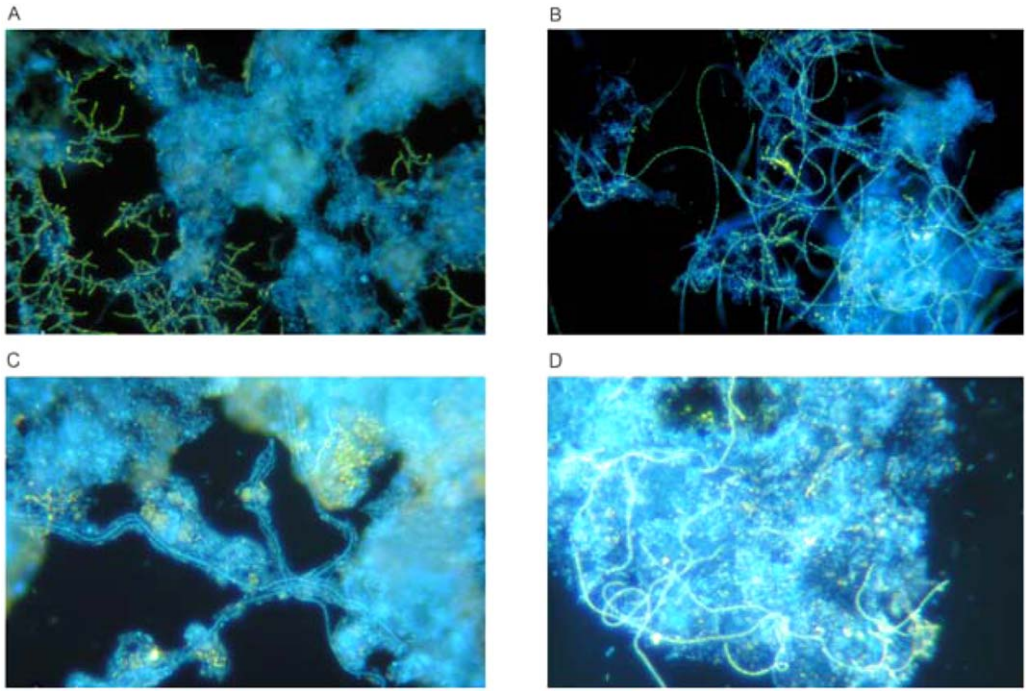

Fig. 3. DAPI-stained mirographs of foam samples in conventional (a) and membrane (b) reactors, and of mixed liquor in conventional (c) and membrane (d). Blue are DNA containing structures and Greenish-yellow inclusions are polyP granules in cells.

richness, entropy and evenness despite marked differences in community compositions between sludge types. DGGE profiles for the influent samples remained very similar throughout the study (Supporting Information S4) and population changes due to varying species in the influent can be considered minor.

Community dynamics and system performance

Time-constrained analysis was used as an objective procedure to detect significant changes in community composition along the time series of DGGE profiles (Supporting Information S4 and S5). Measurements of population dynamics were done for the aligned mixed liquor data of the membrane and conventional systems using four different similarity indices (Table 2). Such similarity indices can be used to compare temporal changes in community composition (Gutierrez & Fey 1980). Time-constrained cluster analysis was used to segment the time series in clusters of similar profiles (Supporting Information S9). The length of time constraints profiles can be interpreted as a measurement of the rate at which the community composition changed over time. The high evenness (Fig. 1) indicates that there was low variability in the relative abundance of DGGE bands and that the changes in community composition were mainly due to presence and absence of species. Analyses were therefore continued using the Sørensen similarity coefficient (S_8), which is a binary and asymmetrical coefficient compatible with the structure of this data (Table 2). The magnitude of changes was evaluated for different alpha levels and indices (Supporting Information S9 and S10).

Dynamics of bacterial community composition can be used to test hypotheses about mechanisms structuring the community in WWTPs. This is possible because different mechanisms operate at different time-scales (Wells *et al*. 2011). After the reactor ran for 300 days (Supporting Information S2) it was decided to improve phosphate removal efficiency by adding Mg^{2+} (Fig. 2). However, phosphate removal declined immediately after Mg^{2+} addition, and significant rapid changes in community composition occurred concurrently (Fig. 1). Therefore, rather than stabilizing functionality of the systems, addition of magnesium appears to have disturbed it. This disturbance was reflected in the response of populations. Phosphate removal slowly recovered again after magnesium addition was stopped (Fig. 1, Supporting Information S2). An intriguing result was that the time series segmented in both reactors at the point at which the addition of magnesium was stopped. Clusters B and C occurred over very similar times in both reactors. The relatively long B Clusters occurred before the stop of Mg^{2+} addition, while Clusters C occurred after the supplementation of Mg^{2+} and are only half as long as Clusters B. The data suggest that the phosphate removal recovery was accompanied by a significant shift in the microbial community composition. Both C Clusters break at the point when the phosphate removal efficiency stabilized after a period of low efficiency,

Table 3 Best Blast hits of sequenced bands classified within the phylum *Actinobacteria*[a] *Bacteroidetes*[a] and *Proteobacteria*[a]

Source of band	Bp length	%	Blast hit	Class/Suborder[b]	Source of Blast hit
Actinobacteria					
Foam	514	99	*Gordonia amerae*	*Corynebacterineae*	Foam formers
Foam	518	99	*Gordonia terrae*	*Corynebacterineae*	Identification studies
Mixed liquor/foam	514	99	*Mycobacterium mucogenicum*	*Corynebacterineae*	Nontuberculous Mycobacterium
Mixed liquor	525	95	*Terrabacter sp*. DFA1	*Corynebacterineae*	Dibenzofuran-degrading
Mixed liquor	519	94	Clone: Run-SP116	*Acidimicrobineae*	PAOs in EBPR processes
Foam	529	94	*Candidatus* Microthrix parvicella	*Acidimicrobineae*	Filaments in wastewater
Foam	518	95	*Candidatus* Microthrix calida	*Acidimicrobineae*	Filaments in wastewater
Proteobacteria					
Foam	509	92	Caulobacter sp.	*Alphaproteobacteria*	Bottled Mineral
Mixed liquor	540	82	Clone AKYH1112	*Deltaproteobacteria*	Geothermal ecosystem
Foam	443	93	Sludge bacterium A9	*Deltaproteobacteria*	Nitrifying-denitrifying sludge
Mixed liquor	448	94	Clone AKYH1112	*Deltaproteobacteria*	Diverse microbial communities
Influent	528	99	Moraxella sp. D30C2A	*Gammaproteobacteria*	Bottled water
Foam	517	97	Betaproteobacterium	*Betaproteobacteria*	Identification of PAOs
Mixed liquor	531	95	Clone SBR1021	*Betaproteobacteria*	Identification of PAOs
Foam	492	98	Clone PE03-7G18	*Betaproteobacteria*	Freshwater Sediment
Mixed liquor	520	92	Clone DR-10	*Betaproteobacteria*	Denitrifying bioreactor
Mixed liquor	528	94	Clone LKC3_102B.28	*Betaproteobacteria*	Filamentous microbial mats fromsulfidic springs
Mixed liquor	444	92	*Paucibacter toxinivorans*	*Betaproteobacteria*	Cyanobacterial hepatotoxins
Influent	535	100	Clone: 13C-A24	*Betaproteobacteria*	Acetate and Methanol-Utilizingdenitrifyers
Bacteroidetes					
Influent	509	98	*Riemerella sp*. Lo3	*Flavobacteria*	Bacterial Flora in the Mucus
Influent	523	98	Bergeyella CCUG 47293	*Flavobacteria*	Municipal wastewater
Mixed liquor/foam	534	96	Clone PHOS-HE28 16S	*Flavobacteria*	Phosphorus-removal ecosystem
Mixed liquor	519	94	Clone EUB72-2	*Flavobacteria*	Granular sludge in wastewater
Mixed liquor/foam	405	91	Clone Ebpr3	*Sphingobacteria*	PolyP/PHA-accumulating traits
Foam	502	92	Clone HP1A28	*Sphingobacteria*	EBPR in sludge
Foam	530	98	Clone PHOS-HE35	*Sphingobacteria*	phosphorus-removal ecosystem
Foam	527	97	Clone CYCU-0281	*Sphingobacteria*	Membrane bioreactor
Foam	494	97	Clone:TB003-97	*Sphingobacteria*	Activated sludge

Phylogenetic trees with these sequences can be found in supplementary materials (Figure S6, S7 and S8).

[a]Bergey's Manual of systematic bacteriology (Boone D.R. and Castenholz R.W.C, 2001) was used for classification as follows: 1. bacteria (domain), b) phylum (*Actinobacteria*); c) class (*Actinobacteria*). 2. Bacteria (domain), b) phylum (*Bacteroidetes*), c) class (*Flavobacteria and Sphingobacteria*) 3. bacteria (domain), b) phylum (*Proteobacteria*), c) class (*Alphaproteobacteria Betaproteobacteria,Gammaproteobactria* and *Deltaproteobacteria*).

[b]Higher phylogenetic level that exhibited a differential classification of the bands.

suggesting an association between the community composition and a change in phosphate removal efficiency. Our results may be in contrast with other studies that found changes in bacterial community structure could be related to periodic pulse additions of gelatine, but these changes did not alter the removal efficiencies (Carrero-Colón *et al*. 2006).

Several mechanisms may be involved in the observed deterioration of phosphate removal efficiency by magnesium addition and the accompanied community composition shift. Magnesium can precipitate with a number of chemical nutrient and limit their availability (Lee *et al*. 2002; Zillers *et al*. 2002a; Beer *et al*. 2005). Another mechanism can be the role of divalent cations such as Mg^{2+} in the formation of bacterial flocs and their structural density (Tezuka 1969; Morgan *et al*. 1990), which might have increased the local retention time of bacteria, leading to a sudden biomass washout after magnesium addition was stopped.

Cell structure and polp-P in mixed liquor and foam

Microscopic observation showed a clear trend of more filamentous organisms in the foam (Fig. 3a,b) and fewer filaments in the underlying mixed liquor (Fig. 3c,d). In the mixed liquor round shaped bacteria appeared to be yellow indicating polyP granules in PAOs.

However, the filaments also showed also in the DAPI staining yellow granule type structures hence indicating that filaments are capable of accumulating polyP. *Gordonia* species have the polyphosphate kinase gene (Hiessl *et al*. 2012) and can hence store phosphate for growth, but they cannot actively uptake and release phosphate as do PAOs (Smolders *et al*. 1994). Thus they do not contribute significantly to phosphate removal, as suggested by earlier studies comparing the P-release rate from mixed liquor and foam from the

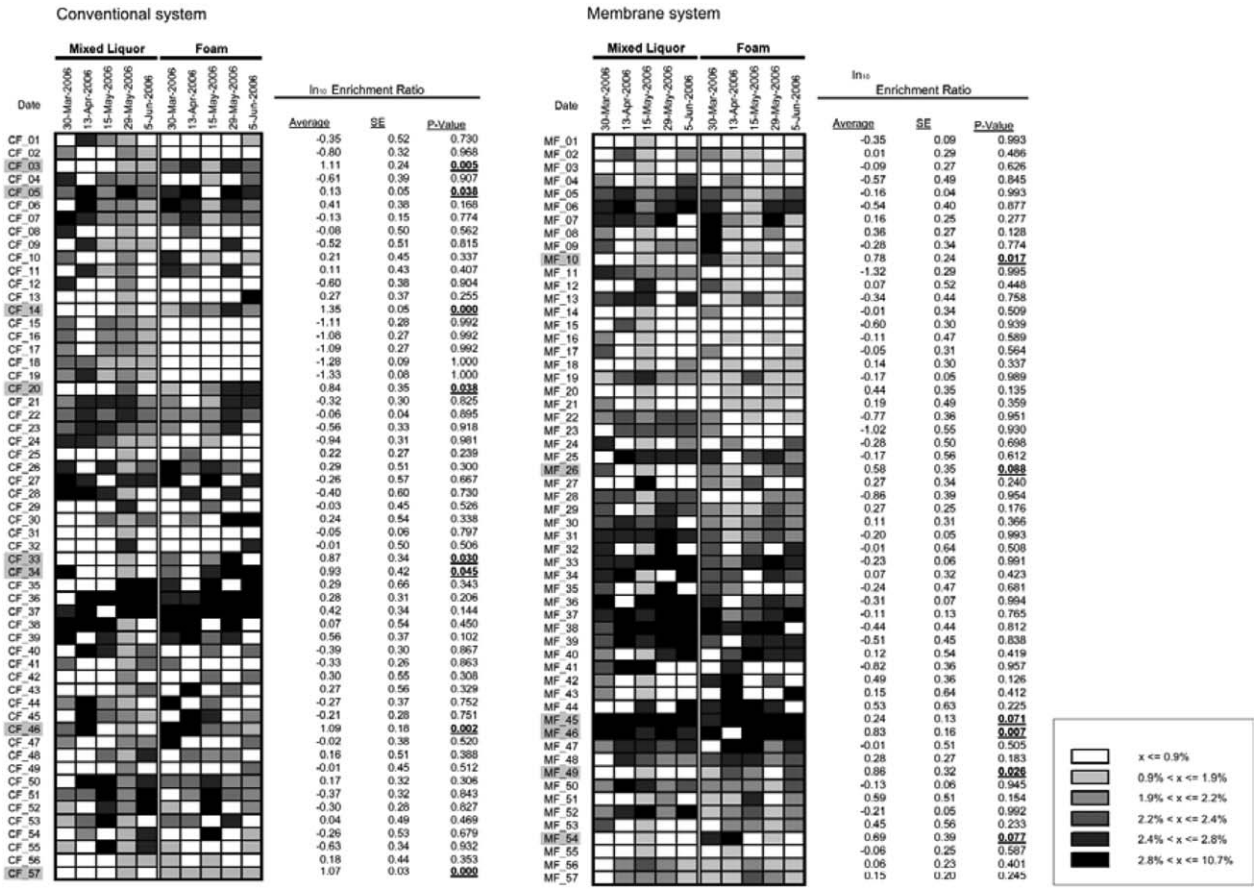

Fig. 4. Heatmap representations of the DGGE profiles obtained for mixed liquor and foam samples from the conventional reactor (left) and membrane reactor (right). The lowest relative abundance of an observed band was 0.9%, and all the bands not detected were coded white (<0.9%). The rest of the data were equally distributed amongst five intervals shown in the legend. Average of the log of the enrichment ratio and associated standard error (SE) are reported for each band. A positive average log of the enrichment ratio indicates a higher relative abundance in the foam, and a negative value indicates a higher relative value in the mixed liquor. The probability of no difference in relative abundance between the foam and the mixed liquor is also reported (*P*-value).

Table 4 Sequenced bands from putative foam-forming populations

Blast hit	Bp length	%	Suborder	Source of blast hit	locus
Gordonia amerae	514	99	*Corynebacterineae*	Foaming in activated sludge and digesters	AF020329
Gordonia terrae Mycobacterium	518	99	*Corynebacterineae*	Identification studies	AY771337
mucogenicum	514	99	*Corynebacterineae*	Nontuberculous Mycobacterium species	AY457073
Candidatus Microthrix parvicella	529	94	*Acidimicrobineae*	Filaments in wastewater	DQ147285
Candidatus Microthrix calida	518	95	*Acidimicrobineae*	Filaments in wastewater	DQ147288

A phylogenetic tree of the putative foam formers can be found in supplementary materials (Figure S6).

UBC pilot plant (Hall *et al*. 2011). However, magnesium and potassium have been shown to be necessary for polyP uptake in filamentous cultures (Machnicka 2006) and the magnesium addition might have promoted their phosphate uptake in this study, which might have negatively impacted the growth of PAOs. In addition, Mg^{2+} can lead to chemical precipitation (Pastor *et al*. 2008) and cause struvite formation (Diwani & Rafi 2003), potentially increasing the inactive fraction in the reactor and negatively impacting phosphate removal efficiency.

Phylogenetic identity of the activated sludge reactors

Throughout the study, bands with the highest relative abundance were affiliated with *Actinobacteria* (Fig. 3 and

Table 3). Blast hits of two sequenced bands from the mixed liquor were affiliated with *Betaproteobacteria* and were most similar to reference sequences of the genus *Rhodocyclus* (Supporting Information S7), an organism implicated in EBPR (Bond *et al*. 1999; Crocetti *et al*. 2000). Two additional bands were affiliated with the clones PHOS-HE45 and Epbr-17, both of which were present in phosphate removal processes (Dabert *et al*. 2001; Liu *et al*. 2001). One band, which was identified as a *Gammaproteobacteria* was closest to *Moraxella* sp., which was an influent specific band, since it had a high relative abundance in the influent and was absent in mixed liquor and foam samples suggesting that fluctuations in influent do not necessarily change the community in the reactor.

Determination of foam-forming populations

Biological foaming affects activated sludge treatment plants around the world (Blackall *et al*. 1989; de los Reyes & Raskin 2002) and was also an issue in the current pilot-scale reactor experiment. Therefore, part of this study aimed to identify the microorganisms responsible for biological foam accumulation.

In the current study, the putative foam-forming populations were identified by comparing DGGE banding patterns of five sampling days from the mixed liquor and foam samples (Fig. 4). Both systems suffered from foaming, but this was worse in the membrane system than in the conventional system. By the means of statistical analysis six bands were identified as putative foam formers in the membrane reactor and eight bands in the conventional reactor (Fig. 4), whereof five bands were identified by sequencing analysis as typical foam formers such as *Gordonia* and *Microthrix* species (Table 4). The bands which were statistically calculated to be foam forming bands were also identified to be foam formers by sequencing analysis, showing the robustness of the used model (Klein *et al*. 2007).

Conclusions

It is essential to undertake careful molecular community analysis of activated sludge systems to assess their functionality, structure, and dynamics. In this study, methods were developed to detect significant changes over time in bacterial community composition of membrane and conventional systems. Both systems had distinct communities, but similar evenness and temporal dynamics. Magnesium addition caused a change in community composition and reduced phosphate removal efficiency. It was investigated that the community of the membrane system and conventional system behaved differently. A model predicted some bands as foam forming bands, which were identified as foam formers after sequencing. The model is very simple and could be used by other researchers to identify most important foam formers in their system. The techniques used in this study are of use for long-term monitoring and can be used to determine the relative magnitude of the changes in community composition that are associated with variations in operating conditions and system performance.

Conflicts of Interest

There are no conflicts of interest.

Acknowledgements

The authors would like to thank Thomas Hug and Fred Koch for useful discussions and Hemanth Srinivas for the analytical data. Mari Winkler was supported by the Wasser Stipendium Rhur, Rhurverband, Essen, Germany.

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