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ENVIRONMENTAL BIOTECHNOLOGY

Microbial diversity differences within aerobic granular sludge and activated sludge flocs

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Abstract In this study, we investigated during 400 days the microbial community variations as observed from 16S DNA gene DGGE banding patterns from an aerobic granular sludge pilot plant as well as the from a full-scale activated sludge treatment plant in Epe, the Netherlands. Both plants obtained the same wastewater and had the same relative hydraulic variations and run stable over time. For the total bacterial population, a similarity analysis was conducted showing that the community composition of both sludge types was very dissimilar. Despite this difference, general bacterial population of both systems had on average comparable species richness, entropy, and evenness, suggesting that different bacteria were sharing the same functionality. Moreover, multi-dimensional scaling analysis revealed that the microbial populations of the flocculent sludge system moved closely around the initial population, whereas the bacterial population in the aerobic granular sludge moved away from its initial population representing a permanent change. In addition, the ammonium-oxidizing community of both sludge systems was studied in detail showing more unevenness than the general bacterial community. *Nitrosomonas* was the dominant AOB in flocculent sludge, whereas in granular sludge, *Nitrosomonas* and *Nitrosospira* were present in equal amounts. A correlation analysis of process data and microbial data from DGGE gels showed that the microbial diversity shift in ammonium-oxidizing bacteria clearly correlated with fluctuations in temperature.

Keywords Aerobic granular sludge · DGGE · Diversity · Functional stability · *amoA*

Introduction

The functional stability (e.g., nutrient removal efficiency) in biological wastewater treatment systems has been proposed to be correlated to species richness (number of bacteria) and evenness (distribution of bacteria in a population) (Stirling and Wilsey 2001; Naeem and Li 1997; Wittebolle et al. 2009). Species diversity is reported to have functional consequences because the number and kinds of species determine ecosystem processes (Bell et al. 2005; Tilman et al. 1997). Diversity is generally assumed to be positively correlated to functional stability because independent species can degrade similar compounds or create a competition network of many species (Cardinale et al. 2002; McCann 2000; Rowan et al. 2003), whereas mono- and highly enriched cultures are reported to negatively influence ecosystem functioning (Chapin et al. 2010; Wilsey and Potvin 2000). Even though most of the studies confirm the positive diversity–stability relationship, the opposite has been described as well (Jiang et al. 2011; Manefield et al. 2002;

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Temudo et al. 2008). Diversity is thought to be positive for 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 and Loreau 1999).

Aerobic granular sludge is a new wastewater treatment strategy in which the same functional groups of bacteria as present in a conventional sludge floc (nitrifiers, denitrifiers, and phosphate-accumulating organisms) are present. Flocs and granules are clearly different in structure, shape, and substrate availability (Fig. 1). It can therefore be questioned whether aerobic granular sludge or activated sludge has a higher diversity and if a different diversity offers a more stable system performance. Ecological niches are important for the maintenance of species diversity (Levine and HilleRisLambers 2009). Granular sludge provides many ecological niches due to substrate gradients, shear stress phenomena (Nicolella et al. 2000; Xavier et al. 2007) as well as protozoa grazing on the outer layers of the granules (Huws et al. 2005; Winkler et al. 2012). In addition, there is also segregation of biomass over the sludge bed column. The microbial population in the bottom of the reactor will be different from those present in the top (Winkler et al. 2011a, 2011b). Also, granules can harbor slow-growing organisms due to higher biomass retention time for the biomass in the deeper parts of the granule (de Kreuk and van Loosdrecht 2004). One might argue that granular sludge selection leads to a lower biodiversity since only organisms capable of forming granular sludge might be enriched, which might decrease diversity-based evenness. On the other hand, the substrate gradients in granular sludge might lead to more ecological niches and in turn a higher biodiversity. Clearly, there are pros and cons why aerobic granular sludge should hold a more or less diverse microbial population and hence offer more/less freedom for functional stability than it is the case for an activated sludge floc. Studies have shown that the community composition within a reactor changes despite a functional stability of the reactor (Carrero-Colon et al. 2006; Fernandez et al. 1999; Lee et al. 2002). The diversity within one and among different aerobic granular sludge reactors has been described earlier (Ebrahimi et al. 2010; Li et al. 2008), but a comparison of the bacterial population of an aerobic granular and a suspended sludge system has not been described.

From an engineering point of view, it is appealing to correlate microbial shifts to system performance. Studies have already attempted to quantify community shifts over time and partly correlated these changes to system performance (Ayala-Del-Río et al. 2004; Cai-Yun et al. 2011; Pholchan et al. 2010; van Nostrand et al. 2011); however, studies showing a temporal pace of microbial and operational changes within full-scale systems are sparse (Wells et al. 2011). In this study, two industrial reactor system (granules and flocs) were investigated for their microbial community composition. The reactor operation of both systems was clearly different, but both systems were run on the same municipal wastewater and experienced the same seasonal changes. We then made a direct comparison of population dynamics within both treatment systems and correlated these changes to changes in operational conditions (N–P–chemical oxygen demand (COD) removal as well as

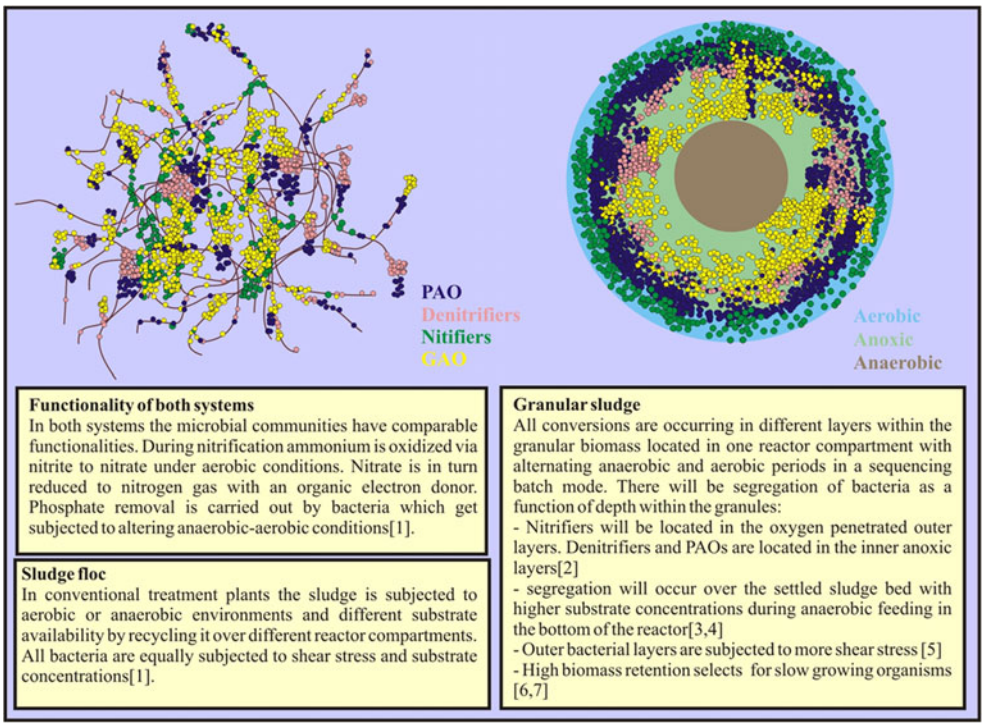

Fig. 1 Structural and functional difference in aerobic granular sludge and a conventional sludge floc [1](Henze et al. 2008); [2](Xavier et al. 2007); [3.4](Winkler et al. 2011a, 2011b); [5](Picioreanu 2000); [6](de Kreuk and van Loosdrecht 2004); [7](de Bruin 2004)

temperature) to investigate potentially interesting conclusions from an ecological and engineering point of view.

Material and methods

Sample collection operational data

Samples from an aerobic granular sludge pilot plant in Epe and from a full-scale activated sludge plant were collected over a period of 400 days (November 2008 to January 2010). The granular reactor ran as a sequencing batch reactor similar to our lab reactors (Winkler et al. 2011a), whereas the flocculent system ran in a continuous mode. Despite the difference in operational conditions, both installations were fed with the same wastewater, experienced the same temperature fluctuations, and were operated on the same relative hydraulic flow variations. The aerobic granular sludge pilot plant was operated by the company DHV, situated in the Netherlands. At the moment sampling was started, the granular system already ran stable for a period of 2 years. The flocculent sludge treatment plant was operated by the municipal wastewater treatment authorities of Epe, The Netherlands. Throughout the sampling period, influent parameters of COD, ammonium, and temperature from both reactors as well as effluent values were collected on a daily basis. Information on operational conditions is given online in the Electronic supplementary material (ESM) S1.

DNA extraction

Total genomic DNA from the reactor sludge of both systems was extracted using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The manufacturer's protocol was applied, based on the principle of combined mechanical/chemical cell lysis (chemically enhanced bead beating). The DNA extraction was tested by electrophoresis on a 1.5 % Agarose Gel, at 90 V for 32 min with Syngene Gel Doc G:BOX (Syngene, Cambridge CB4 1TF, UK) and the software Gene Snap (Syngene). The DNA extract was checked on quantity and quality by gel electrophoresis.

PCR procedure

General primers

A set of universal primers, Bac341f and Bac907rM (Muyzer and Smalla 1998), was used targeting the 16S rRNA gene fragment including the hypervariable regions V3 to V5 (Neefs et al. 1990). The PCR mixture contained 0.25 μl (50 μM) of each of the primers, 12.5 μl Qiagen PCR Taq master mix (1.25 units Taq Polymerase, dNTPs, buffer), 11.5 μl PCR water (Qiagen), and 0.5 μl DNA template, with a content of 1.5 mM $MgCl_2$. The whole reaction mix and one positive control (*Escherichia coli*), as well as a negative control, were loaded in a Biometra T-Gradient Thermocycler (Biometra, Goettingen, Germany). The following program was used for PCR: 5 min denaturing at 95 °C followed by 32 equal cycles of 30 s denaturing at 95 °C, 40 s at 57 °C for annealing, and 40 s at 72 °C for extension. An extension step of 30 min at 72 °C followed after which the final resident temperature was set to 12 °C. The quality and quantity of amplicons of all the dilutions were tested on a 2 % agarose gel.

Functional primers

For the ammonium-oxidizing bacteria, the following specific ammonia monooxygenase (*amoA*) primers were used: *amoA*-1F_deg6 (5′-GGGGHTTYTACTGGTGGT-3′)+GC (Rotthauwe et al. 1997) and *amoA*r-i (5′-CCC CTCIGIAAAICCTTCTTC-3′) (Hornek et al. 2006; Stephen et al. 1996). The PCR mixture contained 0.25 μl (50 μM) of forward and reverse primer, 12.5 μl of Qiagen PCR Taq master mix (Taq Polymerase, dNTPs, buffer), 0.25 μl (5 units/μl) extra Taq polymerase, 0.5 μl $MgCl_2$ (50 mM), 10.75 μl PCR-H_2O (Qiagen), and 0.5 μl extracted DNA template (undiluted). The resulting reaction mix of 25 μl contained 2.5 units Taq polymerase, 0.5 μM of each primer, and 2.5 mM $MgCl_2$. The following program was used for the PCR: 5 min denaturing at 95 °C followed by 41 equal cycles of 30 s denaturing at 95 °C, 40 s at 50 °C for annealing, and 40 s at 72 °C for extension. An extension step of 30 min at 72 °C followed, and the procedure concluded with a final resident temperature of 12 °C. The reaction mix and one positive control (a mixture of *Nitrosomonas europaea* 43 μg/ml, *Nitrosomonas oligotropha* 41.9 μg/ml, *Nitrosospira multiformis* 42 μg/ml, 1,000 or 20,000 times diluted), as well as a negative control, were loaded in a Biometra T-Gradient Thermocycler (Biometra, Goettingen, Germany).

Denaturing gradient gel electrophoresis

The gel was prepared in order to obtain 6 % acrylamide-bis-acrylamide mix content (*w*/*v*) in a 1× TAE buffer and a gradient of 20–70 % of denaturant. The electrophoresis was run for 16 h in a 1× TAE buffer at 100 V and 60 °C. The running conditions were set to 5 h and 100 V. The best fitting dilutions of PCR products were chosen by the densitometry estimation in order to load 200–300 ng DNA per slot. A loading dye of 2.4 μl (10× concentrated "Blue juice," Invitrogen) was added as well as PCR water to fill up to a volume of 24 μl/lane. The lanes on the edges of the gel were filled with 25 μl anti-smiling liquid (anti-smiling solution,

Ingeny, Netherlands). After electrophoresis, the gel was stained with SYBR gold solution in 1× TAE and visualized in the Syngene Gene Doc by a digital camera.

Construction of heatmaps

The image processing was accomplished to convert the data stored as a digital camera picture of the denaturing gradient gel electrophoresis (DGGE) gel into numerical values in order to use them for statistical analysis. In this research, the main steps of image processing for the alignment of bands were done using the software GelCompar II® (version 5.0) from the company "Applied Maths NV". Heatmaps of DGGE gels were created with Visual basics and are based on band intensities derived from GelCompar II®. The heatmap for the general primer sets is given in Fig. 1, and the heatmap of the functional primer sets (*amoA*) is given online in ESM S2. The code used for the creation of the heatmaps can be found online in ESM S3.

Sequencing of amoA bands from aerobic granular sludge

Bands were cut from the ammonia monooxygenase (*amoA*)-denaturing acrylamide gels using flame-sterilized blade. The cut material was soaked in 40 μl 1× Tris buffer (pH 8) for 2 days at 4 °C. For re-amplification, the *amoA*-1F_deg6 primer was used without GC clamp. The PCR procedure was kept as previously described, however reducing the cycle number to 25 and using 1.25 units polymerase per vial and 0.5 μl of eluted DNA. The resulting PCR products were purified and sequenced (Macrogen Inc., South Korea). The obtained *amoA* gene sequences were imported into the ARB software (http://www.arb-home.de) version 5.1 and aligned by using the integrated aligner. All accession numbers for the nucleotide sequence and corresponding name of the band picked are listed in Table 1. The alignment was further verified and corrected manually. A phylogenetic tree was generated using a maximum likelihood algorithm (RAxML).

Table 1 Name and accession number of sequenced bands of *amoA* gene DGGE gels as presented in Fig. 3

Name of band	Accession number
Conventional system (flocs)	
CS_01	JX291223
CS_02	JX291224
CS_03	JX291225
CS_04	JX291226
CS_05	JX291227
CS_06	JX291228
CS_07	JX291229
CS_08	JX291230
CS_09	JX291231
CS_10	JX291232
CS_11	JX291233
CS_12	JX291234
CS_13	JX291235
CS_14	JX291236
CS_15	JX291237
CS_16	JX291238
Granular system	
AGS_1	JX291205
AGS_2	JX291206
AGS_3	JX291207
AGS_4	JX291208
AGS_5	JX291209
AGS_6	JX291210
AGS_7	JX291211
AGS_8	JX291212
AGS_9	JX291213
AGS_12	JX291214
AGS_13	JX291215
AGS_14	JX291216
AGS_15	JX291217
AGS_16	JX291218
AGS_17	JX291217
AGS_18	JX291220
AGS_19	JX291221
AGS_21	JX291222

Statistical analysis

Entropy and Pareto–Lorenz

For statistical analysis, two evenness measurements were used. Firstly, the evenness according to Pielou was calculated which is 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 and Legendre 1979), where q is the number of bands. The base of the logarithms was chosen to be 2 for both entropy-based measures. The measurement of zero order (H_0) is the logarithm of the number of bands (richness) in each DGGE lane. The Pielou evenness is defined as $J=H_1/H_0$ (Pielou 1981). The second measure of evenness is derived from the Pareto–Lorenz curve (Naeem 2009; Wittebolle et al. 2009). The reason to choose these two classes of measures is that they have been often used in ecological literature (Buzas and Hayek 2005).

Time constraint analysis

In our study, we have chosen to use asymmetrical indices only, i.e., only the bands in the DGGE gel are compared. Symmetrical indices were not considered because in these indices, the absence of a band (double zero) in two different patterns is also considered as a factor, increasing the similarity between these two patterns. Resemblance analysis for 16S primer sets was checked with chord and Ruzicka dissimilarity index (Legendre and Legendre 1979). Only the Ruzicka index was used for data representation. All banding patterns were converted into a binary (presence/absence) and quantitative (band intensities) matrix to compare if species abundance or presence/absence changes the community more.

Tree clustering and MDS of banding patterns

For tree clustering, the average dissimilarity (Ruzicka) and chord distance were calculated (Legendre and Legendre 1979). Multi-dimensional scaling was performed based on the chord distance. All statistical analyses were performed using the Vegan package in the software program R! (http://www.r-project.org/). The programmed code for the construction of MDS plots is available online as ESM S4.

Correlation of functional and process data

A correlation coefficient R was calculated to determine the relationship between functional data y (COD, total N, and temperature) and microbial data x (similarity matrix from DGGE gels of *amoA* and bacterial primers) for the aerobic granular sludge system over a time period of 400 days. All similarity indices (chord, Ruzicka) were tested for quantitative and binary data sets. The correlation was calculated by $r(x,y) = \frac{\sum (x-\overline{x})(y-\overline{y})}{\sqrt{\sum (x-\overline{x})^2 (y-\overline{y})^2}}$. Operational data were averaged according to the time elapsed between samples taken for DGGE analysis, and the plots on operational data are given online in ESM S1. To test for statistical significance for each data set, the P value was calculated (Table 2). At a P value of $P \leq 0.05$, a statistically significant correlation exists, whereas a value of $P \leq 0.1$ is reviewed to be of marginal significance, and P values of $P \geq 0.1$, no statistical significant is given (Lipson et al. 2006).

Results

Heatmaps and sequencing

DGGE gels of bacterial 16S DNA gene from aerobic granular sludge (AGS) and activated sludge were aligned for cluster analysis in GelCompar. The data were extracted from GelCompar and were used to present the data in a heatmap by the usage of visual basics (ESM S3). Each band on one height is assumed to represent the same operational taxonomic unit (OTU; Fig. 2). In addition, the same procedure was applied to DGGE gels from functional *amoA* primers. To check for specificity of functional primer sets, bands were picked and sequenced. Sequencing results from selected bands are represented in Table 1 as well as in the *amoA* heatmap (ESM S2). The sequenced bands from DGGE gels clustered in the tree close to reference sequences of *Nitrosomonas* sp. and *Nitrosospira* sp. related bacteria (Fig. 3).

Evenness estimation

The evenness according to Pielou and the Pareto–Lorenz curve was calculated based on banding patterns of 16S DNA DGGE gels from both treatment system (Figs. 4 and 5). Both methods measure evenness and indicate how heterogeneous the value of a measured variable in a sample series is. The evenness according to Pielou is a concentration measurement which will take its maximal value (1) when all species are equally represented (Pielou 1981). The Pielou index of the microbial community is expressed in one value

Table 2 Correlation analysis and corresponding P values between changes in operational data (COD, N, effluent and influent concentrations as well as temperature) and changes in microbial community differences derived from DGGE gels of *amoA* and bacterial primer sets from aerobic granular sludge samples only over a time period of 400 days

Treatment system	Aerobic granular sludge				Conventional sludge			
Primer set	Bacterial		*amoA*		Bacterial		*amoA*	
Parameter	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Influent								
COD (mg/l)	0.02	0.49	−0.18	0.37	0.64	0.08	0.46	0.18
Temperature (°C)	0.07	0.45	0.78	0.03	−0.07	0.45	0.79	0.03
Effluent								
NH_4 removal (%)	−0.12	0.43	−0.59	0.10	n.a	n.a	n.a	n.a
N removal (%)	−0.67	0.11	−0.08	0.44	−0.82	0.02	0.08	0.44

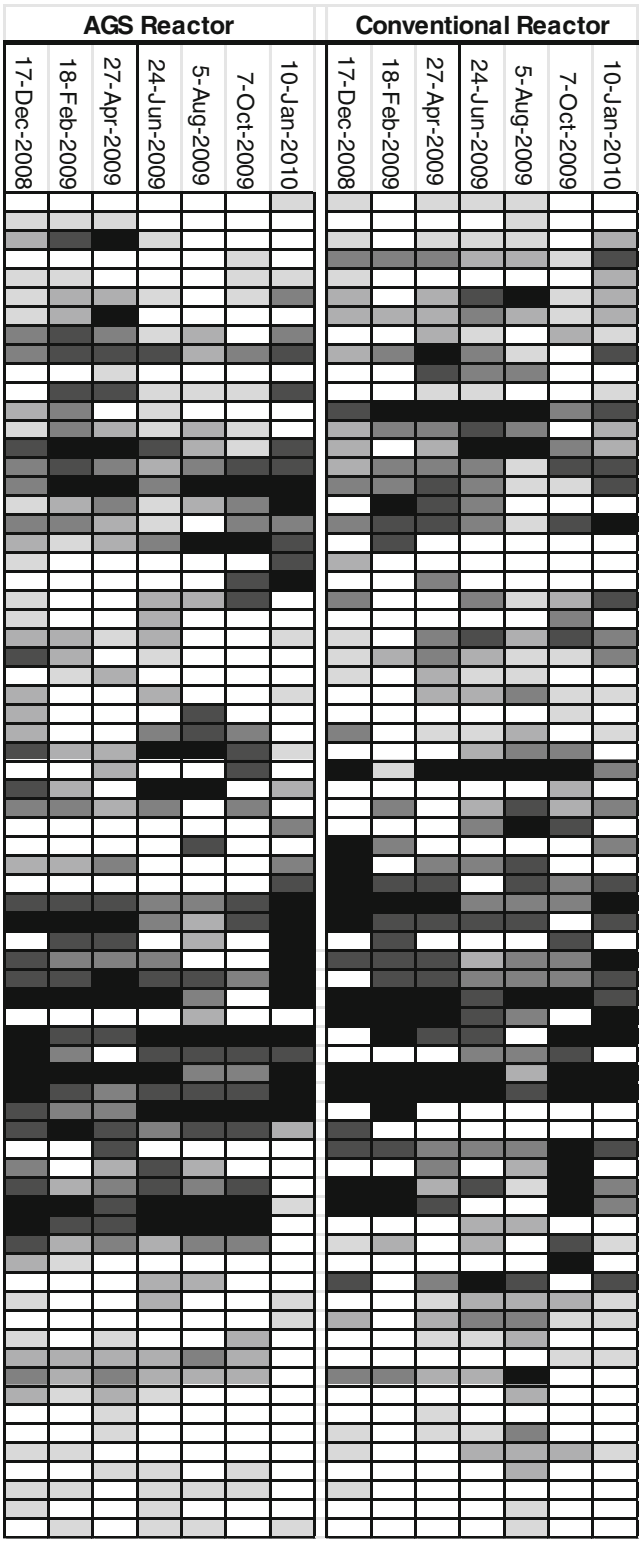

Fig. 2 Heatmaps derived from constructed densitometric curves (GelCompar) of the bacterial 16S DNA DGGE gel of the aerobic granular sludge (*AGS*) system and flocculent sludge treatment system (conventional system) from seven samples taken over a period of 13 months. The *grey scale* corresponds to bins of intervals of relative band intensity

at a certain time point resulting from zero (or maximum) and first-order entropy measurements (Fig. 4). Both systems show similar evenness for the bacterial primer sets. The average number of bands was 42 for the conventional system and 43 for aerobic granular sludge with a standard deviation of 6 for both systems. For both systems, the evenness according to Pareto–Lorenz was also evaluated for *amoA* and bacterial primer sets. The average number of bands for the ammonia-oxidizing bacteria (AOB) community was 14±5 for flocculent sludge system and 9±2 for aerobic granular sludge. The Pareto–Lorenz curve belongs to the category of concentration statistics which shows the relation between the cumulative proportions of band intensities (abundance) and cumulative proportions of number of bands (richness; Fig. 5) (Naeem 2009; Wittebolle et al. 2009). The reference line in the graph corresponds to a perfect evenly distributed community composition. In this case, all bands would have the same abundance/band intensity. Results reflect the same trend as the Pielou index (Fig. 4) and show that the general bacterial community of both systems was more even than the AOB community, in particular in the aerobic granular sludge (Fig. 5).

Time constraint analysis

The Pielou index (Fig. 4) and Pareto–Lorenz curve (Fig. 5) give an indication on how uniform a population distribution is in a certain sample. However, the linkage between the sampling dates is lacking. For instance, if from 1 month to the next the whole population is replaced by other species while their abundance and richness remain comparable, the evenness measurement will not shift. In similarity measurements, which are based on a comparison of community data, the population of one sampling day is compared with the population on the following sampling dates and is hence showing a relation among them. A population of two sampling dates, having everything in common, is identical and has a similarity of 1, whereas a population which does not have anything in common has similarity of zero. Here, we addressed this with the Ruzicka similarity index the question what is changing the community more, either species richness (presence and absence of a band) or species abundance (band intensity). There was not a strong trend observable, but we can report that in the flocculent sludge system, the similarity based on species richness [presence (1); absence (0)] changed in time more significantly than it was the case for the data taking species abundance into consideration, whereas the reverse was true for the granular sludge system (Fig. 6).

Cluster analysis

Clustering analysis for the DGGE band patterns was conducted because it has the advantage of not only showing the

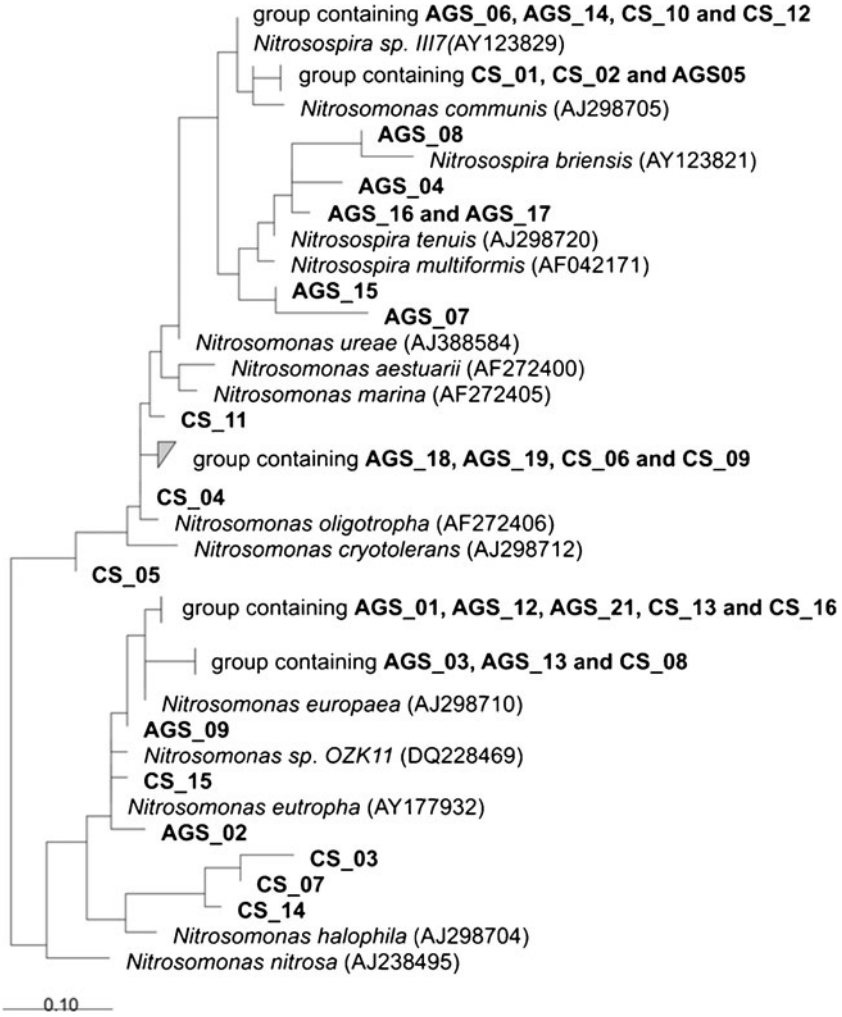

Fig. 3 The tree was constructed using the full *amoA* gene length; the cultured ammonium-oxidizing bacteria were used as references with the addition of unknown sequences (*AGS_#* for granules and *CS_#* for flocs) derived from DGGE gel (heatmaps in additional material). Corresponding accession numbers can be found in Table 1. For the calculation of the tree, the maximum likelihood algorithm (RAxML) was used. A total of 94 amino acid positions were used for analysis. The sequence of *Nitrosococcus oceani ATCC 19707* (AF047705) was used as an outgroup, but was pruned from the tree

relation within a community between one sampling point to the next but also compares the similarity among all samples and clusters them into groups (Fig. 7). Here, we present the results of one dissimilarity measurement (1–similarity) (Ruzicka) and one distance measurement (chord) on both systems. Measurements on populations which have no species in common can have distances that depend on the number and abundance of species. Therefore, distance measurements are not bounded to one or zero, and in chord distance, the highest possible distance will be $\sqrt{2}$ (=1.414). Results revealed that the flocculent and granular systems are very different. They share only a similarity of approximately 30 % (1–0.7) and a distance of 1 (Fig. 7).

Multi-dimensional scaling analysis

In a tree diagram, the populations (bands) from different sampling dates are compared according to their similarity

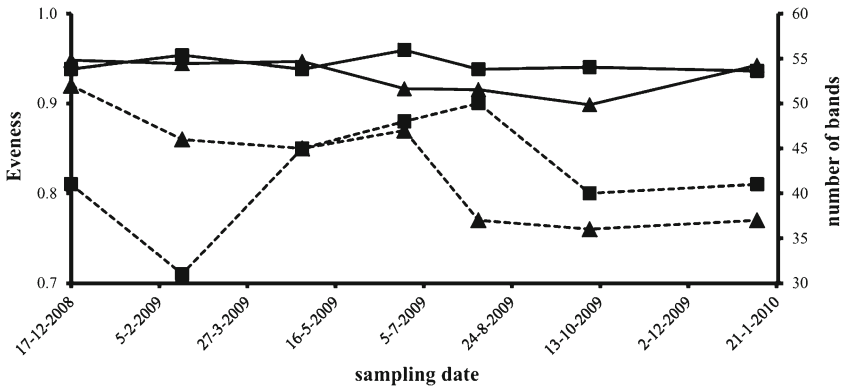

Fig. 4 Profiles of evenness according to Pielou (1981) of general bacterial 16S DNA DGGE gels (*solid line*), the number of bands on the gels (*dashed line*) both for flocculent sludge (*black squares*) and aerobic granular sludge (*black triangles*). Each sampling *point* represents the banding patterns (species) of one lane (sampling day) on the DGGE gel

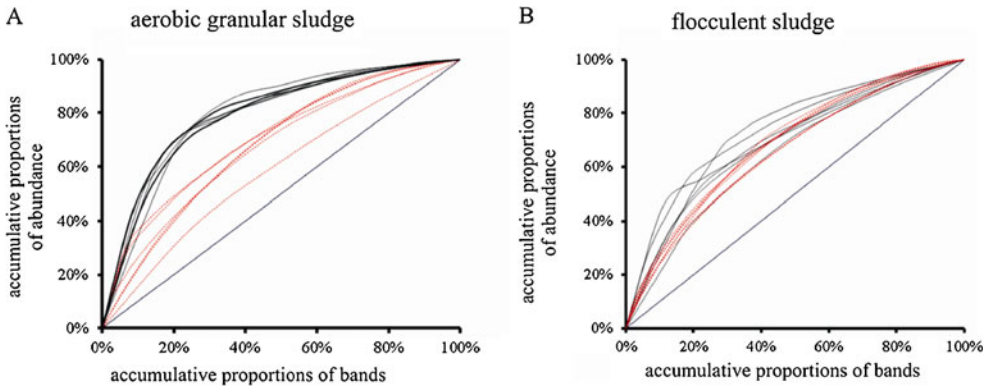

Fig. 5 Relation between the cumulative proportions of band intensities (abundance) and cumulative proportions of number of bands (richness) of **a** aerobic granular sludge and **b** conventional sludge floc from general bacterial 16S DNA DGGE gels (*red dashed lines*) and *amoA* DGGE gels (*black solid lines*); each *line* is for one of the samples in Figs. 2, 3, and 4. The reference *line* in the graph corresponds to a theoretical perfect evenly distributed community composition

and are then clustered together in groups hence showing the relation among different sampling dates (Fig. 7). However, when clustering is applied, some information is lost after certain groups (sampling points) are clusters to each other. This can be overcome with a multi-dimensional scaling analysis. In a multi-dimensional scaling analysis, all objects (species in a population) are ordered in a multivariate graph, with as many axes as there are descriptors (sampling dates). The data representation is only possible in a reduced dimension (2D, two axes) and shows the distance between all objects as well as all descriptors (Legendre and Legendre 1979). It therefore shows the direction in which a population shifts and also shows the distance of the population shift. An eigenvalue analysis was conducted, and more than 50 % of the information from the microbial data set was condensed in dimension 1 and 2 with 42 % variability represented in the first and 14 % in the second dimension, respectively. The same analysis with random numbers spread less than 50 % of the variability on the first four dimensions (dim) with values of 14 % (dim1), 13 %(dim2), 10 %(dim3), and 9 %(dim4). Since the major variability of the microbial data was represented in these first two dimensions, they were used for data interpretation within this study. The multi-dimensional scaling analysis based on Euclidean distance revealed that the microbial populations of the flocculent sludge system moved around the initial population, whereas in the aerobic granular sludge, the bacterial population moved away from its initial populations (Fig. 8).

Correlation analysis

Correlation analysis was conducted to express the degree of relationship between the microbial community composition (x) of a DGGE similarity matrix and operational data (y). For the microbial community composition, all indices (chord and Ruzicka) were tested, and the best fit to operational data gave the binary data from the Ruzicka similarity

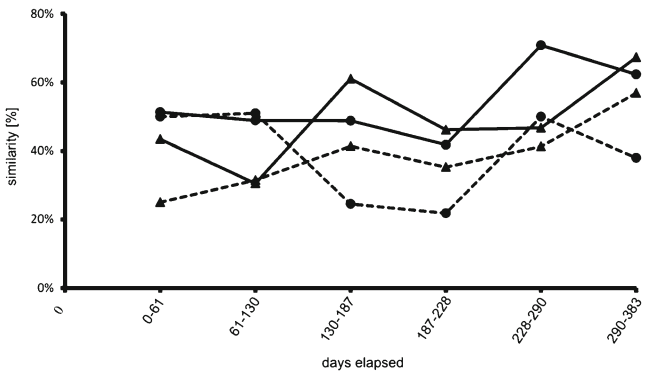

Fig. 6 The times constraint similarity measurement based on the Ruzicka index for binary data (*dashed line*) and quantitative data (*solid line*) from flocculent sludge (*black circles*) and aerobic granular sludge (*black triangles*) 16S DNA DGGE band patterns

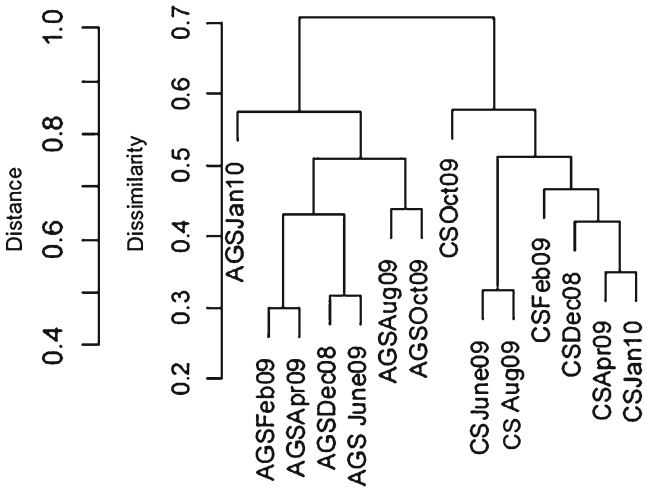

Fig. 7 Tree cluster diagram showing the average Ruzicka dissimilarity (1–similarity) and chord distance between samples of flocculent sludge treatment plant (*CS*) and aerobic granular sludge treatment plant (*AGS*) on quantitative data set (16S DNA DGGE gel) at different time points

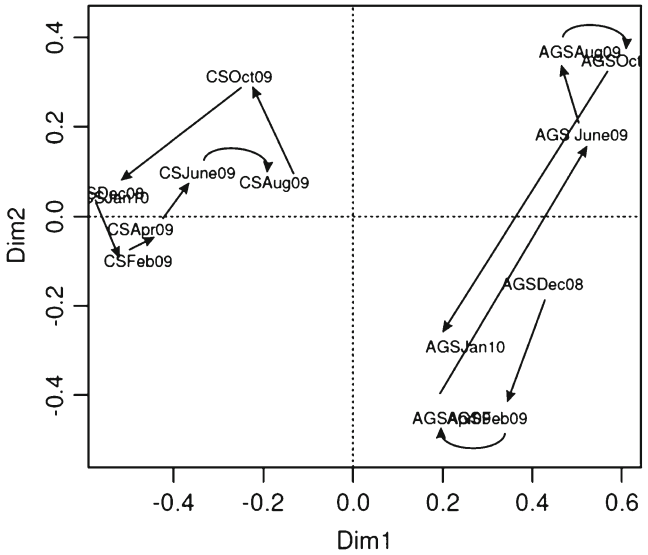

Fig. 8 Multi-dimensional scaling analysis of the distance matrix based on the Euclidean distance matrix of aerobic granular sludge (*AGS*) system (*right*) and the conventional system (*CS*) of the flocculent sludge (*left*). The points (*a*, *c*) represent the centroid of the clusters determined by time-constraint cluster analysis

indices, which are presented in Table 2. The analysis shows to what extent two variables show similar or equivalent information about subjects of these two variables *x* and *y*. The *P* value shows the statistical significance of a correlation. A correlation analysis between functional data and microbial data was conducted for both systems of the entire community as well as the ammonium-oxidizing bacterial population. Results showed that there was no statistically significant correlation between total phosphate and ammonium influent concentrations and microbial community changes (data not shown). None of the fluctuations measured in influent concentrations resulted in a statistically significant correlation to microbial data sets ($P>0.1$) except for COD, which correlated statistical marginal significantly with shifts in bacterial population of flocculent sludge ($r=0.64$, $P=0.08$). The ammonium-oxidizing bacteria showed in both systems a statistically significant positive correlation to temperature shifts (granules, $r=0.78$, $P=0.03$ and flocs $r=0.79$, $P=0.03$) and a negative correlation with fluctuations in ammonium removal efficiencies (granules, $r=-0.59$, $P=0.1$ and flocs n.a). The bacterial population negatively correlated with fluctuations in total nitrogen removal efficiency (granules, $r=-0.67$, $P=0.11$ and flocs $r=-0.82$, $P=0.02$; Table 2).

Discussion

Here, we compared the microbial community changes derived from DGGE banding patterns from an aerobic granular sludge pilot plant in Epe (NL) with the community changes occurring in a full-scale activated sludge plant over a period of 400 days. Despite the fact that both systems are structurally different, it must be emphasized that both systems received the same wastewater (composition and variation in flow rate) and were subjected to the same seasonable variations. The strongest conclusions within this study could be drawn from the similarity tree, the evenness measurements as well as the multi-dimensional scaling analysis. The similarity cluster analysis showed that the community composition within the reactors was very dissimilar and distant (Fig. 7). This can be explained by the very different operational conditions and structural differences of aerobic granular and flocculent sludge technology (Fig. 1). Despite the difference derived from our cluster analysis, general bacteria community assessment showed that both systems had on average highly similar species richness, entropy, and evenness (Figs. 2, 4, and 5), suggesting that in both systems different bacteria had the same functionality. The multi-dimensional scaling clearly showed that the populations of conventional and flocculent sludge were separated in two distinct groups. The microbial populations of the flocculent sludge system moved closely around the initial population, whereas the bacterial population in the aerobic granular sludge moved away from its initial population representing a permanent change (Fig. 8). Beside these clear results, we also conducted more analysis, which yielded less obvious conclusions as it was the case for the similarity tree, the evenness measurements as well as the multi-dimensional scaling analysis.

We addressed the question if species abundance or presence and absence is causing more shifts within the population. Our analysis on binary (presence–absence) and quantitative (abundance) data suggested that community changes in aerobic granular sludge were rather affected by a change in species abundance than due to appearance and disappearance of bacteria, whereas the opposite was true for the flocculent sludge system (Fig. 6). This is also in line with the multi-dimensional scaling analysis and hence shows a different development in time of the microbial communities in both systems which is likely due to the different process characteristics as presented in Fig. 1. In order to test if a subpopulation is distributed in a similar way, a DGGE was run for AOB of both systems. Sequencing results yielded in only AOB strains showing the specificity of used primer sets (Fig. 3). Similar to other research conducted on granular sludge systems, the sequencing results indicated the presence of sequences highly similar with *Nitrosospira* sp. and *Nitrosomonas* sp. (Bassin et al. 2011). In the flocculent sludge system, *Nitrosomonas* sp. and not *Nitrosospira* sp. was the dominant AOB, whereas in the granular system, sequences equally clustered within both AOB types (Fig. 3). *Nitrosospira* has been shown to

be negatively impacted at higher dissolved oxygen concentrations and potentially suppressed by *Nitrosomonas* (Wells et al. 2009), which is in line with other research showing mainly the appearance of *Nitrosospira* in environments low in DO (Park et al. 2002). In the granular sludge, diffusion limitation is more severe, and finding organisms more adapted to low DO environments is therefore not surprising. The AOB population of both systems showed less evenness than the bacterial population (Fig. 4). This can be explained by the fact that nitrifiers in a granule can only grow in the outer oxygen-penetrated layers, which are subjected to more shear stress than bacteria in the inner core (Fig. 1). However, in principle the fluctuations in community composition were behaving very similar to what has been reported in other nitrifying bioreactors (Egli et al. 2003; Falk et al. 2009; Wittebolle et al. 2008), suggesting that a lower evenness of this microbial group might be related to a narrow substrate spectrum (only ammonium) as opposed to a heterotrophic organism (different electron donors and acceptors) (Curtis and Sloan 2006; McGuinness et al. 2006). It must be mentioned that only minor OTUs were unique for either the AGS or the activated sludge. Several studies have attempted to quantify community shifts over time, and they partly correlated these changes to system performance (Cai-Yun et al. 2011; Pholchan et al. 2010). Therefore, one of our attempts was to correlate microbial data with process data. Both processes in this study were reported to run without process disturbances and were removing COD, nitrogen, and phosphate to effluent levels as required by the European standards (European-Water-Framework-Directive 2000). Temporal fluctuations of the influent (nutrient availability and temperature) can alter population growth rates, potentially resulting in shifts in population dynamics driven by for instance species competition or stress adaptation (Müller et al. 2000; Carrero-Colon et al. 2006). The combination of chemical and microbial community data showed a coherent correlation between system performance and community changes. This was indicated by our correlation analysis, which showed a statistically significant ($P<0.05$) positive correlation between temperature shifts and AOB population (Table 2). Since the growth rate of ammonium-oxidizing bacteria is significantly lowered at lower temperatures, a positive correlation to species richness is hence in line with general expectations (Gujer 1977). The correlation between the ammonium removal efficiency and the microbial shifts in the AOB population was with a P value of 0.1 less evident. A weak correlation was measured between the similarity shifts derived from bacterial primer sets and total nitrogen removal efficiency ($r=-0.59$, $P=0.1$). Since AOBs account for ammonium removal and most of general bacteria are capable of denitrification, in both cases, a negative correlation is expected and is hence reported here despite of a P value higher than 0.05.

Here, we demonstrated that statistical and ecological modelling of molecular microbial community data can be used to identify differences between processes with the same functionality. Especially the similarity tree, evenness measurements as well as the multi-dimensional scaling analysis offered clear results showing that statistical analyses are worth to be conducted. Our study gives evidence that statistical and ecological model approaches can couple shifts in microbial communities with process data (Table 2). We have explored many different similarity measures and methodologies, and the choices made here are those we evaluated as the optimal ones.

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