Microbial ecological processes in MBBR biofilms for biological phosphorus removal from wastewater

Knut Rudi, Inger Andrea Goa, Torgeir Saltnes, Gjermund Sørensen, Inga Leena Angell and Sondre Eikås

ABSTRACT

Phosphorus is both a major environmental pollutant and a limiting resource. Although enhanced biological phosphorus removal (EBPR) is used worldwide for phosphorus removal, the standard activated sludge-based EBPR process shows limitations with stability and efficiency. Recently, a new EBPR moving bed biofilm reactor (MBBR) process has been developed at HIAS (Hamar, Norway), enabling a phosphorus removal stability above 90% during a whole year cycle. To increase the knowledge of the HIAS (MBBR) process the aim of the current work was to characterize the MBBR microbiota and operational performance weekly for the operational year. Surprisingly, we found a major succession of the microbiota, with a five-fold increase in phosphorus accumulating organisms (PAOs), and major shifts in eukaryote composition, despite a stable phosphorus removal. Temperature was the only factor that significantly affected both phosphorus removal and the microbiota. There was a lower phosphor removal during the winter, coinciding with a higher microbiota alpha diversity, and a lower beta diversity. This differs from what is observed for activated sludge based EBPR. Taken together, the knowledge gained from the current microbiota study supports the efficiency and stability of MBBR-based systems, and that knowledge from activated sludge-based EBPR approaches cannot be translated to MBBR systems.

Key words | MBBR, microbiota, phosphorus, rRNA, wastewater

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INTRODUCTION

Phosphorus is an essential and limiting macronutrient in agriculture that is critical for maintaining a secure food supply for the growing human population (Amundson *et al.* 2015). Phosphorus lost to the environment represents both a major eutrophication problem and a challenge since phosphorus is already a limiting resource in which about 86% of the phosphorus used in agriculture is lost (Rittmann *et al.* 2011). Traditionally, chemical precipitation has been used to remove phosphorus from wastewater; unfortunately, this results in a low bioavailability for phosphorus. Therefore, new biological approaches that both remove and recover phosphorus from wastewater are urgently needed. (Stratful *et al.* 1999; Gilbert 2009).

A major hurdle for a stable and efficient biological phosphorous removal from wastewater is that the process requires oscillation of microorganisms between aerobic and anaerobic zones, since phosphorus accumulating organisms (PAOs) accumulate phosphorus aerobically and release it anaerobically (Srinath *et al.* 1959). Unfortunately, standard activated sludge-based EBPR approaches are not very efficient or stable, and require additional chemical precipitation (Coats *et al.* 2017). Moving bed biofilms (MBBR)-based approaches, on the other hand, offer the possibility of low process volume, are cost efficient and give a stable phosphorus removal (Helness & Ødegaard 1999). This is the reason why the Hamar municipality wastewater treatment plant (HIAS, Hamar, Norway) has developed a new MBBR process (HIAS process) for biological phosphorus removal.

The HIAS process utilizes a novel active transport of biofilm carriers between aerobic and anaerobic zones, as schematically outlined in Figure 1. The process has demonstrated better performance compared to other activated sludge-based EBPR processes. However, the microbiota composition associated with the HIAS process has not yet been determined. Without this knowledge, the process will

doi: 10.2166/wst.2019.149

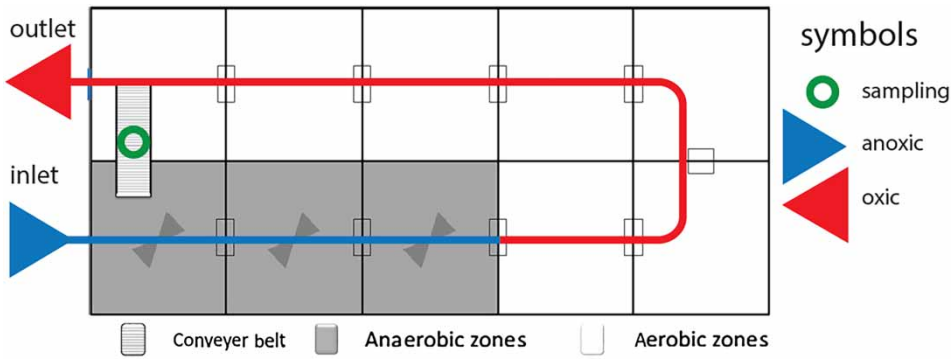

Figure 1 | Schematic presentation of the Hias process. First, the water flows into the anaerobic (grey) zone with 60% biofilm content. Secondly, the biofilms flow with the water flow into the aerobic (white) zone. Finally, the treated water flows out of the plant and the biofilms are transported mechanically out of the water and back into the anaerobic zone (Saltnes *et al.* 2017).

be hard to control and never be able to reach its full potential (Saltnes *et al.* 2017). Although it is known that factors such as temperature (Ong *et al.* 2014) and organic carbon load influence phosphorus removal, the association between how these factors affect the microbiota function and composition and thus phosphorus removal is not well understood (Coats *et al.* 2017). Furthermore, how eukaryotes affect the biofilm composition and function remains largely unexplored (Angell *et al.* 2017).

The aim of our work was therefore to determine the HIAS process temporal shift in both phosphorus removal and microbiota composition/quantity through a whole year cycle. This was done by determining the eukaryote/prokaryote composition/quantity in relation to phosphorus load/removal, organic carbon load/removal and temperature.

METHODS

Chemical measurements

The wastewater in the HIAS process plants were analyzed in parallel with biofilm carriers for correlation with the biofilm microbiota composition. The samples for chemical analyses were filtered through 1.2 μm fiberglass filter and analyzed for dissolved phosphorus and soluble chemical oxygen demand (SCOD) with a NOVA spectroquant 60 spectrometer (Merck). In addition, the inlet volume and the temperature of the wastewater was recorded for correlation analyses.

Sampling of biofilms

The biofilms were stabilized for seven months, with the phosphorus removal being stable for about four months prior to the sampling. The HIAS MBBR biofilms were collected approximately once a week from 27.10.16 to 06.09.17, and immediately stored at −20 °C before further processing. The material collection took place at the end of the aerobic zones (outlined Figure 1).

Cell lysis and DNA extraction

The biofilms were cut to fit 2 mL test tubes (Sarstedt, Germany) containing glass beads (Sigma Aldrich, Norway, 0.25 g < 106 μM) and 500 mL STAR buffer (Roche, Germany). The samples were mechanically disrupted 2 × 40 s in FastPrep96 (MP Biomedicals, USA) at 1,800 rpm with 5 min break between the runs. DNA was isolated by the KingFisher flex robot (Thermo Fisher Scientific, USA) using the MagMidi LGC kit (LGC Genomics, UK) with 50 μL lysate, as previously described (Angell *et al.* 2017).

Illumina sequencing

The sequencing was done as previously described with 16S and 18S rRNA genes as targets (Angell *et al.* 2017). The sequences went through pre-processing which involved demultiplexing, truncating primers and quality filtering using QIIME (Caporaso *et al.* 2010). The following step involved operational taxonomic unit (OTU) processing using USEARCH v8 (Edgar 2010), where the OTUs were first clustered at a 97% homology level creating an OTU-table, and then the OTUs were taxonomically assigned using the SILVA database (Quast *et al.* 2013). The composition of functional bacteria was determined by comparing the EBPR and MiDAS databases, with functions being assigned based on taxonomic matches to the OTUs.

Data analyses

Basic statistical analyses were done using MINITAB 18 (MINITAB Inc.). Multivariate and ecological analyses were done in the Matlab programming environment, with PLS Toolbox (Eigenvector Inc.) for multivariate analyses and the Fathom toolbox for ecological analyses (www.marine.usf.edu/user/djones/matlab/matlab.html). Graphical visualizations were created using Illustrator CC 2015 (Adobe).

Data deposition

Raw reads from the prokaryote and eukaryote SSU gene sequencing are available in the Sequence Read Archive (SRA) database with the accession number PRJNA513239.

RESULTS AND DISCUSSION

Wastewater treatment efficiency

For the whole experimental period, the average phosphorus removal efficiency was $94 \pm 0.5\%$ [mean ± std], and SCOD removal $66 \pm 0.07\%$ [mean ± std]. Surprisingly, we found no association between phosphorus or SCOD load and the respective removal efficiencies (results not shown). Temperature, however, was positively correlated with both phosphorus and SCOD removal (Pearson correlation = 0.49, $p = 0.001$). A summary of the operational characteristics for the MBBR pilot plant is presented in Supplementary Table 1 (available with the online version of this paper).

Microbiota

A total of 4,406,897 16S rRNA sequences satisfied the quality filtering criteria, being represented by 1,678 OTUs. For comparative analyses 9,000 sequences were randomly chosen from each sample. The final rarefied 16S rRNA gene OTU-table contained 113 samples, with triplicate or duplicate samples from 40 sampling time points. For 18S rRNA we obtain 2,931,910 sequences representing a total of 835 OTUs. The OTU-table was processed with 2,000 randomly chosen sequences, with the final OTU-table containing 99 samples from 38 time-points.

The prokaryotes were mainly composed of Proteobacteria, with the phosphorus accumulating Beta proteobacterium *Candidatus accumulibacter* being the most dominant (Figure 2(a)). For eukaryotes there was a clear dominance of *Rhogostoma*, belonging to the structure-activity relationship (SAR) cluster (Figure 2(b)). The temporal changes were much larger for the eukaryotes than prokaryotes (Figure 3), with *Rhogostoma* showing a clear dominance from week 25 and onwards (Figure 3(b)). For the prokaryotes there was a steady increase of *Cantidatus* Accumulibacter throughout the whole experimental period (Figure 3(a)). The quantity of both prokaryotes and eukaryotes also increased during the experimental period, with a leap in prokaryotes corresponding with the increase in *Rhogostoma* (Figure 3). For the prokaryote functional associations both the PAOs and glycogen accumulating organisms (GAOs) increased through the year cycle, while fermenters decreased (Figure 4(e)), with the ratio between PAOs and GAOs being constant at 33.9 ± 11.2 [mean ± std]. Unfortunately, for eukaryotes, we do not have sufficient knowledge for reasonable functional assignments.

In addition to the time related succession, the bacterial composition showed a clear temperature association (Figure 4(a)). *Chrysobacterium* was associated with low temperature, while *Flavobacteriales* was associated with both high and low temperatures (Figure 4(c)). Alpha and beta diversity showed apparent opposite trends, where high alpha diversity was associated with low beta diversity (Figure 4(d)).

Associations of microbiota function with wastewater treatment efficiency

We finally compared wastewater treatment efficiency with functional characteristic of the prokaryote microbiota. These comparisons showed that temperature was central, as it was related to treatment efficiency and composition, phosphorus and SCOD removal. Alpha diversity was positively correlated, while beta diversity was negatively correlated (Figure 5).

Difference between MBBR and activated sludge

The current EBPR microbiota knowledge has mainly been derived from activated sludge (Yang *et al.* 2017) in which the microbiota composition is largely determined by co-diversification processes (Leventhal *et al.* 2018). For MBBR, on the other hand, we found co-diversification low, with biofilms sharing more of the same bacteria (decreased beta diversity) with increasing biofilm diversity (increased alpha diversity). Furthermore, both the positive correlation between GAOs and PAOs and the positive association between temperature, phosphorus and SCOD removal for our MBBR were opposite from that expected for activated sludge (Erdal *et al.* 2003). It has been suggested that GAOs have a competitive advantage over PAOs at high temperature due to growth rate (Seviour *et al.* 2003). Since growth rates in biofilms are intrinsically slow, high growth rate potential will

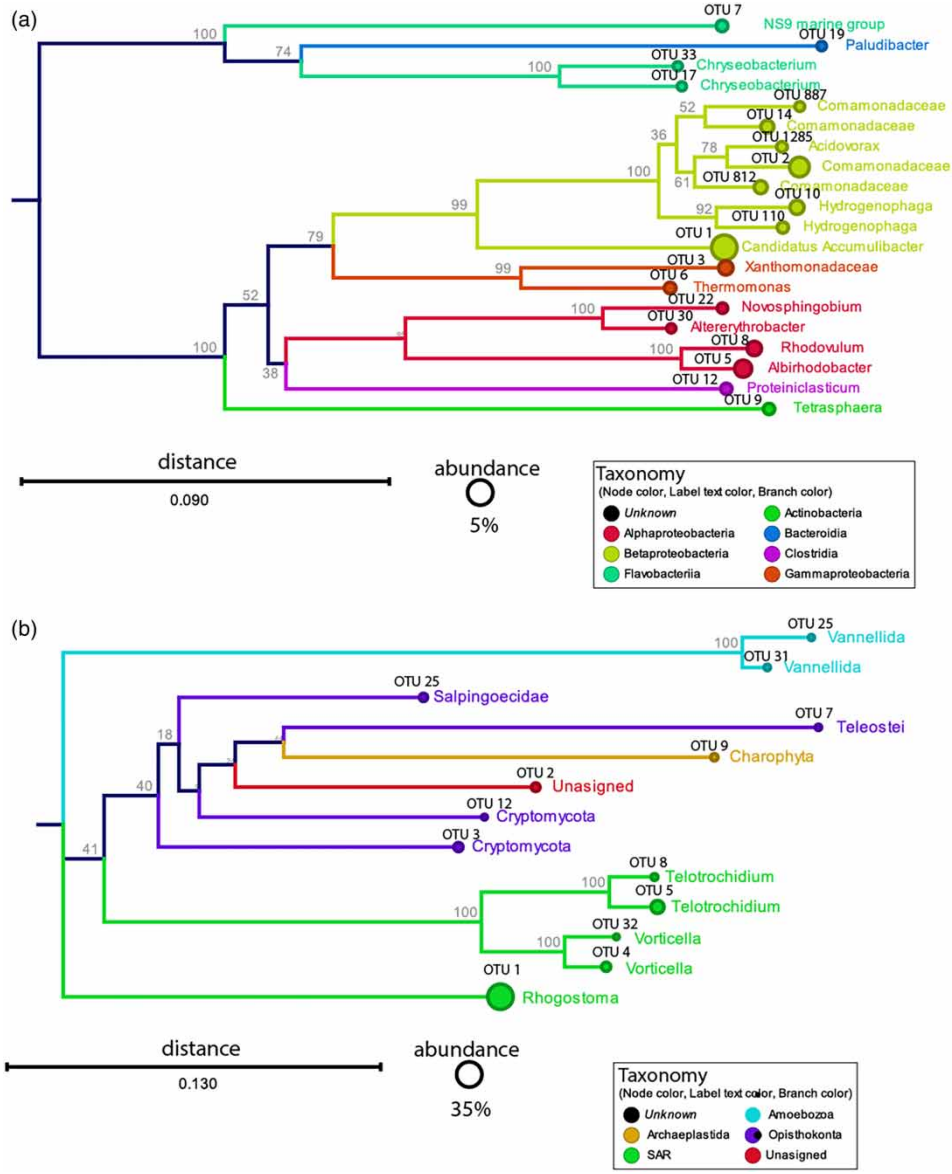

Figure 2 | Phylogeny of the dominating OUTs for prokaryotes (a) and eukaryotes (b). The OTUs with an average abundance >1% are included. The phylogenetic trees were made using the neighbor joining algorithm with Kimura 80 distances. The numbers at the nodes indicate bootstrap support based on 500 replicates.

not be an advantage in biofilms (Flemming *et al.* 2016), potentially explaining why GAOs do not have an advantage at high temperature in MBBR biofilms.

Lack of association between PAO and phosphorus removal

The lack of association between PAO level and phosphorus removal, however, was similar for activated sludge and MBBR (Coats *et al.* 2017). The potential explanation could be that the PAO phosphorus uptake potential greatly exceeds the actual phosphorus levels (Saltnes *et al.* 2017). The apparent excess of PAOs in mature biofilms could also help to explain the stable MBBR phosphorus removal, despite fluctuating phosphorus levels in wastewater. Unfortunately, in the literature there is very little information on the stability, energy storage and development of mature biofilms in fluctuating environments (Flemming *et al.* 2016).

Influence of eukaryotes

Eukaryotes are also commonly ignored in microbiota studies (Flemming *et al.* 2016). Here, we showed that a

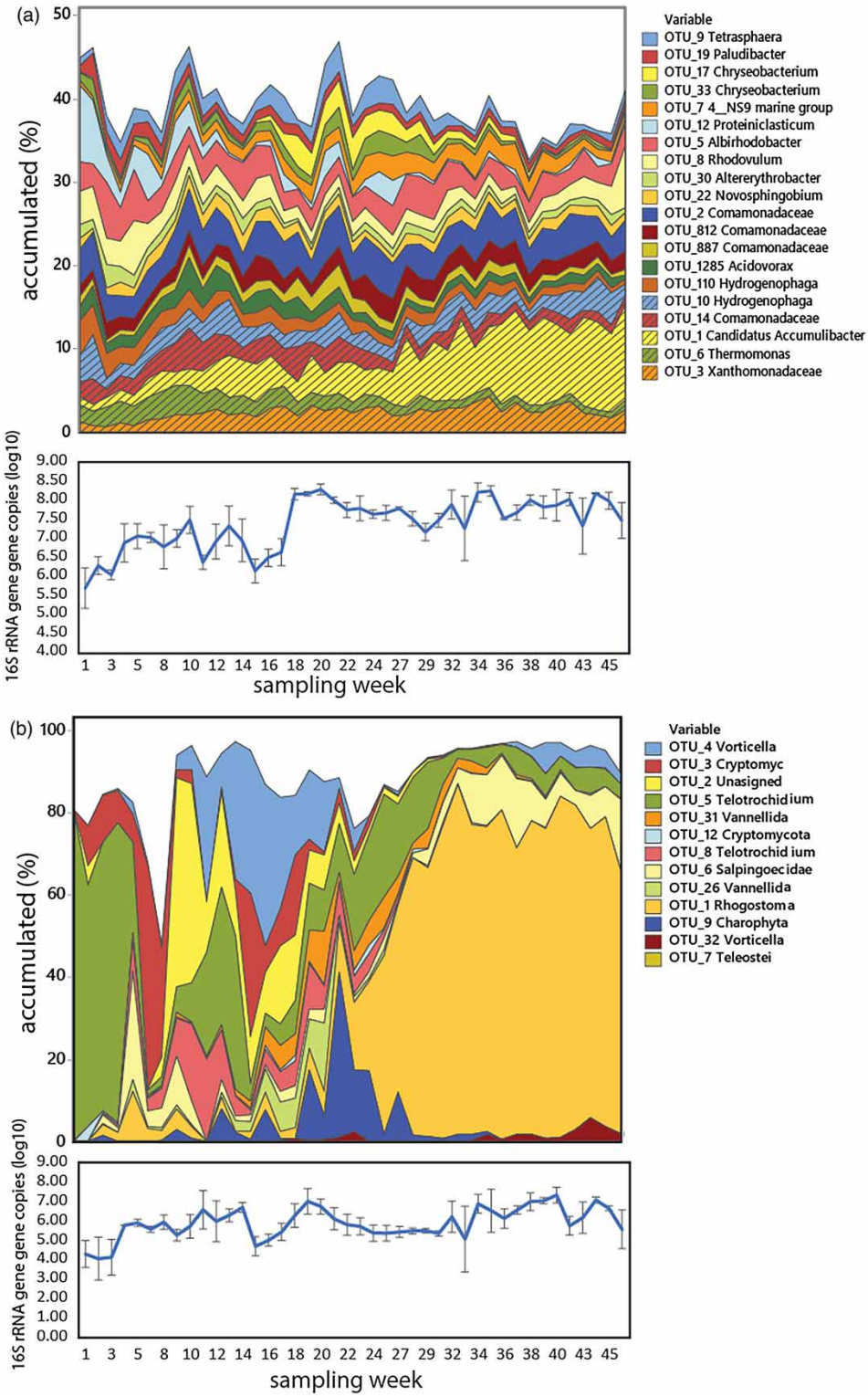

Figure 3 | Temporal development of biofilm composition and quantity for (a) prokaryotes and (b) eukaryotes. The temporal development is shown for the OTUs present on average in >1% of all the samples. The quantitative information is represented by the mean and standard deviation from three independent bio-carriers.

shift in eukaryote composition corresponded to an increase in bacterial load, but not in composition or function. Several eukaryotes are also known phosphorus accumulators (Yang *et al.* 2017), but these were not observed in our

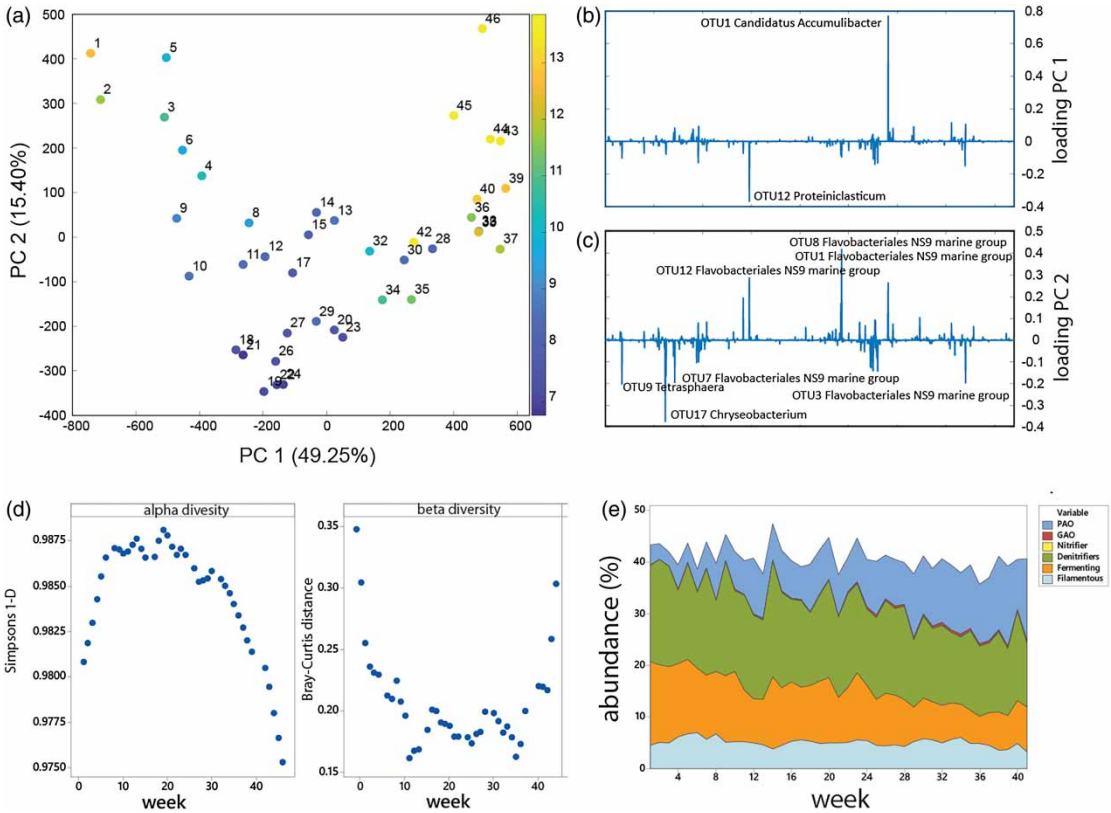

Figure 4 | Prokaryote biofilm composition and function. (a) PCA score plot for the average weekly microbiota (week is marked with number), with the corresponding temperature indicated with a color code. (b) and (c) Loading plots for the two first principal components, respectively. (d) Alpha and beta diversity associated with temporal development and (e) temporal development of functional groups. Please refer to the online version of this paper to see this figure in color: http://dx.doi.org/10.2166/wst.2019.149.

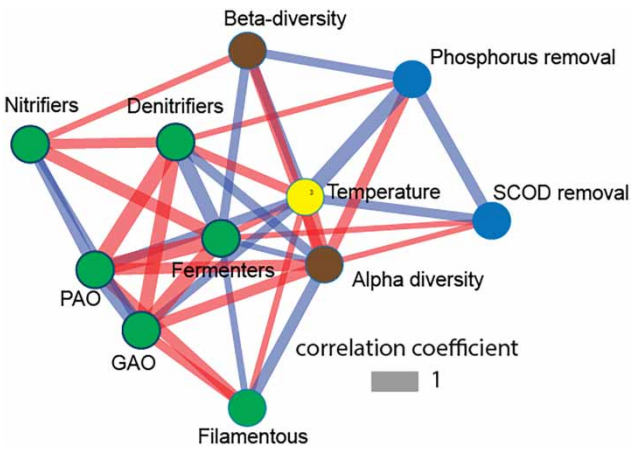

Figure 5 | Associations between functional groups, diversity, temperature and treatment efficiency. Associations with Spearman correlation p-value <0.05 are shown. The thickness of the lines represent the strength of the correlation coefficient, while the color reflects the direction, with blue lines representing positive correlations and red lines negative correlations. The color code for the spheres are the following; green – functional groups, brown – diversity measures, blue – chemical parameters, and yellow – temperature. Please refer to the online version of this paper to see this figure in color: http://dx.doi.org/10.2166/wst.2019.149.

study. We have previously proposed eukaryotes as a diversifying factor for the prokaryote microbiota composition under aerobic conditions (Angell *et al.* 2017). Whether this is the case for MBBR based EBPR, with cycling between aerobic and anaerobic zones, remains unknown.

CONCLUSION

In conclusion, we found a major increase in PAOs during the year cycle despite a stable phosphor removal. Temperature was the main factor affecting the microbiota composition and phosphor removal. Beta diversity showed a negative association and alpha diversity a positive association with low temperature during the winter, coinciding with lower phosphor removal.

ACKNOWLEDGEMENTS

We wish to thank NMBU and HIAS for their financial support.

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First received 9 January 2019; accepted in revised form 12 April 2019. Available online 23 April 2019