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Seasonal bacterial community dynamics in a full-scale enhanced biological phosphorus removal plant

Jason J. Flowers^{a,b,*}, Tracey A. Cadkin^b, and Katherine D. McMahon^{b,c}

^a Department of Civil and Environmental Engineering, University of Washington, Seattle, WA 98105, USA

^b Department of Civil and Environmental Engineering, University of Wisconsin–Madison, Madison, WI 53706, USA

^c Department of Bacteriology, University of Wisconsin–Madison, Madison, WI 53706, USA

Abstract

Activated sludge is one of the most abundant and effective wastewater treatment process used to treat wastewater, and has been used in developed countries for nearly a century. In all that time, several hundreds of studies have explored the bacterial communities responsible for treatment, but most studies were based on a handful of samples and did not consider temporal dynamics. In this study, we used the DNA fingerprinting technique called automated ribosomal intergenic spacer region analysis (ARISA) to study bacterial community dynamics over a two-year period in two different treatment trains. We also used quantitative PCR to measure the variation of five phylogenetically-defined clades within the *Accumulibacter* lineage, which is a model polyphosphate accumulating organism. The total bacterial community exhibited seasonal patterns of change reminiscent of those observed in lakes and oceans. Surprisingly, all five *Accumulibacter* clades were present throughout the study, and the total *Accumulibacter* community was relatively stable. However, the abundance of each clade did fluctuate through time. Clade IIA dynamics correlated positively with temperature ($\rho = 0.65$, $p < 0.05$) while Clade IA dynamics correlated negatively with temperature ($\rho = -0.35$, $p < 0.05$). This relationship with temperature hints at the mechanisms that may be driving the seasonal patterns in overall bacterial community dynamics and provides further evidence for ecological differentiation among clades within the *Accumulibacter* lineage. This work provides a valuable baseline for activated sludge bacterial community variation.

Keywords

Enhanced biological phosphorus removal; “*Candidatus Accumulibacter phosphatis*”; Activated sludge

*Corresponding author. Civil and Environmental Engineering, University of Washington, Seattle, WA 98105, USA. Tel.: +1 206 543 5454. jflowers002@gmail.com (J.J. Flowers)..

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2013.07.054>.

1. Introduction

Environmental engineers studying activated sludge systems have long investigated the activity and composition of bacterial communities that comprise activated sludge, initially using culture-based methods and more recently using molecular-based tools (Kaewpipat and Grady, 2002; Seviour and Nielsen, 2010; Wagner et al., 2002). Several of these studies have drawn broad conclusions using single or a few samples collected from wastewater treatment plants (WWTPs) (Kwon et al., 2010; Snaird et al., 1997). While such studies have identified several significant bacterial community members, they provide a somewhat myopic view of activated sludge bacterial community composition (BCC) because they do not capture variation in community structure over time scales of months to years. Investigations into the bacterial community dynamics in lakes (Shade et al., 2007) and oceans (Fuhrman et al., 2006) across several years have revealed within-year community shifts that exhibit a repeated and predictable seasonal pattern. Thus, we might expect similar patterns to occur in activated sludge communities.

A few researchers have performed long term studies of the bacterial community in activated sludge (Dabert et al., 2001; Frigon et al., 2006; Gilbride et al., 2006; Victorio et al., 1996), but they either did not track the community with fine enough temporal resolution for longer than a few months or they changed operational conditions in the middle of the study, which limited their conclusions about community dynamics occurring at WWTPs under normal, unperturbed conditions. A recent study conducted using fluorescent in situ hybridization (FISH) suggested that polyphosphate accumulating organism (PAO) associated with enhanced biological phosphorus removal (EBPR) in activated sludge were surprisingly stable over three years in a set of 28 Danish WWTPs sampled roughly quarterly (Mielczarek et al., 2013). Wang et al. (2010) and Wells et al. (2011) each explored bacterial community dynamics in full-scale activated sludge WWTPs over the course of one year by taking regular samples every 15 or 7 days, respectively. Using Terminal-Restriction Fragment Length Polymorphism (T-RFLP), they both found that while the system performed stably throughout the study, the bacterial community was more dynamic than expected. While their dataset did provide some insight into patterns of community dynamics, their studies only lasted one year, making it difficult to conclude if activated sludge communities change in similar ways each year.

EBPR is an effective process to remove phosphorus from wastewater by sequestering excessive amounts of phosphorus inside bacteria as polyphosphate. While the process is used extensively, it has been shown to have periodic upsets (Neethling et al., 2005). A better understanding of the communities important for this process and how they are affected by the dynamic environmental conditions experienced in WWTPs should enable more rational design and operation, leading to greater stability. While several types of bacteria are known to be important in EBPR (Beer et al., 2006; Kong et al., 2005; Nielsen et al., 2011), *Candidatus Accumulibacter phosphatis* (hereafter referred to as Accumulibacter) is a model organism for the process because it has been detected in high abundance in full-scale WWTPs (Zilles et al., 2002) and it is easily enriched for in lab-scale bioreactors that simulate EBPR performance (McMahon et al., 2010). The Accumulibacter lineage can be divided into two main “Types” and further into several distinct clades within these Types,

using the polyphosphate kinase 1 (*ppk1*) locus (Peterson et al., 2008). Recent work exploring the genomic differences between Accumulibacter Clade IA and IIA that were enriched in lab-scale EBPR bioreactors revealed that the genome compositions of these two clades were quite distinct including apparent differences in phage resistance, but they did still contain the essential functions for EBPR metabolism (Flowers et al., 2013). Eco-physiology based studies of different Accumulibacter populations growing in lab-scale EBPR bioreactors have suggested different nitrate reduction capabilities among Accumulibacter clades (Carvalho et al., 2007; Flowers et al., 2009). He et al. (2007) explored the Accumulibacter distribution in several full-scale WWTPs in the United States (in single time-point samples), and observed differential distributions of the clades. Some plants, such as the Nine Springs WWTP in Madison, WI, contained a roughly even representation of the five clades, while others were dominated by only one or a few clades. Similarly, Mielczarek et al. (2013) used qualitative *ppk1*-PCR on a single time point to better resolve the Accumulibacter community detected using FISH in the same 28 Danish WWTPs discussed above. Accumulibacter Clade IA and IIC were found in nearly all the plants while Clade IIA and Clade IID were found in only a few plants. In both studies, however, the phylogenetically resolved analysis was conducted on samples collected only on a single day, and no information was available regarding the variability in Accumulibacter clade abundances over time. Thus, it is not currently possible to draw conclusions about “representative” Accumulibacter clade composition in any particular WWTP.

Because several key WWTP design factors including sludge biomass yield and specific substrate utilization rates can vary even among closely related bacteria, community dynamics could influence WWTP performance. The aim of this study was to explore activated sludge BCC dynamics over a two-year period at a full scale WWTP. We used a bacterial community fingerprinting technique called automated ribosomal inter-genic spacer region analysis (ARISA) to quantify temporal dynamics in two different treatment trains of an activated sludge process during a two year period at the Nine Springs WWTP in Madison, WI, USA. By exploring the population dynamics over the two year period, we were able to observe repeating patterns of community change. In addition, we measured the abundance of Accumulibacter clades in a subset of these samples using clade-specific qPCR primers to estimate the range of variability normally found within the Accumulibacter lineage.

2. Materials and methods

2.1. Wastewater treatment plant characteristics and sampling information

The Nine Springs WWTP (Madison, WI, USA, 43°2'9"N 89°21'28"W) treats 159,000 cubic meters of wastewater per day. The plant configuration includes two separate primary and secondary treatment trains classified as the East and West Trains. The East Train contains two Anaerobic/Oxic (A/O) and four University of Cape Town without nitrate recycle (UCT) activated sludge tanks. The return sludge from the six tanks in the East Train are mixed before returning to the head of the aeration tanks. The West Train contains only four UCT activated sludge tanks.

A total of 144 activated sludge samples were collected over two years (approximately every 1.5 weeks) from the two different treatment trains at the Nine Springs WWTP (Table S1). Seventy samples were collected from the West Train and 74 were collected from the East Train. Samples from the East Train were collected from one of the A/O tanks in the system. Samples from both trains were collected from the same location at the end of the aerobic zone for each date, transported to the lab, centrifuged, decanted, and stored in the -80°C freezer within 2 h of sampling. Plant performance data including total phosphorus, total Kjeldhal nitrogen, ammonia, and BOD₅ was provided by the Nine Spring WWTP plant personnel. Total phosphorus was analyzed using EPA Method 365.4 (US-EPA, 1983). Ammonia and Total Kjeldhal Nitrogen was analyzed by EPA Method 350.1 and 351.2, respectively (US-EPA, 1993). BOD₅ was determined by Standard Methods 5210 A&B (APHA, 1999).

2.2. DNA extraction and ARISA analysis

DNA was extracted from previously frozen biomass using the PowerSoil DNA Extraction Kit (MoBio, Carlsbad, CA) following the manufacturer's instructions. The BCC was analyzed from the extracted DNA samples using ARISA as previously described (Jones and McMahon, 2009). Briefly, we used a fluorescently labeled forward primer targeting the bacterial 16S rRNA gene (1406f: 5'-TGYACACACCGCCCGT-3', 5' labeled with the phosphoramidite dye 6-FAM) and an unlabeled reverse primer targeting the 23S rRNA gene (23Sr: 5'-GGGTTBCCCCATTCRG-3') for PCR to amplify intergenic spacer regions from the entire bacterial community (Jones and McMahon, 2009). Duplicate PCR reactions were performed for each sample and each DNA amplification reaction was performed with approximately 10 ng of template extracted DNA. The amplified products were separated by capillary electrophoresis on a 3730xL ABI analyzer at the University of Wisconsin–Madison Biotechnology Center. An internal ROX-labeled size standard (BioVentures, Inc, Murfreesboro, TN) was used to determine the size of the resulting fragments in each sample. GeneMarker (SoftGenetics, State College, PA) was used to visually quality control each fingerprint. After quality control of each fingerprint, ARISA fragment bins were identified using aligned ARISA profiles. To identify the presence and magnitude of every ARISA fragment in each sample, all peak heights above the mean by two standard deviations were determined to be “signal” and removed from the analysis. Then a new mean height was determined from the remaining pool of peaks, and all peaks that were two standard deviations above this new mean were determined to be “signal” and removed from the analysis. This iteration continued until no peaks were found to be greater than two standard deviations above the mean, and therefore the remaining peaks were considered noise (Jones and McMahon, 2009). For all samples, the relative peak height was calculated for every peak determined to be “signal”. To minimize variations in PCR, ARISA PCR was performed in replicate for each sample, and the relative peak height was averaged between replicated samples prior to further analysis. In addition, three separate biomass samples were collected as complete biological replicates on the same day and processed to evaluate variation due to sampling and methodology (i.e. DNA extraction, PCR, electrophoresis, and profile analysis).

2.3. Quantification of *Accumulibacter* clade-specific *ppk1* gene abundance

We analyzed the abundance of five *Accumulibacter* clades for both trains using qPCR targeting the *ppk1* gene, in 35 samples collected between September 2006 and March 2008. To prepare *ppk1* clones for controls to use with qPCR, partial *ppk1* gene amplicons (105–408 bp in length, depending on clade) from several EBPR full-scale WWTPs (He et al., 2007) were cloned with the Topo TA Cloning Kit (Invitrogen, Carlsbad, CA). These clones were phylogenetically analyzed to assign clade identification and used in qPCR analysis (He et al., 2007). Clones from –80 °C storage were re-grown in LB with 50 µg mL⁻¹ Kanamycin before five mL of solution was centrifuged to collect biomass. Plasmids containing the desired *ppk1* gene insert were extracted using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Plasmids were then linearized with the ScaI restriction enzyme (Promega, Madison, WI) before purification with the AxyPrep PCR Cleanup Kit (Axygen, Union City, CA). One modification was made to the AxyPrep PCR Cleanup Kit: the final, linearized plasmid was eluted in 1X TE. The purified plasmid DNA concentrations were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) in order to calculate gene copy number mL⁻¹ to be used as standards for qPCR. Samples were stored undiluted in a –20 °C freezer until use.

To detect the *Accumulibacter* clades present in the Nine Springs WWTP, the qPCR procedures developed by He et al. (2007) were used with the following changes: the qPCR annealing temperature for the clade IIA reaction was changed from 61 °C to 63 °C, the primer concentration for Clade IID was reduced to 250 nM from 400 nM, and the betaine concentration for Clade IID was reduced from 0.7 M to 0.5 M (Table 1). Briefly, 2X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was mixed with primers, betaine, water, and sample for a 25 µL total reaction volume. Five ng of genomic DNA was added to each sample reaction. Positive controls were used to generate a standard curve by serially diluting each positive control in 1X TE between 10³ and 10⁸ gene copies per reaction. One negative control, which was a representative clone from another clade with the fewest mismatches to the primer set (between one to four mismatches) was used to ensure specificity (10⁶ gene copies per reaction) (He et al., 2007), while water blanks were used to ensure there was no contamination. Technical replicates were analyzed to ensure more accurate quantifications. Each standard curve was duplicated within each 96-well PCR plate (plate). Samples were replicated across two 96-well plates, but not within them based on our previous work with qPCR that indicated that the highest amount of inter-reaction variability was introduced across plates, and that within-plate variation among replicates was generally less than 5% (data not shown).

When more than one abnormal peak was seen in the qPCR melt curve and could not be attributed to small amounts of negative control amplification, gel electrophoresis of the sample was performed to verify a single band in each sample. In many cases, there was some amplification of the negative control; however, the negative amplified below detection on the standard curve and sample averages were near 10⁶ gene copies per reaction. Therefore, it was concluded that this low amount of amplification seen in the negative control had no impact on the quantification values.

Quantification of *ppk1* gene copy number per reaction from cycle threshold values was performed with iCycler iQ Optical System Software Version 3.0a. In order to make these data comparable to previous studies, the relative fluorescence threshold was set to 50 (He et al., 2007). Quantification values were relativized to one-mL mixed liquor suspended solid (MLSS). PCR efficiency was calculated from the standard curve using equation (1).

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1 \quad (1)$$

Results are reported only for those reactions with efficiencies between 90 and 110%.

One deviation from the methods of He et al. (2007) was the use of the primer set that targets Clade IIC to the exclusion of clone NS D3 (GenBank accession number EF559355). Previous analyses on Nine Springs sludge samples based on *ppk1* genes identified an operational taxonomic unit (OTU) named NS D3. Phylogenetically, this OTU is most closely affiliated with Clade IIC, but does not cluster as closely to other taxa in Clade IIC. This outlier makes it difficult to use qPCR primers to target all of Clade IIC; therefore, two primer sets were designed previously for IIC: one that includes OTU NS D3 and one that excludes NS D3. Due to difficulties with non-specific amplification, only the primers targeting Clade IIC to the exclusion of NS D3 were used.

We also analyzed four biological replicates on a single sampling date, to further evaluate sources of variation in our overall qPCR assay. We found that calculated clade abundance varied by up to 20% around the mean, and therefore only consider variation greater than 20% of the mean to be true measurable variation in clade abundance.

2.4. Statistical analysis

To assess the differences between communities from the ARISA analysis, the relative peak heights for each ARISA PCR amplicon in each sample were compared using the Bray–Curtis dissimilarity metric (Legendre and Legendre, 1998). Differences between predefined sets of samples including the train of origin, season, year, or month were determined using analysis of similarity (ANOSIM) (Clarke, 1993) measurements. The ANOSIM test evaluates the differences between these groupings by providing an R test statistic that indicates the level of separation. To identify any community patterns throughout the study, the relative peak height data for all samples was ordinated using Correspondence Analysis (CA) (Legendre and Legendre, 1998). In addition, the environmental variables measured at the Nine Springs WWTP were fitted to the CA data to observe any correlations to the community patterns. Because samples for total phosphorus, ammonia, total kjeldhal nitrogen, and BOD₅ were not collected every day, results from the closest sample date, which was at most 3 days apart, were assumed to be the same for the bacterial community sample date.

To assess the variability within groups of samples, beta diversity was assessed using the PERMDISP2 test (Anderson et al., 2006), and the overall community alpha diversity was assessed using the Shannon Index (Shannon and Weaver, 1949). All statistical analysis was performed using the vegan 1.13 within the R software package (Oksanen et al., 2008).

We also compared *Accumulibacter* clade abundances measured with qPCR to plant performance metrics as well as with each other. For these comparisons, the Pearson's Product Moment was used with $\log(x + 1)$ transformed data. This correlation coefficient value (ρ) ranges between -1 and $+1$ depending on the strength of the correlation. We considered correlations to be significant when $p < 0.05$. Additionally, $\log(x + 1)$ transformed data was used to assess both abundance (T-test) and variation differences (f-test) between clades.

3. Results

3.1. Nine Springs operational data

The Nine Springs WWTP has two separate primary and secondary treatment systems that are referred to as the East and West Train. The East Train employs both UCT and A/O activated sludge processes while the West Train includes only the UCT process (Fig. 1). Both processes are designed for carbon oxidation, nitrification, and enhanced biological phosphorus removal (EBPR). The Nine Springs WWTP performed well over the course of the two-year study with no serious performance failures (Fig. 2). The measured effluent temperature did observe a predictable seasonal change with temperature ranging from 10 to 22 °C. The total plant flow averaged around 167,000 cubic meters per day throughout the study with six wet weather events, which all took place in the second year, that resulted in plant flow exceeding two standard deviations over the average flow rate. Throughout the study, biochemical oxygen demand (BOD) and total phosphorus in the effluent never exceeded 8.5 and 2.1 mg/l, respectively. The average effluent ammonia level was below 0.15 mg/l as N with no date above 1 mg/l as N.

3.2. Seasonal BCC dynamics

Based on seasonal BCC dynamics that have been observed in freshwater lakes (Shade et al., 2007), we hypothesized that the BCC in activated sludge systems would exhibit similar patterns of change. To test this hypothesis, activated sludge samples were collected approximately weekly from both the East and West Trains over a two-year period.

In total, 144 samples were collected from the two plants during the two-year study, and the BCC for each was assessed using ARISA (Table 2 and S1). Winter was defined as December–February. Spring was defined as March–May. Summer was defined as June–August, and Fall was defined as September–November. A total of 116 unique ARISA fragments (operational taxonomic units, OTUs) were identified across all the samples. We compared the communities present in winter, spring, summer and fall. Six OTUs were never found to be present during Winter, twelve OTUs were absent from the Fall Season, and five were absent from Summer (Fig. 3). Surprisingly, Spring was the season during which all OTUs were detected.

Interestingly, when we compared the communities in the East and West Trains, only one OTU was never present in the East Train throughout the study; however, all of the 116 OTUs were present at least once in the West Train. The one OTU missing from the East Train was only present in 17% of the West Train samples with a max relative abundance of

only 1%. These results are not particularly surprising since both trains have similar processes and they have the same influent composition.

To further explore the changes in the bacterial community within each train, we examined the occurrence frequency for each OTU for both trains versus the average relative abundance (Fig. 4). In both trains, only ~10% of the OTUs were present in all samples. Additionally, only ~50% of the OTUs were present in 50% of the samples. Interestingly, those OTUs that were present in nearly all samples were most likely the dominant members of the BCC, but at most they were only 10% of the community. These results highlight how transient the bacterial community can be in a WWTP.

3.3. Overall community comparison and dynamics

To assess how different the overall bacterial communities were in the time series (beta diversity), a pair-wise Bray–Curtis similarity analysis was performed for all samples (Legendre and Legendre, 1998). When samples across both trains were included, the minimum and maximum similarity between samples were 38% and 90%, respectively, and the mean similarity was 62%. In contrast, when we average the pairwise comparison of the samples on the East and West Train collected on the same date, they have an average similarity of 70%. On one occasion, triplicate samples were collected on the same day to explore the variability due to sampling, DNA extraction, and PCR amplification. The samples had an average similarity of $90 \pm 2\%$ across true biological sample replicates, which suggest that nearly all samples can be considered to be statistically distinct since the maximum similarity in all samples was 90%.

The overall community dynamics of both trains throughout the study was explored by ordinating the BCC data for all samples using correspondence analysis (CA) (Fig. 5). The communities grouped on the ordination based on season. While the general pattern was cyclical with samples from each season mapping into one quadrant irrespective of the sampling year, we could not detect a consistent directional trajectory within seasons. Analysis of the mean distance to the centroid (PERMDISP2) (Anderson et al., 2006) for each seasonal grouping found that the summer months were the most variable for both the East and West Train (data not shown). Evaluating the correlation of environmental variables to the BCC changes revealed that effluent temperature and effluent BOD were the two variables that correlated significantly ($R > 0.5$, $p < 0.05$). Not surprisingly, the higher effluent temperatures were correlated to summer and fall samples ($R = 0.92$ (East Train) and 0.91 (West Train)). Additionally, high effluent BOD values were correlated with cold weather samples ($R > 0.7$ for both East and West Train, $p < 0.05$).

To assess if the bacterial communities were different across Months, Years, Seasons, or Trains, ANOSIM analysis was performed (Table 3). Surprisingly, bacterial communities were distinct from each other when classified by season as well as by month across the years. This result was surprising considering the variability in temperature, precipitation, and other environmental parameters across the two years for a given month or season. In contrast, dividing communities into groups by year or train showed little distinction.

3.4. Seasonal diversity effect

It was previously suggested that creating a community with high diversity was advantageous to promote a stable ecosystem (McCann, 2000), particularly in activated sludge systems (Siripong and Rittmann, 2007). To determine how community alpha diversity changed across the time series, we calculated the Shannon Index for Diversity for each sample (Fig. 6). The Shannon index considers not only species richness, but also the relative abundance of each species (measured here with the proxy of relative peak height). During the first year of the study the alpha diversity in both trains exhibited a sinusoidal pattern that loosely correlated with temperature (Pearson Correlation = 0.65 (West Train) and 0.46 (East Train), $p < 0.05$). This correlation coefficient implied that the East Train was more variable in diversity during the first year than the West Train. In the second year when there were more storm events, this correlation was markedly weaker (Pearson Correlation = 0.27 (West Train) and 0.15 (East Train)).

3.5. Accumulibacter clade dynamics

To assess the variability in clade composition within the Accumulibacter lineage, clade abundances were calculated from qPCR results using Accumulibacter *ppk1* clade-specific primer sets and relativized to one-mL MLSS homogenized sample from an aerobic tank from samples collected between September 2006 and March 2008. Since each Accumulibacter cell contains a single copy of the *ppk1* gene (Garcia Martin et al., 2006), the sum total of clade abundances measures should represent the total Accumulibacter abundance. Clade abundances varied with time, but seemed to hover around an average abundance (Figure S2). Additionally, each clade was present within the plant at every time point, indicating that these populations were relatively stable. Interestingly, we observed similar abundance patterns across the two trains. In both the East and West Train, Clades IIA and IID were statistically less abundant than the other three clades (t -test, $p < 0.001$); however, the Clade abundances across the two trains did not correlate well with only Clade IA having a significant abundance correlation across the East and the West Train (Pearson correlation = 0.561, $p < 0.001$).

To discern whether the clade average abundances really were stable across time or trended over time, running averages of five sample points were calculated and plotted against their median sample point (Fig. 7). Clade IIB was relatively stable through time in both the East and West Trains of the plant with its variance statistically significantly lower than Clade IA, IIC, and IID in both the West and East Train (f -test, $p < 0.05$). Clade IA was more prominent in the winter months and less prominent in the summer months, and Clade IIA, IIC and IID were observed to have differing periods of growth and decline throughout the study. However, no other significant abundance variance pattern for any other clade was detected in the East and West Train. Inter-clade correlation analysis for both the East and West Train found rather strong correlation between Clade IIA and Clade IID in the West Train ($\rho = 0.78$, $p < 0.001$). A few other moderately strong correlations between clades were observed in the West Train ($\rho > 0.50$, $p < 0.001$) including Clade IIA with Clade IIB and IIC as well as Clade IA with Clade IIB. For the East Train, only Clade IIC and Clade IID had a moderately strong correlation ($\rho = 0.54$, $p < 0.001$). Interestingly, there were no significant

negative correlations, which suggest that no inter-clade interactions were a result of direct competition for identical resources.

3.6. Clade correlation to plant performance metrics

This study aimed to determine whether individual clade population abundances were impacted by (or predictive of) plant performance metrics. Any emergent relationships might shed light on physiological differences among clades and allow engineers to operate a plant to select for specific clades associated with good system performance.

The strongest correlation was observed between Clade IIA and temperature in the East section ($\rho = 0.65$, $p < 0.05$). Other moderately strong (and significant) correlations were negative correlations between Clade IA and temperature (in West ($\rho = 0.35$, $p < 0.05$) and East ($\rho = -0.36$, $p < 0.05$)), which agrees with the observed trend of Clade IA abundance increase during winter. This might be one explanation for previous observations of better EBPR performance at colder temperatures than warmer temperatures (Erdal et al., 2008; Oehmen et al., 2007). It also suggests that Clade IA and Clade IIA occupy different temperature niches and that previous studies regarding PAO temperature preferences (Brdjanovic et al., 1998) may not encompass the entirety of the PAO community. Clade IIB also had a moderately strong correlation with flow rate (in the West ($\rho = -0.33$, $p = 0.05$)). The remaining correlations were weak ($|\rho| < 0.3$) or not statistically significant. The correlations between Clades IA and IIA, and temperature are apparent when examining the running averages of clade abundance (Fig. 7).

4. Discussion

This is the first study that investigated the normal bacterial community dynamics in a full-scale activated sludge WWTP over a multi-year period using such a highly resolved community analysis method (ARISA). Wang et al. (2010) explored the bacterial community of two WWTPs using T-RFLP over one year, and discovered that the bacterial communities changed while the performance of both WWTPs was stable, and Wells et al. (2011) performed a similar study over a year on a single WWTP. Because the current study explored community dynamics over a two-year period, patterns that were observed in the first year could be verified during the following year. Similar to Wang et al. (2010) and Wells et al. (2011), we observed relatively stable performance despite a markedly variable community. This suggests that functional redundancy leads to consistent performance despite changes in the BCC.

In comparison to other studies that explored the long-term community dynamics in other systems (e.g. lakes and oceans), this ecosystem was relatively unique because of the large biomass concentration (i.e. large population size, $\sim 10^9$ cells mL^{-1} (Manti et al., 2008)) and the relatively stable and rich supplies of carbon source, nutrients, and oxygen. The only measured variable that changed directionally over the course of the study was temperature. Not surprisingly, temperature was the environmental variable that correlated best with bacterial community changes. It is likely that there are certain groups of bacteria that have a narrow temperature range within which they grow optimally; therefore, temperature swings outside this range will allow for other groups to out-compete them. For example, the

Accumulibacter Clade IA and Clade IIA populations varied significantly with temperature based on the qPCR results in this study. As a result, the bacterial communities cycled through a predictable and repeatable seasonal pattern each year. While these results were not entirely unexpected, this is the first time that a two-year study has been performed to confirm the repeating community pattern that exists in the activated sludge system. The openness of the system to invasion by bacteria either in the influent or from the atmosphere makes the level of seasonal and even monthly similarities across the communities during the two years of the study somewhat surprising (Sloan et al., 2006). Whether this type of cyclical pattern would exist in a climate with less seasonal change to force strong selection is not known.

Initial inspection of Accumulibacter clade abundances suggested that the populations varied to some extent with time, but maintained a relatively consistent average. All five clades were present in every sample, indicating that characteristics of the Nine Springs WWTP maintain high clade diversity. However, we note a caveat that should be considered when considering these results. As previously mentioned, the exclusion of NS D3 was necessary due to non-specific amplification of the negative control and low reaction efficiency (86–89%). A portion of the Clade IIC population was comprised of OTU NS D3, estimated at approximately 25% as determined by the single time-point analysis of Nine Springs in 2004 (He et al., 2007). Therefore, Clade IIC abundance measure is almost surely an underestimate due to the exclusion of OTU NS D3.

To better visualize longer-term trends and smooth fine-scale temporal variation, a running average was used to reveal clade boom and bust cycles. These periods of population increases and decreases were not consistent for all clades; individual clade cycles staggered across the year and had no consistent pattern. Statistical analysis of these populations revealed only some statistical differences between clades including that Clade IIB was statistically less variant than the other clades and that Clade IIA and Clade IID were statistically less abundant than the other clades. These results are somewhat consistent with the work by Mielczarek et al. (2013), which found through qualitative *ppk1*-PCR on a single date that Clade IIA and Clade IID were found in only a few plants. This suggests a general less important role of these particular clades in the overall process. Not surprisingly, two clades, Clade IA and IIA, did show moderately strong correlations with temperature. Interestingly, there were some strong positive correlations between different clade pairings in the two trains with a rather strong correlation occurring between Clade IIA and Clade IID in the West Train. The lack of significant negative correlations as well as each clade having its own unique pattern of growth suggests that the clades were responding to different plant characteristics and again supporting previous hypotheses of differences in clade physiologies (He et al., 2007). That is, if all five clades were identical in their physiologies, we might predict that they would vary synchronously in time.

The Nine Springs WWTP has a long history of stable performance that maintains highly active EBPR communities. In a previous survey of ten WWTPs, Nine Springs was the only plant surveyed that contained all five clades (He et al., 2007). Our results lend support to the hypothesis that a higher diversity of Accumulibacter clades can maintain a stable effluent P concentration through functional redundancy, as has been suggested in activated sludge

communities containing ammonia oxidizing bacteria (Siripong and Rittmann, 2007; Whang et al., 2009). However, since we had no significant upsets in our system, our results do not definitively support this idea.

In the same way that the overall community composition changed with temperature, community diversity as defined using the Shannon Index correlated with temperature. This trend was stronger during the first year when there were fewer storm events (compare Fig. 1A with Fig. 6). The drop in diversity during winter might have been due to a drop in the growth rate of community members to the extent that they were washed out of the system. Despite this drop in diversity, the plant performance was not affected. In the second year, there six significant rain events that caused the plant flow to increase two standard deviations higher than the average while no such events occurred during the first year. During these rain events, the diversity in both trains increased, which is contrary to what might be expected since the extra flow should wash out organisms with slower growth rates. It is possible that the increase in diversity was due to increased transport of non-activated sludge bacteria in the collection systems into the WWTP. The observed drop in diversity thereafter could have been caused by acclimated activated sludge populations subsequently out-competing these immigrants. An alternate explanation relates to niche space diversity where storm events might deliver novel substrates to the WWTP that provide transient niches for otherwise rare or absent populations.

We observed little difference in the BCC between the East and West Train. Nearly every OTU was observed in both trains, and samples were determined to be statistically indistinct when they were grouped based on the train of origin. In addition, both trains observed similar cyclical seasonal change in the overall BCC, and both trains had similar changes in diversity indexes throughout this study. While the East Train did have two tanks that were operated in the A/O configuration, the remaining four tanks in the East Train were identical to those present in the West Train. As a result, the East Train was a mix of communities selected from both the A/O and UCT process, rather than being distinct from the West Train UCT community. These results seem to suggest that the temperature, influent, and pre-treatment processes may have a greater impact on the community structure and dynamics than the process configuration. The similarities in the *Accumulibacter* assemblages of the East and West Trains seem to support this; however, further more targeted study on this topic is required to prove this hypothesis.

Similarly, the plant configuration did not appear to have a significant influence on the *Accumulibacter* populations with both locations within the plant containing all five clades on every sample date. This suggests that the treatment systems do not select for particular clades; it is possible that the A/O process could select for certain clades, but since the return sludge streams for A/O and UCT variant systems are coupled in the East Train, it is difficult to separate effects of the different process configurations. That is, the A/O process could be selecting for particular clades, but the UCT variant recycle stream could be continually reseeding with different clades. Despite any differences seen between treatment systems, the clade abundances and variation across time were comparable across locations within the WWTP. Visually, the box and whisker plot analysis of clades both for abundance

measurements and for percent of total *Accumulibacter* seemed consistent across the East and West sides in both median values and extent of variation (Figure S1).

5. Conclusion

In this study, patterns of bacterial community change in two different activated sludge treatment trains were compared over the course of a two-year period. The study revealed that while there was little difference in the BCC between the two treatment trains, there were significant differences between the seasons and months with temperature being the biggest variable correlating with BCC changes. Additionally, we discovered that the BCC diversity fluctuated significantly over the seasons with higher diversity associated with warmer weather. However, wet weather events during any season coincided with a transient marked increase in the community diversity. Overall, the total bacterial community appeared to be rather transient, but there appears to be a repeating seasonal pattern in the overall community succession. Whether these same repeating patterns of succession is observed over many years in other plants still needs to be determined. Surprisingly, the *Accumulibacter* populations were rather stable with all clades being present throughout the study, but each *Accumulibacter* clade population exhibited different cycles of growth and decline throughout the study. This suggests that they are responding to some distinct ecological cues and may have distinct eco-physiologies. Temperature appeared to shape some of the *Accumulibacter* population with Clade IA and IIA negatively and positively correlating with water temperature, respectively. Also, none of the clades in the two trains had negative correlations with each other, which suggests that they are not in direct competition for the same resources. More work needs to be performed to reveal the dimensions of physiological diversity among the clades and the impact on the overall performance and stability of the system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Flow Diagram of the A/O (A) and the variant of the UCT (B) activated sludge processes that were sampled at the Nine Springs Wastewater Treatment Plant. The West Train was entirely composed of the variant of the UCT process (B) while the East Train contained a mix of the A/O and the variant of the UCT process (A and B).

Fig. 2.

Five-point rolling average influent and effluent characteristics from Nine Springs Wastewater Treatment Plant throughout the study period. (A) Total Plant Flow, (B) Influent BOD₅, (C) Influent Total P, (D) Influent TKN, (E) Effluent Temperature, (F) Effluent BOD₅, (G) Effluent Total P, and (H) Effluent Nitrate and Ammonia.

Fig. 3. Summary of OTU occurrence across seasons showing the number of OTUs observed in different seasons.

Fig. 4. OTU percent occurrence (percent of samples that each OTU was detected) versus OTU average abundance for all samples for East (A) and West (B) Train. The OTUs are ordered from the most frequently occurring on the right and the least frequently occurring to the left.

Fig. 5.

Correspondence analysis of the ARISA profiles for the East (A) and West (B) Train over the entire two-year sampling period. Each point represents an ARISA profile for each date with the distance between any two points representing the dissimilarity between two ARISA profiles. Different symbols for each ARISA profile point are used based on the season it was sampled. The arrow labels “Effl BOD₅” and “Effl Temp” represent the effluent BOD₅ and temperature measured for each sample date at the Nine Springs WWTP, and the length and direction of each arrow indicate the degree of correlation with the axes.

Fig. 6. Three-point rolling average of East and West Train BCC diversity calculated based on the Shannon Index on ARISA profiles over the two year sampling period and corresponding effluent temperature at the Nine Springs WWTP.

Fig. 7. Five-point running average of *Accumulibacter* clade abundance over time for the West (A) and the East (B) Train; sampling began in September 2006 and occurred every two weeks for approximately eighteen months.

Table 1

Primers and conditions for qPCR analysis of *Accumulibacter* population.

Primer ^a	Sequence (5' - 3')	Target	T _a (°C)	Betaine conc (M)	Primer conc (nM)
Acc-ppk1-763f	GACGAAGAAGCGGTCAAG	Acc-I ppk1	61	0	500
Acc-ppk1-1170r	AACGGTCATCTTGATGGC				
Acc-ppk1-893f	AGTTCAATCTCACCGAGAGC	Acc-IIA ppk1	63 ^a	0.7	550
Acc-ppk1-997r	GGAAC TTCAGGTCGTTGC				
Acc-ppk1-870f	GATGACCCAGTTCCTGCTCG	Acc-IIB ppk1	61	0	400
Acc-ppk1-1002r	CGGCACGAACTTCAGATCG				
Acc-ppk1-254f	TCACCACCGACGGCAAGAC	Acc-IIC ppk1	66	1	400
Acc-ppk1-460r	CCGGCATGACTTCGCGGAAG				
Acc-ppk1-1123f	GAACAGTCCGCCAACGACC	Acc-IIC ppk1 excluding OTU NS D3	63	0.7	500
Acc-ppk1-1376r	ACGATCATCAGCATCTTGGC				
Acc-ppk1-375f	GGGTATCCGTTTCCTCAAGCG	Acc-IID ppk1	63	0.5 ^a	250 ^a
Acc-ppk1-522r	GAGGCTCTGTGAGTACACGC				

^a Modified from previously published conditions (He et al., 2007).

Table 2

Description of samples collected and average influent characteristics for each seasonal period.

Season	No. of east samples	No. of west samples	Flow, MGD	Temp, oC	Influent BOD ₅ , mg/l	Influent TKN, mg N/l	Influent total P, mg P/l
Spring 06	2	4	43	15	234	37	6
Summer 06	12	10	41	20	230	36	6
Fall 06	11	11	41	18	244	39	6
Winter 07	9	9	39	12	266	39	6
Spring 07	12	11	44	14	235	36	6
Summer 07	11	9	44	20	234	34	6
Fall 07	6	6	44	18	233	36	6
Winter 08	5	4	42	12	242	38	6
Spring 08	5	5	50	13	203	32	5
Summer 08 ^a	1	1	65	17	168	26	5

^a Summer 2008 average influent results are from only through the last sample date of June 13, 2008.

Table 3

Comparison of factors structuring the bacterial community composition at the Nine Springs WWTP using ANOSIM analysis with $p < 0.001$.

Train	ANOSIM R-value			
	Year	Season	Month	Train
East	0.261	0.523	0.617	-
West	0.174	0.532	0.635	-
Both trains	0.167	0.465	0.547	0.214