

# Survey of Full Scale Side-Stream EBPR Facilities and Comparison with Conventional EBPR: Process Stability, Kinetics and Microbial Ecology

Annalisa Onnis-Hayden<sup>1</sup>, Varun Srinivasan<sup>1</sup>, Nicholas B. Tooker<sup>1</sup>, Guangyu Li<sup>1</sup>, Dongqi Wang<sup>1</sup> and April Z. Gu<sup>1,2,\*</sup>

<sup>1</sup>Northeastern University, Boston, Massachusetts

<sup>2</sup>Cornell University, Ithaca, New York

The first two authors contributed equally and can be considered as co-first authors.

\*Email: [aprilgu@cornell.edu](mailto:aprilgu@cornell.edu).

## ABSTRACT

Side-stream EBPR process (S2EBPR) is a new alternative to address the common challenges in EBPR related to weak wastewater influent and to improve EBPR process stability. A systematic evaluation and comparison of the process performance and microbial community structure between four S2EBPR with conventional EBPR configurations in US was conducted. The statistical analysis suggested higher performance stability in S2EBPR than the conventional EBPRs, although possible bias is recognized due to variations in the target permit levels and plant-specific factors among the plants. Total and known PAOs and GAOs abundance and identities were investigated with FISH, DAPI, 16S rRNA gene sequencing and Raman microspectroscopy. The results suggested comparable relative PAO and *Candidatus* Accumulibacter abundances in S2EBPR and conventional EBPR systems. *Tetrasphaera*, a putative PAO, was also found at similar abundance in S2EBPR as in conventional facilities, whereas the relative abundance of known GAOs was lower in S2EBPR than those typically seen at conventional EBPRs. Microbial community analyses via 16S rRNA gene amplicon sequencing revealed differences in the community phylogenetic fingerprints between S2EBPR and conventional plants. Shannon and Inverse Simpson indices, which are combined measures of

richness and evenness evaluation of the microbial communities, suggested that the microbial diversity in S2EBPR plants were higher than those in conventional EBPRs.

## KEYWORDS

EBPR, PAOs, GAOs, Side Stream EBPR.

## 1. INTRODUCTION

The increasingly stringent nutrient permit limits at water resource recovery facilities (WRRFs) demands for more efficient and stable phosphorus removal and recovery technologies. Although enhanced biological phosphorus removal (EBPR) process is considered a potentially efficient process with economic and environmental advantages compared to traditional chemical phosphorus removal, the benefits are often offset, in practice, by the needs to have standby chemicals for achieving reliable and consistent performance. EBPR facilities often suffer from inconsistent performance with unpredicted upsets and performance fluctuations (Barnard and Abraham, 2006; Gu et al. 2008; Neethling et al. 2006). Week influent carbon to phosphorus ratio, external disturbances such as high rainfall, excessive nitrate loading to the anaerobic reactor are among the common factors that contribute to the performance fluctuations. It is recognized that maintaining conditions favoring the proliferation of the key agent-polyphosphate accumulating organisms (PAOs)- over glycogen-accumulating organisms (GAOs) is critical for the stability of EBPR process (Gu et al. 2008, Christensson et al. 1998). Different studies have linked the high relative GAO to PAO abundance with the deterioration, suboptimal operation and even failure of the EBPR process performance (Cech and Hartman 1993, Satoh et al. 1994, Saunders et al. 2003). Factors that have been shown to influence the competition between PAOs and GAOs include influent COD (chemical oxygen demand) to bio-available P ratio, solids

retention time (SRT), substrate type, hydraulic retention time (HRT), temperature, pH, dissolved oxygen, feeding strategy etc. (Filipe et al. 2001, Oehmen et al. 2005a, Oehmen et al. 2005b, Rodrigo et al. 1996, Wang et al. 2006, Whang and Park 2006).

Evaluation of full-scale EBPR performance data reported by Gu et al (2008) indicated that process stability was positively correlated with the influent carbon to phosphorus ratio (bCOD or BOD/P) and they hypothesized that although the excessive amount of available carbon can harbor the proliferation of GAOs, stable process can be maintained as long as the operational conditions are controlled to kinetically favor the growth of PAOs over GAOs. External carbon addition via commercially purchased organic chemicals or on-site primary sludge fermentation has often been used to stimulate and enhance EBPR for facilities with influent characteristics that are deemed unfavorable. External carbon addition increases the cost and adds carbon footprint to the process. In addition, primary sludge fermentation may not be feasible for all cases, particularly for facilities with no primary treatment. For facilities with sludge dewatering liquor P recovery, it has also been recognized that those processes suffer as a result of sporadic metal salt addition used to improve EBPR reliability.

Side-stream EBPR process (S2EBPR) is a new alternative to address the common challenges in EBPR related to weak wastewater influent and to improve EBPR process stability. S2EBPR refers to the modified EBPR configurations that includes diversion of a portion of RAS or anaerobic mixed liquor to a side-stream reactor, where VFA production via sludge fermentation and PAO activity-related P release and carbon uptake occur. S2EBPR has proven successful at number of facilities worldwide; currently, there are about 80 full-scale facilities that have implemented various forms of the S2EBPR process (Barnard et al. 2010; Stevens et al. 2015; Stroud and Martin, 2001; Vale et al. 2008; Vollertsen et al. 2006; Copp et al. 2012). A few recent

studies that compared the EBPR activities, rates and microbial populations between S2EBPR and conventional EBPR plants suggested observable differences between the two configurations (Lanham et al. 2013a, Mielczarek et al. 2013, Stockholm-Bjerregaard et al. 2017). Lanham and colleagues (2013a) compared two S2EBPR with 3 conventional systems and observed that S2EBPRs exhibited a higher level of glycolysis activity and more efficient biological P removal than conventional. In addition, a S2EBPR facilities had a slightly higher abundance of *Tetrasphaera* and *Candidatus (Ca.) Accumulibacter phosphatis* (hereafter referred to as Accumulibacter) and a lower abundance of GAOs the based-on fluorescence in situ hybridization (FISH) data (Mielczarek et al. 2013). Stockholm-Bjerregaard et al. (2017) employed 16S rRNA gene amplicon sequencing to analyze the microbial population at over 20 Danish facilities and found lower relative abundance of *Tetrasphaera* and *Ca. Competibacter* in S2EBPR facilities and similar level of Accumulibacter among the different configurations.

While the full-scale processes demonstrated the potential promises and advantages of S2EBPR, the existing knowledge gap in fundamental understanding of the biochemical mechanisms and microbial ecology involved in S2EBPR hampers its wider application and implementation. This technology is not yet widely applied in US, with only few plants currently in operation. A systematic evaluation and comparison of the process performance and microbial community structure between S2EBPR with conventional EBPR configurations in US has not been reported. The objectives of this study are to: 1) Provide an updated, comprehensive review of the current status of S2EBPR, with focus on operation, performance and microbial ecology findings from previous studies; 2) Conduct a survey to evaluate removal efficiency and reliability of four S2EBPR systems and compare with conventional EBPR systems in US; 3) Investigate and

compare the process rates, kinetics and microbial community structures between S2EBPR systems and conventional EBPR processes.

## 2. METHODOLOGY

A comprehensive review of previous studies on S2EBPR facilities was conducted and, influent characteristics, operation conditions and performances, when available, were summarized and compared. In addition, a survey study was performed by evaluating the four S2EBPR facilities in full-scale operation in US, representing four variations of S2EBPR configurations (Figure 1): side-stream RAS fermentation (SSR) at the South Cary Facility, in Apex, North Carolina, side-stream RAS fermentation with supplemental carbon addition (SSRC) at the Westside Regional facility, in West Kelowna, British Columbia, side-stream mixed liquor suspended solids (MLSS) fermentation (SSM) at the Cedar Creek facility in Olathe, Kansas, and unmixed in-line MLSS fermentation (UMIF) at the Kurt R. Segler Water Reclamation Facility, herein referred as Henderson (Henderson, Nevada).

### 2.1. S2EBPR survey: data collection and analysis

For this study, information over a three-year period for each of the S2EBPR plants were collected, including influent and effluent characteristics, plant process configurations, and operational details and performance monitoring data. Average, arithmetic mean and standard deviation were assessed. For effluent phosphorus, additional analysis and statistical evaluation were performed for the full data set including the arithmetic average (mean), geometric mean, standard deviation, skew, minimum, and maximum. To better assess reliability and variability, effluent P concentration values were plotted against probability according to the approach proposed by previous studies (Neethling et al. 2006; Neethling et al. 2009). To calculate the probabilities of various effluent P levels, the raw data were ranked, and the Weibull probability

was calculated according to:  $P = \text{rank}/(n+1)$ , where  $P$  is the probability and  $n$  is the number of data points in the data set.

Samples of activated sludge from the aerobic zone were also collected, over the period 2016-2017 and subjected to phosphorus uptake and release testing and microbial population analysis at the four S2EBPR facilities. Three conventional EBPR facilities (KalisPELL, MT, Rock Creek, OR and Upper Black Stone, MA) were evaluated for comparison. In addition, performance information on EBPR plants in US from previous studies (Neethling et al. 2006, Gu et al. 2008, He et al. 2008) were also included in this survey study.

## 2.2. P-release and P-uptake batch tests

To evaluate EBPR activity, P release and uptake kinetics tests were conducted on the collected activated sludge samples in accordance with previously described protocols (Gu et al. 2008). During the execution of the batch tests, samples were collected at regular interval for the determination of acetate (HAc), orthophosphate ( $\text{PO}_4^{3-}\text{-P}$ , OP), nitrate ( $\text{NO}_3^-\text{-N}$ ), glycogen (Gly), poly- $\beta$ -hydroxyalkanoates (PHA), mixed liquor suspended solids and volatile suspended solids (MLSS and VSS, respectively). The results were used to determine: maximum acetate uptake rate, anaerobic P-release rate, aerobic P-uptake rate, as well as stoichiometric ratios, such as anaerobic P/HAc, Glyc/HAc and PHA/HAc ratios and aerobic P/PHA and Glyc/PHA ratios.

## 2.3. Chemical Analysis

Acetate, orthophosphate, nitrate, MLSS and MLVSS were analyzed in accordance with Standard Methods (APHA 2013). Glycogen was extracted from freeze-dried sludge samples using a 2 hour digestion time and 0.9 M hydrochloric acid and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in accordance with previously described methods

(Lanham et al. 2012). Poly- $\beta$ -hydroxyalkanoates were extracted from freeze-dried sludge samples using a 3 hour digestion time and a 3% sulfuric acid concentration, and analyzed using gas chromatography – mass spectrometry (GC-MS) (Lanham et al. 2013b).

#### 2.4. Microbial Communities Analysis

**Fluorescence in situ hybridization (FISH).** FISH was applied for quantification of known and functionally relevant microorganisms in EBPR systems. Mixed liquor samples collected at the end of the P-release and P-uptake batch tests, were used for FISH analysis.

The FISH protocol, hybridization conditions and quantification have been described previously (He et al. 2008; Onnis-Hayden et al. 2011; Zilles et al. 2002). FISH probes used in this study were EUBMIX (mixture of probes EUB 338, EUB338-II and EUB338-III) for *Bacteria* (Amann, 1995; Daims et al. 1999); PAOMIX (equal amounts of PAO462b, PAO651 and PAO 846b) to target the *Betaproteobacteria* *Accumulibacter* spp. (Crocetti et al. 2000, Zilles et al. 2002); Tet1-266, Tet2-892, Tet2-174 and Tet3-654 for the *Tetrasphaera* clade 1, 2A, 2B and 3 spp., respectively (Nguyen et al. 2011); GAOMIX (equal amounts of GAOQ989, GAOQ431 and GB742) for the *Gammaproteobacteria* *Ca. Competibacter* spp. (Crocetti et al. 2002; Kim et al. 2010); DF1MIX (TFO\_DF218 plus TFO\_DF218) for the *Alphaproteobacteria* from Cluster 1 *Defluviicoccus* spp. (Wong et al. 2004); DF2MIX (DF988, DF1020 with helper probes H966 and H1038 in equal amounts) for Cluster 2 *Defluviicoccus* spp. (Meyer et al. 2006); and Prop207 for the *Betaproteobacteria* *Ca. Propionivibrio aalborgensis* (Albertsen et al. 2016).

**Amplicon sequencing and Analysis.** 16S rRNA gene amplicon sequencing was performed to obtain more detailed information of microbial community structures in both S2EBPR and conventional EBPR systems. Genomic DNA was extracted from activated sludge samples using the Fast-DNA Spin kit for Soil (MP Biomedicals, Vista, CA, USA). The extracted DNA was sent

to the University of Connecticut-MARS facility for PCR amplification and sequencing targeting the V4 region using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplicons were sequenced on the Illumina Mi-Seq with V2 chemistry using paired-end (2 X 250) sequencing. The sequencing data were analyzed using the mothur pipeline (Kozich et al. 2013) and clustered into OTUs at sequence similarity cutoff of 97% using the OptiClust method (Westcott and Schloss. 2017). The sequences were then classified using the Naïve Bayesian Classifier (80% confidence threshold) using the Silva database (v123) and consensus taxonomy of OTUs was determined using the 80% cutoff using the MiDAS (v123) database (McIlroy et al. 2015). Amplicon data was rarefied to the minimum total sequence count (14646 sequences) across all samples. To identify putative PAOs and GAOs (defined as organisms with glycogen-accumulating capability and no known phosphate-accumulating capability), the MiDAS field guide website (<http://midasfieldguide.org/en/search/>) was used. All data and statistical analysis were performed in R using the following packages: vegan (Oksanen et al. 2018), ggplot2 (Wickham. 2009), dplyr (Wickham. 2018) and ampvis (Albertsen et al. 2015).

**Raman micro spectroscopy.** Single cell Raman micro spectroscopy was conducted to identify and quantify PAOs and GAOs based on their metabolic characteristics, namely the intracellular polymer fingerprints according to previous studies (Majed et al. 2009; Majed & Gu 2011; Majed et al. 2012).

### 3. RESULTS AND DISCUSSION

#### 3.1 Review of current status and summary of previous studies on S2EBPR

Currently, there are about 80 full-scale facilities that have implemented S2EBPR, including 60 in Europe, 12 in the US, 2 in Canada, 1 in India, and 4 in Australia, as shown in **Error! Reference**

**source not found.**1. In addition to full scale facilities operating as S2EBPR, numerous pilot studies have been conducted in the last 20 years.

**Side-stream RAS (SSR).** The SSR configuration is currently the most widely implemented S2EBPR configuration in full-scale plants. Most of these facilities are located in Europe, primarily in Denmark and the UK. Based on published studies (Vollertsen et al. 2006; Mielczarek et al. 2013; Copp et al. 2012; Stroud & Martin 2001, Petersen, 2002), an improvement in P removal can be realized by diverting a relatively small proportion of RAS (4-30%) to a side-stream reactor for a period between 16-48 hours. In a study that focused on population dynamics of 28 full-scale WRRFs in Denmark, the authors reported (Mielczarek et al. 2013) that S2EBPR facilities that did not utilize an external carbon source or chemical P removal were able to achieve average effluent TP concentrations of less than 0.25 mg P/L, independent of influent COD/P ratio and PO<sub>4</sub>-P release rates, although limited operating and performance data were provided, in this study. For all these facilities, typically, 20 to 30% of the return activated sludge entered the side-stream tank, whereas the other portion was returned to the denitrification tank. The HRTs in these anaerobic tanks were all within 12 to 48 hours.

**Side-stream RAS plus carbon (SSRC).** The SSRC configuration is currently in use at one facility in Canada (Stevens et al. 2015), one in the Netherlands (Lopez-Vazquez et al. 2008), and has been previously pilot tested at multiple facilities. The Westside Regional Facility described by Stevens et al. (2015) has, over the years, modified its operation by first reducing then completely removing primary effluent flow to the anaerobic zone, effectively been transformed into a full-scale SSRC system. The change in operation has been associated with improved phosphorus removal with secondary effluent orthoP concentrations of 0.02 mg P/L or lower with no chemical addition. Another example of full scale SSRC plant is the Haarlem Waarderpolder

plant in the Netherlands described by Lopez-Vazquez et al. (2008). The effluent orthoP was found to be one of the lowest among the investigated facilities (0.1 mg/L) despite having the lowest influent VFA/P and BOD/P ratios and lowest Accumulibacter percentage (5.7%). Furthermore, the microbial population results showed that the plant had the lowest *Ca. Competibacter* percentage (<0.4%) and the highest overall Accumulibacter/*Ca. Competibacter* ratio.

In a full-scale pilot study, the Robert W. Hite Facility (Denver, CO) was operated in a modified SSRC process where approximately 25-30% of the RAS was diverted to a side-stream anaerobic reactor and blended with primary sludge fermentate (Carson, 2012). The HRT of the side-stream reactor was 1.1-1.3 hours during the pilot study and the average secondary effluent PO<sub>4</sub>-P concentration was found to be < 0.2 mg P/L (Carson, 2012).

**Side-stream MLSS (SSM).** In one full-scale Danish WRRF, the effluent TP was reduced from an average of 2.05 mg P/L to < 1.14 mg P/L when the system changed from a conventional EBPR configuration to a SSM configuration (Andreasen et al. 1997). They noted that an additional 15 kg/day of soluble COD (a 14% increase compared to the influent sCOD) was provided to the main-stream process via the side-stream reactor overflow (Andreasen et al. 1997). In another full-scale pilot study, approximately 6% of the anaerobic MLSS was diverted to a side-stream reactor at the McDowell Creek Facility. The side-stream reactor were operated at an HRT of 13.6 hours and an SRT of 2.5 days and achieved an effluent TP of less than 1 mg/L (Tremblay et al. 2005). The effluent PO<sub>4</sub>-P concentration was reduced to < 0.3 mg P/L with no chemical addition during a pilot study at the Sacramento Regional County Sanitation District where 10% of the anaerobic MLSS was diverted to a side-stream reactor for an SRT of 2 days and an HRT of 11 hours (Barnard et al. 2015). The side-stream reactor was mixed intermittently for

approximately 10 minutes per day, which allowed for ORP values of -400 mV to be recorded in the side-stream reactor (Barnard et al. 2015). With the SSM configuration, intermittent mixing in the side-stream reactor appears to be a critical component for improving effluent and optimization of reactor performance. Using intermittent mixing allows for a relatively shorter HRT of < 15 hours, but an increased SRT that can be achieved in the thick sludge layer that settles to the bottom of the side-stream reactor.

***Unmixed in-line MLSS fermentation (UMIF).*** Though the anaerobic reactor in the UMIF configuration is not a “strict” side-stream reactor (i.e., influent or primary effluent passes through the anaerobic reactor), it is possible that some of the mechanisms involved in the SSR process also play roles in this type of configurations. Based on operating data from full-scale and pilot scale operations of the process, it appears that a reduction in effluent PO<sub>4</sub>-P concentrations can be realized using the UMIF operation. During a pilot test at the Pinery Water Facility in Colorado, the average secondary effluent TP concentration was less than 0.5 mg P/L, without chemical addition (Barnard et al. 2010). The intermittent mixing operation at this facility resulted in an estimated SRT in the UMIF reactor of approximately 3 days (Barnard et al. 2016). Two facilities near Minneapolis, Minnesota (Seneca and St. Cloud) achieve average effluent TP concentrations of < 0.6 mg P/L using UMIF configurations without chemical addition (Barnard et al. 2010). The secondary effluent PO<sub>4</sub>-P concentration at the Henderson Facility was 0.1 mg P/L during a full-scale study by Barnard et al. (2012). The UMIF at this facility is operated similarly to the Pinery Water Facility, where the mixers in the second of three anaerobic zones are turned off for all but 5-10 minutes per day, which results in an estimated SRT in the UMIF reactor of approximately 3 days.

The review of previous studies conducted on S2EBPR facilities indicates improved performance at full scale and pilot plants that have implemented S2EBPR, although the detailed design, operational and performance data available is limited or incomplete. A long-term survey of S2EBPR and Conventional EBPR facilities along with consideration of design, operation and kinetic activities could lead to insights into key parameters influencing performance and the microbial ecology of EBPR systems.

### 3.2 S2EBPR Performance Evaluation and Comparison with Conventional EBPRs in US

A systematic evaluation of EBPR facilities in the US was conducted with 4 S2EBPR facilities and conventional EBPR facilities. The average secondary effluent water quality based on operating data collected from 2014-2016 for the four S2EBPR plants are summarized in Table 2, while Figure 2 shows the probability plots. It should be noted that each of the facilities has different permit limits and operational goals, making it difficult to compare process reliability among these facilities. Figure 2 . Table 3 shows the frequency with which the facilities achieved secondary effluent phosphorus concentrations of 0.5, 1.0, 1.5, and 2.0 mg P/L based on routine operating data over a three-year period. In addition, a range of percentile statistics were calculated from the raw data set including the 3.84, 50 (median value), 90, 95 and 99 percentiles of the probability that a given value is less than or equal to the stated concentration.

Compared to the previously reported EBPR performance in US, the reliability of S2EBPR facilities meeting a 0.5, 1 or 2 mg P/L secondary effluent phosphate goal, was statistically higher than conventional EBPR. Moreover, the ratio between the 90<sup>th</sup> and 50<sup>th</sup> percentile (90%/50%) effluent P levels, which indicates the range of effluent P concentration variance, was much greater among the conventional EBPR facilities (ranged from 2 to 24, with average of 11.5) than those of S2EBPR (range of 1.34 to 3.43 and average of 2.75), indicating that performances at

S2EBPR facilities tend to be more stable, and less variable. Note that due to the variations in the influent characteristics, operation conditions and permit goals among these plants, this comparison may be biased. Nevertheless, the data provide a statistical and observational comparison of the S2EBPR and conventional EBPR full scale operating facilities.

Similar results were obtained at the full-scale pilot study at Rock Creek Facility, where two independent treatment trains were operated in S2EBPR and conventional EBPR configurations (Maher, et al. 2017).

Reliability and variability in performance of EBPR facilities has often been associated with secondary influent BOD/P ratio. The correlation of BOD/P ratio and secondary effluent orthoP concentrations was statistically analyzed, and no significant correlation was found among the S2EBPR facilities.

### 3.3 EBPR rates, kinetics and associated functionally relevant populations

Phosphorus removal rates and stoichiometry for the four S2EBPR plants were investigated using phosphorus release and uptake testing with acetate addition and compared to conventional EBPR (Table 4). The phosphorus release and uptake rates are generally within the lower range of those observed in conventional EBPR (Gu et al. 2008, Neethling et al. 2006, He et al. 2008, and Lanham et al. 2013a), and they are within the same range identified at the nine Danish S2EBPR facilities (Mielczarek et al. 2013). The ratios between the phosphorus release rate and uptake rate averaged 3.6 (ranging from 2.4 to 4.3) and are within the range observed at conventional EBPR facilities. Note that there is evidence that S2EBPR processes likely enrich for PAOs that utilize more complex carbon sources generated from sludge fermentation, which the acetate-based phosphorus release and uptake tests may not capture.

The P-release/HAc uptake (P/HAc) ratio is influenced by many factors, such as the pH and carbon source, and it has been often used as indicator of the relative PAO and GAO activities and abundance (Saunders et al. 2003; Schuler and Jenkins, 2003). It has been found to correlate well with the relative abundance of *Accumulibacter* in EBPR sludge (Oehmen et al, 2007). The P-release/HAc uptake ratios varied among facilities ranging from 0.14 mol P/mol C to 0.54 mol P/mol C, which agree with those reported for EBPR systems (0.01–0.93 mol P/mol C) as summarized in the study by Schuler and Jenkins (2003). The theoretical P/HAc was suggested to be 0.5-0.75 mol P/mol C based on acetate-fed lab-scale systems (Table S1). All samples, except Cedar Creek, exhibited lower P/HAc ratios than the theoretical stoichiometry suggested, especially the Henderson facility. Cedar Creek exhibited the highest value of P/HAc ratio close to the theoretical value for acetate-fed EBPR at a similar pH of 6.5 - 7 (Smolders et al. 1994a) (Table S1), suggesting relatively low GAO to PAO abundance ratio. The lower P/HAc ratios in S2EBPR facilities could due to (1) the presence of GAOs that are able to assimilate acetate anaerobically but without polyP transformation and/or (2) presence of PAOs with different metabolisms such as fermenting PAOs which do not use acetate to cycle polyP and hence might not perform EBPR metabolism under the conditions in the acetate-fed uptake release test. Note that theoretical values derived from acetate-fed systems may not be true for propionate-fed systems that exhibited lower theoretical value of 0.35-0.42 (Oehmen et al. 2006).

Anaerobic stage Glyc/HAc ratio provides insights in the extent of utilization of glycolysis pathway versus TCA cycle for the anaerobic metabolism. GAOs rely on glycogen as their sole energy source via glycolysis pathway and they exhibit higher Glyc/HAc ratios than PAOs with a value of 0.7 and 1.1 for GAO propionate or acetate model, respectively (Table S1). A Glyc/HAc ratio indicative of involvement of glycolysis activity for PAOs was reported to be 0.3 and 0.5 for

PAO propionate or acetate model, respectively (Table S1), while a Glyc/HAc ratio close to zero would indicate absence of glycolysis and predominant reliance on the TCA cycle. The Glyc/HAc ratios ranged from 0.15 to 0.45 mol C/mol C for the four S2EBPR plants, suggesting glycolysis activity for PAOs and presence of GAOs, and they are consistent with results obtained for other S2EBPR facilities (Lanham et al. 2013a). The highest Glyc/HAc ratio was observed at the Henderson facility.

The anaerobic PHA/HAc ratios for all the plants ranged from 0.61 to 0.8 molC/molC, which were lower than theoretical values predicted from various models, as often observed in full-scale EBPR facilities (Table 4, Tables S1). This could be partly attributed to the involvement of other PAOs (i.e. *Tetrasphaera*) and other heterotrophic bacteria that may utilize or take up acetate without storing it as PHA. Although its role in EBPR is yet unknown, *Tetrasphaera*-PAOs are able to take up acetate and other substrates in the anaerobic period without forming PHA as a storage compound (Nguyen et al, 2011), which can contribute to the lower PHA/HAc ratio observed.

The aerobic yields (P/PHA and Glyc/PHA), are also reported in **Error! Reference source not found**.4. The P/PHA ratios for Westside Regional and Cedar Creek were higher than the PAO model prediction. Lanham et al. (2013a) observed similar results in Danish S2EBPR facilities. The results for the Henderson Facility however showed a lower value than predicted by the PAO model, which along with the higher anaerobic Glyc/HAc ratios would suggest a higher relative abundance of GAOs in this facility.

### 3.4 Functionally relevant microbial populations

Functionally relevant key populations in the mixed liquor samples were examined using 16S rRNA-based FISH probes targeting known and putative PAOs and GAOs. DAPI staining was

also applied to observe the presence and relative abundance of total PAOs. In addition, mixed liquor samples were analyzed for determination of internal storage polymers using Raman microspectroscopy, which allows for the quantification of total PAO and GAO abundance. Table 5 summarizes the observation of PAOs and GAOs for mixed liquor samples collected during the study.

Abundance of *Accumulibacter* at the studied S2EBPR facilities, according to our FISH results, ranged between 4.6 to 7.6% of total biovolume population, comparable to the level observed at full scale EBPR facilities, in the US and around the world.

Another group of recent interest is *Tetrasphaera*, which was found in relatively high abundance at full-scale EBPR facilities in Portugal and Denmark (Lanham et al, 2013a). Our FISH results show a high relative abundance of *Tetrasphaera* (>15%) at the S2EBPR facilities studied. These microorganisms were suggested to be putative PAOs (Kong et al. 2005; Maszenan et al. 2000) with different physiology from *Accumulibacter* (Kristiansen et al. 2013). They are considered more versatile than *Accumulibacter*, being able to denitrify, ferment glucose, synthesize glycogen as a storage polymer, and accumulate polyP (Kristiansen et al. 2013). However, their activities and roles in full-scale EBPR facilities remain largely unknown. The relative abundance of *Tetrasphaera* quantified by FISH can be biased and overestimated due to the filamentous morphology of some *Tetrasphaera* spp. (Nguyen et al, 2011), as shown in Figure 3. As a result, the sum of *Accumulibacter* and *Tetrasphaera* appear to be higher than the Total PAOs determined via DAPI staining. Raman-based PAO identification showed in general higher percentage of total PAOs compared to the DAPI results. Nevertheless, the discrepancy between total PAO and *Accumulibacter* suggests presence of other PAOs such as *Tetrasphaera* or others and their potential roles in EBPR performance and stability. Based on current understanding of

their metabolic trains, the side-stream reactor where extended anaerobic phases occur with fermentation condition potentially favors PAOs such as *Tetrasphaera* due to their ability to ferment.

Our FISH results showed that candidate GAOs, including *Ca. Competibacter* and *Defluvicoccus* GAOs, are very low in these S2EBPR plants, in comparison to those generally observed in conventional EBPR systems with the exception of the Henderson samples. Low abundance of above candidate GAOs was also found in other S2EBPR systems (Lanham et al. 2013a). Total GAO abundance based on Raman microspectroscopy methods, captures both known and potentially unknown GAOs based on their phenotypic traits of intracellular polymers. Raman results indicated the potential presence of other GAOs at these plants, and confirmed higher abundance of GAOs at Henderson, consistent with the FISH results and the batch testing stoichiometry.

Figure 4 shows the correlation between the relative *Accumulibacter*-PAO/GAOs abundance and the P/HAc ratio determined from acetate-based phosphorus release and uptake batch testing. Among the S2EBPR facilities studied, the Henderson facility has the highest abundance of GAOs and it showed most variability in performance, as indicated by the higher ratio between the 90<sup>th</sup> percentile and the median (Table 3). At the Henderson facility, both phosphorus limiting conditions (BOD:P >40) and high temperature (24.4 °C average) could have led to the proliferation of GAOs. These results suggest that while the presence of GAOs does not necessarily preclude successful EBPR, an increase in the GAO population can indicate less stable EBPR performance, as suggested by Gu et al. (2008). Note that Henderson has a configuration more similar to a mainstream EBPR configuration with mixer off in one of the

anaerobic zones, in comparison with any other of the S2EBPR configurations that have dedicated side-stream reactors where no influent flow is introduced.

### 3.5 Comparison of Microbial Community in S2EBPR and Conventional EBPR

The results of the microbial community analysis using the 16S rRNA gene as a marker show distinct community structures among the four different configurations of S2EBPR facilities at both class and genus levels (Figure 5). Based on amplicon sequencing, *Betaproteobacteria* and *Sphingobacteria* were the dominant classes observed across all S2EBPR configurations; these classes plus *Deltaproteobacteria*, have also been found to be present in high abundance in other EBPR sludge samples (Lv et al. 2014; Zhang et al. 2012).

Differences in overall populations among the communities in different facilities was visualized using a non-metric multidimensional scaling (NMDS) analysis (Figure 6). The three conventional EBPR plants clustered relatively close together, while the S2EBPR plants were rather scattered. The samples analyzed from two different time points from each S2EBPR facility clustered together as generally expected, indicating plant-specific community structure that are relatively stable at the two different time points, and mainly influenced by plant-specific factors including influent characteristic and other operational conditions. This highlights the difficulty and potential bias in microbial structure comparison among S2EBPRs and conventional EBPRs at full-scale. Further investigation with alternative approaches and, perhaps at higher phylogenetic or phenotypic resolution, is needed to reveal the distinction among them.

Various diversity indices, including richness and evenness, were calculated for the facilities evaluated. Richness indices are a measure of the number of organisms present in a sample while evenness indices consider the relative abundance of these organisms. In this study, operational taxonomic units (OTUs) are used as a surrogate for organisms. The Chao1 index is a measure of

the richness of a system, with a higher value indicating a higher number of OTUs present. Interestingly, the “stricter” S2EBPR configuration, namely, SSR had the highest Chao1 index (~9000) compared to the other S2EBPR configurations (Figure 7). Evenness indicates how evenly distributed the different OTUs are; a value of 1 usually indicates that all OTUs are present in equal abundance while a value close to 0 indicates that there is a high disparity in the relative abundance of the various OTUs. The Pielou Evenness index was similar across the different samples with an average value of  $0.74 \pm 0.03$ . This combined with the high Chao1 index suggests the presence of different OTUs with low but comparable relative abundances in the S2EBPR systems. A combined measure of richness and evenness are Shannon and Inverse Simpson indices, which quantify the diversity of the microbial community. Generally, the SSR configuration had a higher diversity while the SSRC configuration had the lowest diversity. Moreover, the diversity of S2EBPR community appeared to exhibit higher range and greater variance than those observed for the conventional EBPR facilities analyzed (Figure 7). Higher diversity has been observed to be associated with increased robustness and resilience of the community due to functional redundancy and niche complementation (Shade et al. 2012). In addition to the elucidation of overall microbial community fingerprints among EBPR facilities with various configurations, the 16S rRNA gene also provides additional insights into the identities and diversity of PAOs and GAOs. By comparing the 16S rRNA gene sequence for known PAOs in the MiDAS database, the relative abundance of gene copies of the four candidate PAOs, including *Ca. Accumulibacter*, *Ca. Accumulimonas*, *Ca. Obscuribacter* and *Tetrasphaera* in the four S2EBPR plants were revealed and they show variance among the plants (Figure 8). *Ca. Accumulimonas* have been observed (using MAR-FISH) to accumulate PHA with uptake of VFAs during anaerobic conditions and uptake  $\text{PO}_4\text{-P}$  under aerobic conditions

(Nguyen et al. 2012), while *Ca. Obscuribacter* have been proposed as putative PAOs due to their genomic capacity for polyphosphate accumulation (Soo et al. 2014). However, their importance in EBPR systems has not been characterized. *Tetrasphaera* were found at an average relative abundance of  $1.81 \pm 0.1$  % and at the highest relative abundance at Westside Regional facility (SSRC). Note that the relative abundances obtained from 16S rRNA gene sequences do not necessarily correspond to the actual population abundance due to PCR biases, variation in the number of 16S rRNA gene copies across different organisms, the presence of legacy DNA from EPS, non-active organisms, etc. Therefore, the relative abundance of organisms revealed by 16S rRNA gene sequencing cannot be directly compared with FISH results that detect active populations and use biomass volume as a metric. Furthermore, the lower relative abundance of *Tetrasphaera* observed in this study could be due to DNA extraction biases against this organism (Albertsen et al. 2015)

The mean relative 16S rRNA gene abundance of *Accumulibacter* was slightly higher in conventional EBPR system ( $1.41 \pm 0.06$  %) compared to S2EBPR systems ( $0.54 \pm 0.06$  %). Other candidate PAOs were also found at higher relative abundances in conventional EBPR systems compared to S2EBPR systems except for *Tetrasphaera* which was found at similar median abundances.

16S rRNA sequencing analysis also led to the identification of four known GAOs, including *Ca. Competibacter*, *Ca. Contendobacter*, *Defluviicoccus* spp. and *Propionivibrio* (Figure 9**Error! Reference source not found.**). Even though *Ca. Competibacter* is considered a model GAO, it was not detected in any of the S2EBPR facilities. However, 16S rRNA genes of *Defluviicoccus* spp. and *Propionivibrio* were detected at mean relative abundances of  $0.04 \pm 0.01$  % and  $0.11 \pm 0.02$  % respectively. This is consistent with the detected GAOs by Raman microspectroscopy.

The GAO abundances found in this study, based on 16S rRNA gene are generally lower than those from a study published on Danish EBPR facilities, especially for the conventional EBPRs (Stokholm-Bjerregaard et al. 2017). This is potentially due to the different characteristics of the influent wastewater in Denmark which had an industrial COD contribution of up to 75 %. A heavy proportion of industrial load has been associated with increased GAO abundances (Mielczarek et al. 2013; Stokholm-Bjerregaard et al. 2017).

## CONCLUSIONS

A comprehensive review of previous studies of S2EBPR, and a detailed evaluation of the performance, kinetics, and microbial population for four full scale S2EBPR facilities in US was performed and compared with conventional EBPRs. The key findings can be summarized as follows:

1. Literature review of previous studies on S2EBPR facilities currently operating in Europe and the US showed various implementation configurations and varying operation parameters. The most widely implemented S2EBPR configuration is SSR, where 5-30% of RAS is diverted to a side-stream reactor with an HRT of 12-48 hours.
2. Survey of four S2EBPR in US, representing four variations of S2EBPR configurations, was performed with three years of performance data. S2EBPR plants seemed to perform better with slightly higher stability. It is noted that the comparison may be biased due to great variation in the plant-specific factors such as influent characteristics, effluent permit and operation goals etc.
3. The EBPR activities, rates, kinetic and stoichiometry among the four S2EBPRs surveyed were consistent with previous studies on S2EBPR and were within the range of values observed at conventional EBPR facilities. The range of rates, stoichiometric ratios and the varying extent

of the involvement of glycolysis pathway among the S2EBPR and conventional EBPR plants suggest the presence of PAOs and GAOs with phenotypic diversity greater than what currently known.

4. Total and known PAOs abundance and identities were investigated with FISH, DAPI, 16S rRNA gene sequencing and Raman microspectroscopy, and the results suggested comparable relative PAO and *Accumulibacter* abundances in S2EBPR and conventional EBPR systems. *Accumulibacter* and *Tetrasphaera* were found in all S2EBPR plants along with other putative PAOs such as *Ca.*, *Obscuribacter* and *Ca.* *Accumulimonas*. More in-depth microbial phylogenetic and phenotypic evaluation beyond 16S rRNA gene sequencing may be needed to further reveal finer-resolution differences in PAO populations between S2EBPR and conventional EBPR.

5. *Tetrasphaera*, a candidate PAO, was found at a higher relative abundance than other PAOs using both FISH and amplicon sequencing. The role in EBPR and the diversity and function of various *Tetrasphaera* spp. found in activated sludge is still unknown and warrants investigation.

6. Total and known GAO abundances and identities were evaluated using Raman microspectroscopy, FISH, and 16S rRNA gene sequencings. The relative abundance of known GAOs identified by FISH and 16S rRNA sequencing was lower in S2EBPR than those typically seen at conventional EBPRs. However, total GAO identified by trait-based Raman method detecting the presence of cell with intracellular glycogen polymer seem to indicate the presence of other GAOs, as suggested by the P/HAc ratios. The presence and identities of other GAOs in S2EBPR needs further investigation.

7. 16S rRNA gene sequencing allowed comparison of community structure fingerprints and community diversity. Shannon and Inverse Simpson indices, which are combined measures of richness and evenness evaluation of the microbial communities, suggested that the microbial diversity in S2EBPR plants were higher than those in conventional EBPRs. The underlying mechanisms and ecological selection forces in the S2EBPR systems that lead to higher community diversity holds the key to better understanding of improved performance with S2EBPR systems and warrants further study.

## Acknowledgements

Funding for this research was provided by Water Environment & Reuse Foundation (project no: U1R13), Hampton Roads Sanitation District, and Woodard & Curran, Inc. The authors thank operators and personnel at participating facilities and the entire WE&RF S2EBPR Project Team.

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**Table 1. Summary of Full-Scale Facilities Currently Operating the Four Types Of S2EBPR Process Configurations.**

Configuration	Number of Facilities	Locations	Effluent Performance	References
Side-stream RAS (SSR)	60+	Denmark, Sweden, UK, Australia, North Carolina	TP < 0.9 mg P/L	(Vollertsen et al. 2006; Mielczarek et al. 2013; Copp et al. 2012; Stroud & Martin 2001)
Side-stream RAS plus carbon (SSRC)	2	British Columbia, Netherlands	PO <sub>4</sub> < 0.1 mg P/L	(Stevens et al. 2015; Lopez-Vazquez et al. 2008)
Side-stream MLSS (SSM)	1	Kansas	TP < 0.9 mg P/L	(Kobylinski et al. 2013)
Unmixed in-line MLSS fermentation (UMIF)	4	Nevada, Minnesota, Colorado	TP < 0.6 mg P/L	(Barnard et al. 2010)

**Table 2. Summary of Average Operational Data and Performance for the Four S2EBPR Facilities during the Period 2014-2016.**

Facility	South Cary [SC]	Westside Regional [WR]	Cedar Creek [CC]	Henderson [Hen]
System Configuration Information				
S2EBPR Configuration	SSR	SSRC	SSM	UMIF
Mainstream Configuration	4-stage Bardenpho	MLE	Modified Johannesburg	Johannesburg
Chemical addition	No	Yes	No	Yes
VFA addition	No	Yes, PFO <sup>1</sup>	No	No
Primary Sedimentation	No	Yes	No	No
Tertiary Filtration	Yes	Yes	No	Yes
TP Permit Limit (mg/L)	2.0	0.25	1.5	0.22 (seasonal) <sup>2</sup>
System Operating Parameters				
Wastewater temperature (°C)	21.8	17.2	16.2	24.4
Mainstream Sludge age (days)	7.3	10	13	6.4
Mainstream HRTn (hours)	23	12	19	16
Sidestream Sludge age (hours)	36	1.3	47	NA
Sidestream HRTn (hours)	2.9	0.9	0.9	0.4
Sidestream HRTa (hours)	36	1.3	13.5	NA
Mainstream MLSS (g/L)	3.4	3.2	2.1	2.0
Sidestream MLSS (g/L)	6.6	8.0	4.5-14.5 <sup>3</sup>	N/A
Influent <sup>4</sup> Parameters				
Influent Flow (MGD)	5.2±0.8	2.6±0.2	3.0±0.9	20.9±1.9
TSS (mg/L)	330±115	94.3±21	246±116	274±50
BOD (mg/L)	284.4±69	240±53	236±92	263±38
TKN (mg/L)	48.2±6.8	44.1±4.8	29.4±11	43.7±5.4
TP (mg/L)	7.1±0.1	6.8±1	2.7±5.4	5.7±0.8
BOD/P (mg/mg)	39±6.6	38.4±23	102±88	46.5±4.2
Alkalinity	140	NA	261	268
pH	7.3	7.3	7.4	7.4
Effluent Parameters				
TSS (mg/L)	5.7±7	2.8±11.4	6.1±3.3	5.2±1.9
BOD (mg/L)	2.8±0.5	NA	8.4±3.9	5.4±2.2
TN (mg/L)	2.1±0.7	5.8±4.3	7.6±3.1	16.4±2.3
TP (mg/L)	0.4±0.4	0.2±0.1	0.9±0.3	0.48±0.3

<sup>1</sup>PFO Primary Fermenter Overflow; <sup>2</sup> The facility has a waste load allocation of 43 lb/day of TP between March and October, which corresponds to a concentration of 0.22 mg/L at the current average flow rate. <sup>3</sup> The variation of the sidestream MLSS is due to the intermittent mixing, lower value for top layer, higher value for bottom layer. <sup>4</sup> Influent to biological process. HRTn: nominal HRT; HRTa: actual HRT.

**Table 3. EBPR reliability to meet secondary effluent orthoP concentrations of less than 2 mg P/L, 1 mg P/L, and 0.5 mg P/L for conventional EBPR and S2EBPR facilities.**

Parameter	S2EBPR					EBPR <sup>3</sup>
	South Cary <sup>1</sup>	Westside Regional	Cedar Creek	Henderson	S2EBPR <sup>2</sup>	
OP < 0.5 mg/L	73%	99%	23%	70%	67%	65%
OP < 1 mg/L	91%	100%	79%	90%	90%	82%
OP < 1.5 mg/L	96%	100%	100%	96%	98%	NA
OP < 2 mg/L	99%	100%	100%	99%	99%	93%
3.84% [mg/L]	0.10	0.01	0.10	0.08	0.08	NA
50% [mg/L]	0.28	0.04	0.82	0.30	0.35	0.26
90% [mg/L]	0.89	0.10	1.10	1.00	0.83	1.6
3.84%/50%	0.36	0.29	0.12	0.28	0.27	NA
90%/50%	3.17	2.39	1.34	3.43	2.75	11.5

1. These values are based on the FE TP value; 2. Average values for the S2EBPR facilities in this study; 3. Data extrapolated from WERF 2006 (Neethling et al. 2006).

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Table 4. Summary of Batch Uptake and Release Testing Results.

Parameter	South Cary	Westside Regional	Cedar Creek	Henderson	EBPR <sup>1</sup>
Configuration	SSR	SSRC	SSM	UMIF	
P-Release [mgP/gVSS/h]	5.3	5.9	4.4	2.85	4.2-31.9
P-Uptake [mgP/gVSS/h]	2.6	2.4	1.6	0.57	1.4-7.6
Acetate uptake [mgHAc/gVSS/h]	13.5	17.4	7.68	24.89	16-42
P-Release/P-Uptake	2.0	2.4	3.2	4.31	1.6-5.0
P/HAc [molP/molC]	0.39	0.38	0.54	0.16	0.11-0.6
Glyc/HAc [molC/molC]	0.15	0.33	0.35	0.45	0.02-0.82
PHA/HAc [molC/molC]	0.72	0.61	0.80	0.75	0.67-2.10
P/PHA [molP/molC]	0.52	0.69	0.73	0.21	0.2-1.9
Glyc/PHA [molC/molC]	0.9	0.75	1.07	2.9	0.2-1.2

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1. Data extrapolated from Neethling et al. (2006), Gu et al. (2008), He et al. (2008) and Lanham et al. (2013b) as well as results from study conducted at Northeastern University with EBPR sludge from 3 different facilities (Kalispell, MT, Rock Creek, OR and Upper Black Stone, MA) at the same time of the S2EBPR testing. P/HAc: P release to acetate uptake ratio; Glyc/HAc: glycogen utilization to acetate uptake ratio; PHA/HAc: PHA production to acetate uptake ratio; P/PHA: PolyP formation to PHA consumption ratio; Glyc/PHA: glycogen formation to PHA consumption ratio; NA: not available.

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Table 5 Summary of PAOs and GAOs Observations at S2EBPR Facilities Studied.							
	Total PAOs (DAPI)	Total PAOs (Raman)	Accumulibacter (FISH)	Tetrasphaera (FISH)	GAOs (FISH)	Total GAOs (Raman)	P-rel/Hac up
South Cary (SSR)	10.5%	22.9%	6.4%	15.3%	0.7%	2.9%	0.39
Westside Regional (SSRC)	13.5%	14.3%	7.6%	18.1%	0.5%	2.9%	0.38
Cedar Creek (SSM)	11.5%	13.0%	6.2%	20.2%	0.3%	2.0%	0.54
Henderson1 (UMIF)	9.2%	16.7%	5.6%	19.7%	3.8%	4.2%	0.16
Henderson2 (UMIF)	8.2%	12.5%	4.6%	18.7%	4.5%	8.3%	0.14
EBPR <sup>a</sup>	5-35%		2-20%	17-30%	1-18%		0.1-0.0.6

770 a. Data extrapolated from Gu et al. 2008; He et al. 2008; Beer et al 2005; Wong et al. 2005; Lopez-Vazquez et., 2009; Nguyen et

771 al. 2011, and Lanham et al. 2013b. Henderson1 and Henderson2, are two different completely independent trains.

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Supporting Information

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Table S1. Metabolic Model Predictions for Key Ratios.					
	Anaerobic			Aerobic	
	P/HAc [molP/molC]	Glyc/HAc [molC/molC]	PHA/HAc [molC/molC]	P/PHA [molP/molC]	Glyc/PHA [molC/molC]
Anaerobic PAO TCA model <sup>a</sup>	0.5-0.75	0	0.9	N/A	N/A
Anaerobic PAO Gly Model <sup>b</sup>	0.5	0.5	1.33	N/A	N/A
Anaerobic PAO propionate Model <sup>c</sup>	0.42	0.3	N/A	N/A	N/A
Anaerobic PAO TCA + Gly Model <sup>d</sup>	0.16	0.7	1.48	N/A	N/A
Anaerobic PAO partial TCA + Gly Model <sup>e</sup>	0.37	0.6	1.4	N/A	N/A
Aerobic PAO model <sup>b</sup>	N/A	N/A	N/A	0.41	0.42
GAO Model <sup>f</sup>	0	1.12	1.86	0	0.65
Anaerobic GAO propionate Model <sup>b</sup>	N/A	0.7	N/A	N/A	N/A

776 a. Comeau et al. 1986, 1987; b. G. J. F. J. Smolders et al. 1994a and 1994b; c. Oehmen et al. 2006; d. Pereira et al. 1996; e.

777 Hesselmann et al. 2000; f. Zeng et al. 2002. P/HAc: P release to acetate uptake ratio; Glyc/HAc: glycogen utilization to acetate

778 uptake ratio; PHA/HAc: PHA production to acetate uptake ratio; P/PHA: PolyP formation to PHA consumption ratio; Glyc/PHA:

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Figure 9. (A) Relative Abundance of Putative GAOs in the Investigated S2EBPR Facilities based on 16S rRNA sequencing. (B) Comparison of Relative Abundance of Putative GAOs between Conventional and S2EBPR Facilities. The Red Point is the Average Relative Abundance from Stockholm-Bjerregaard et al. 2017.

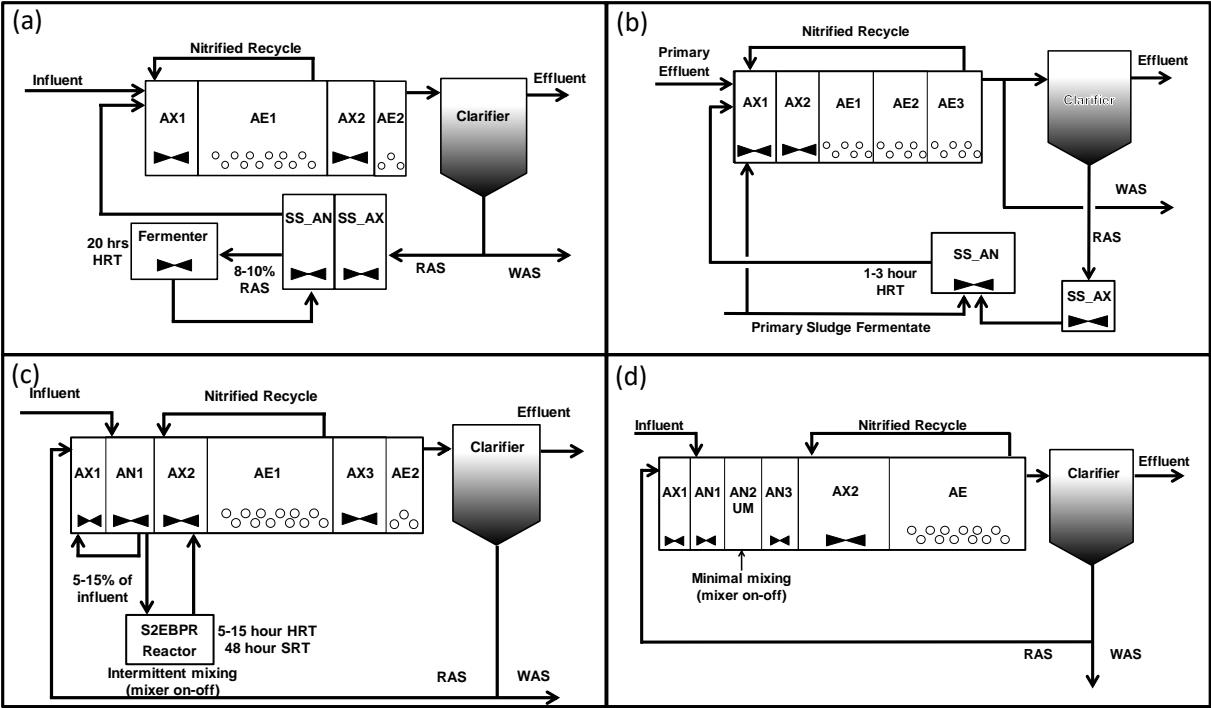


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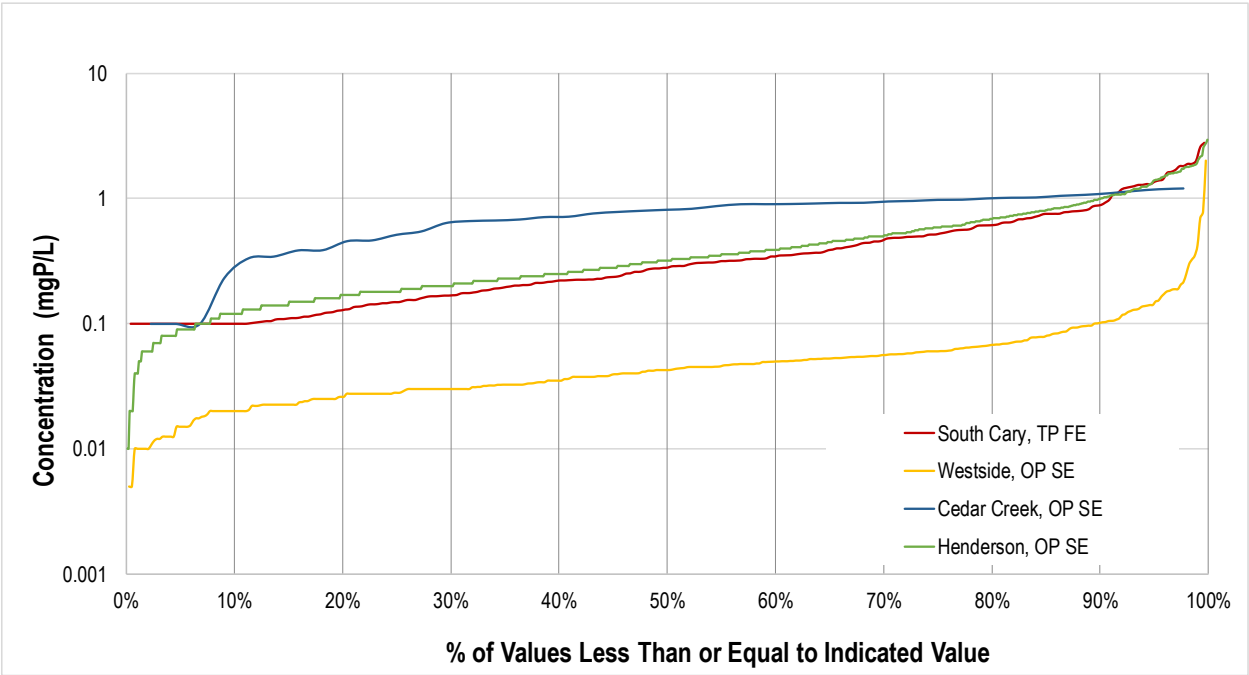


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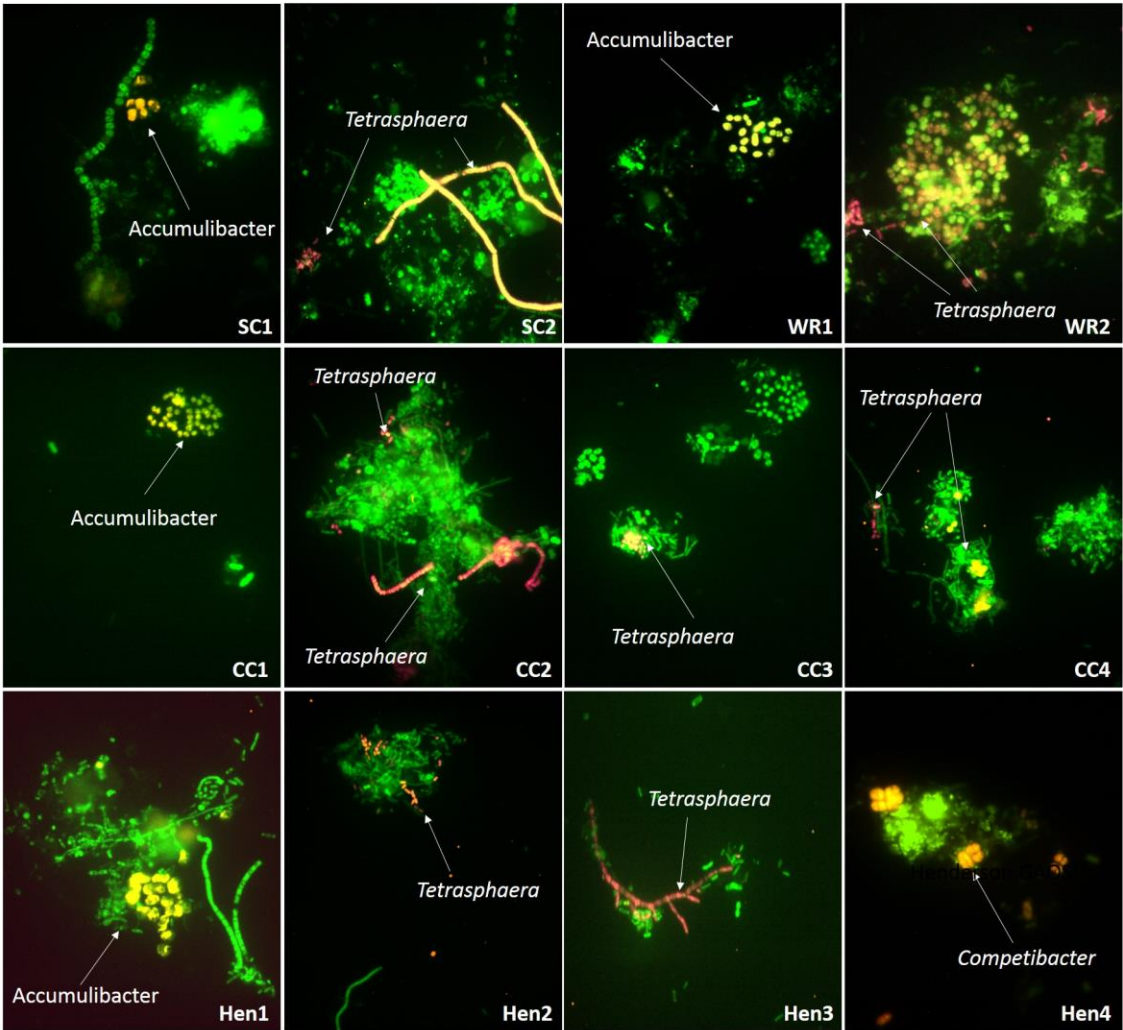


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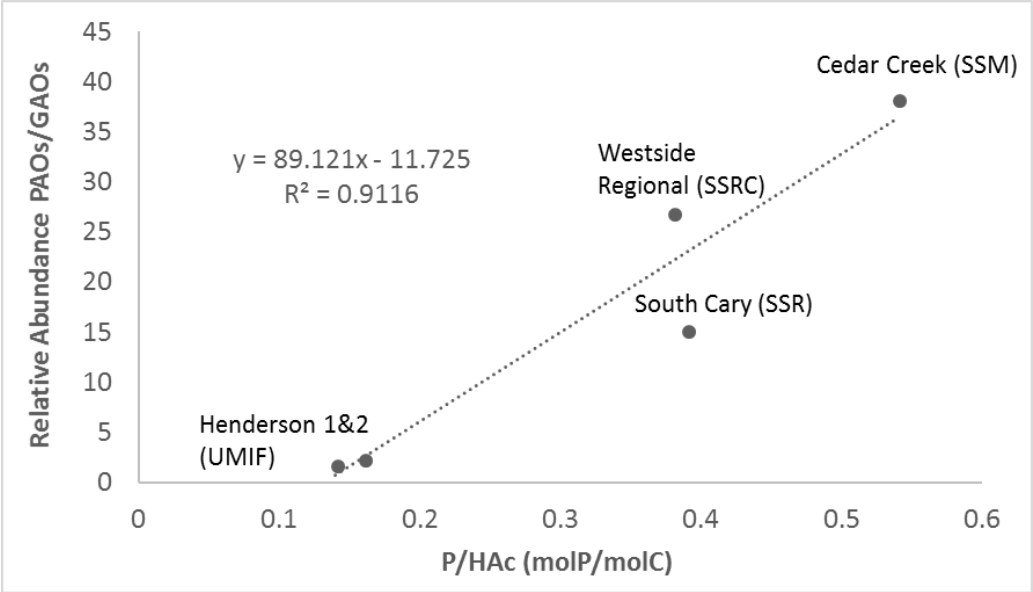


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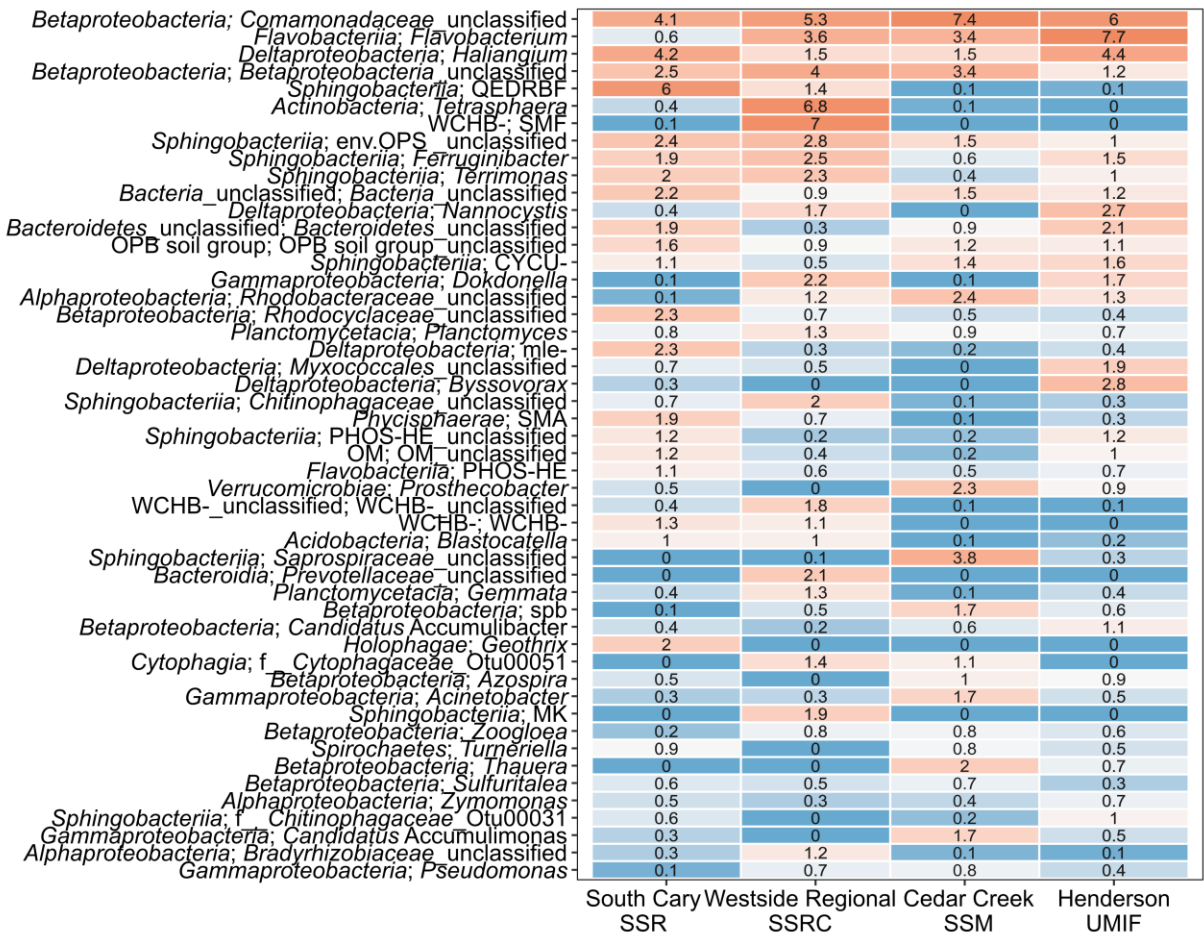


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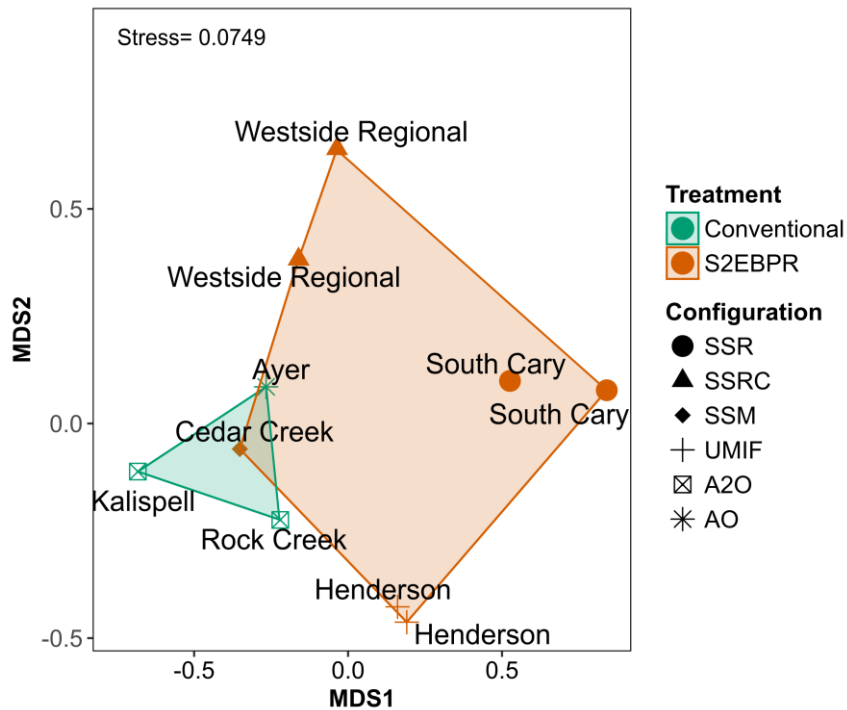


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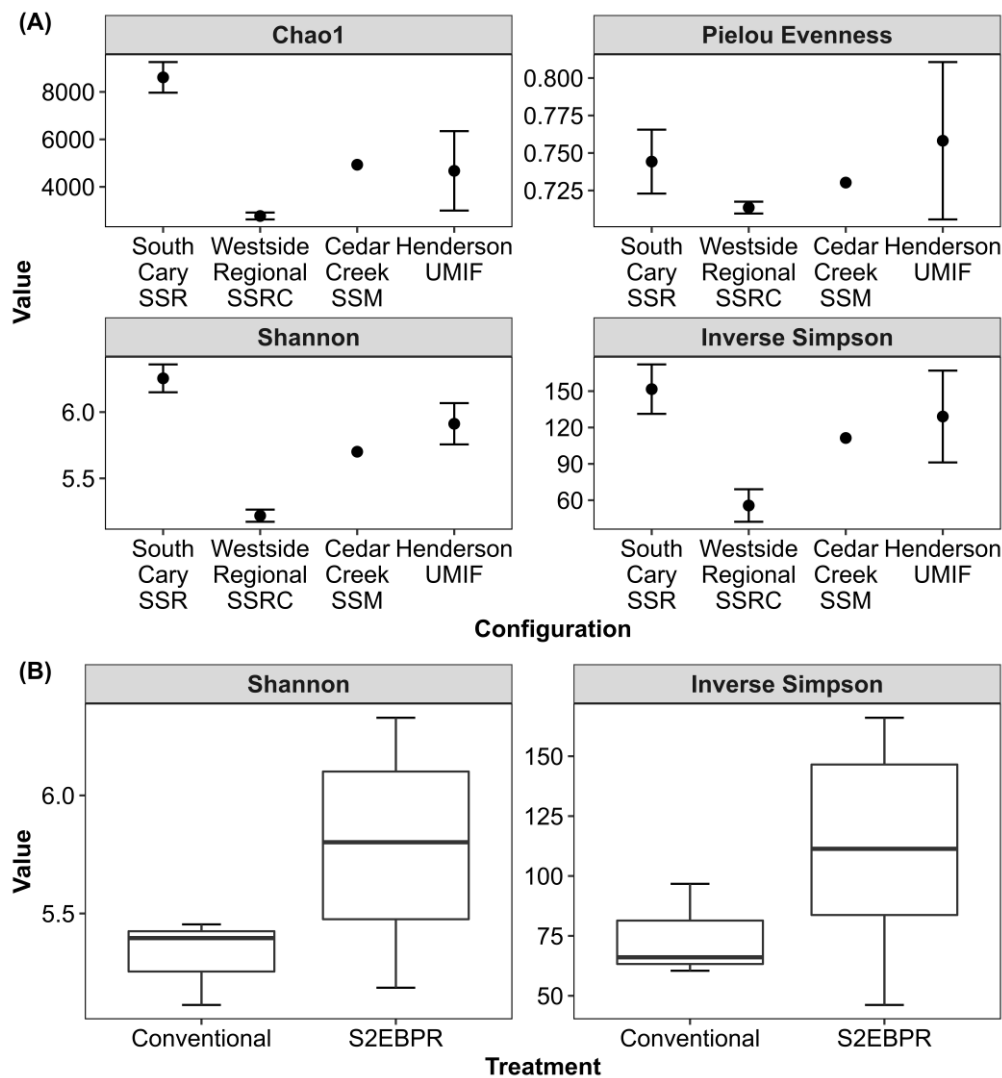


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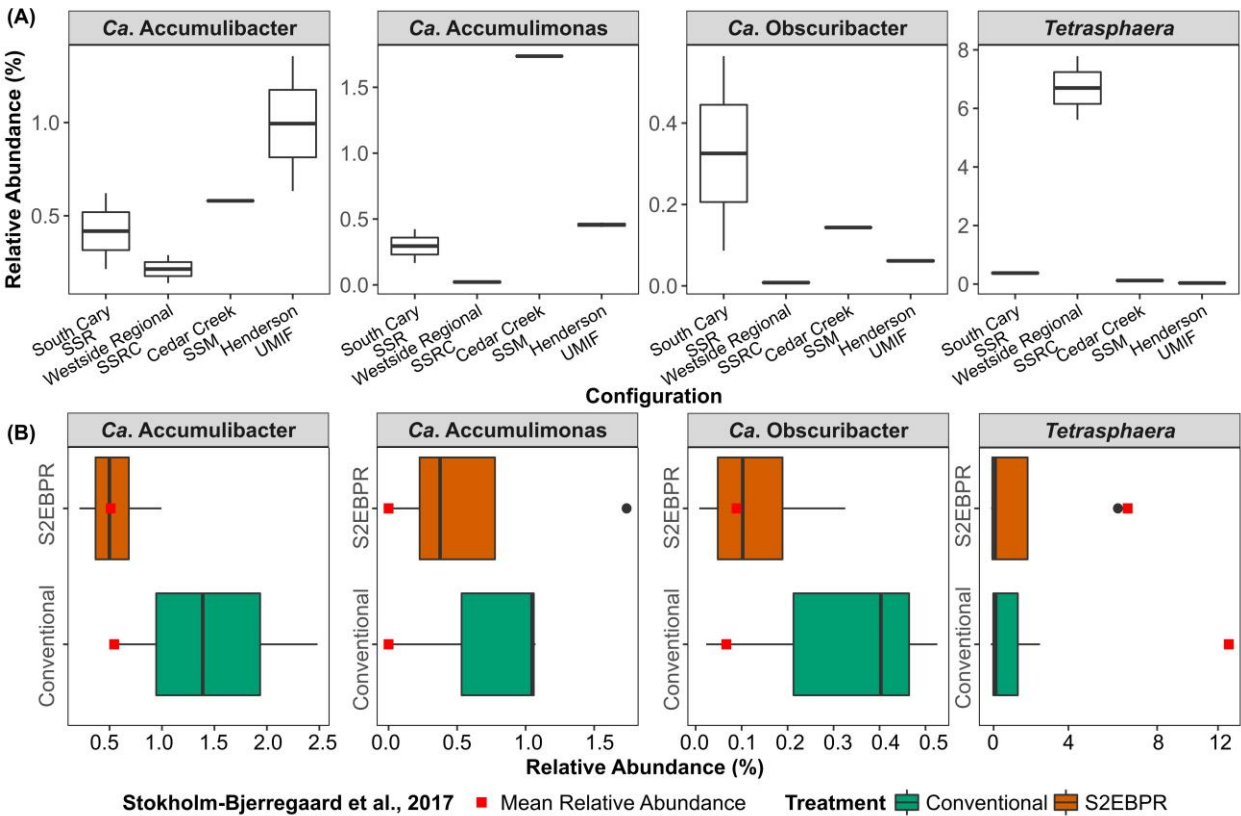


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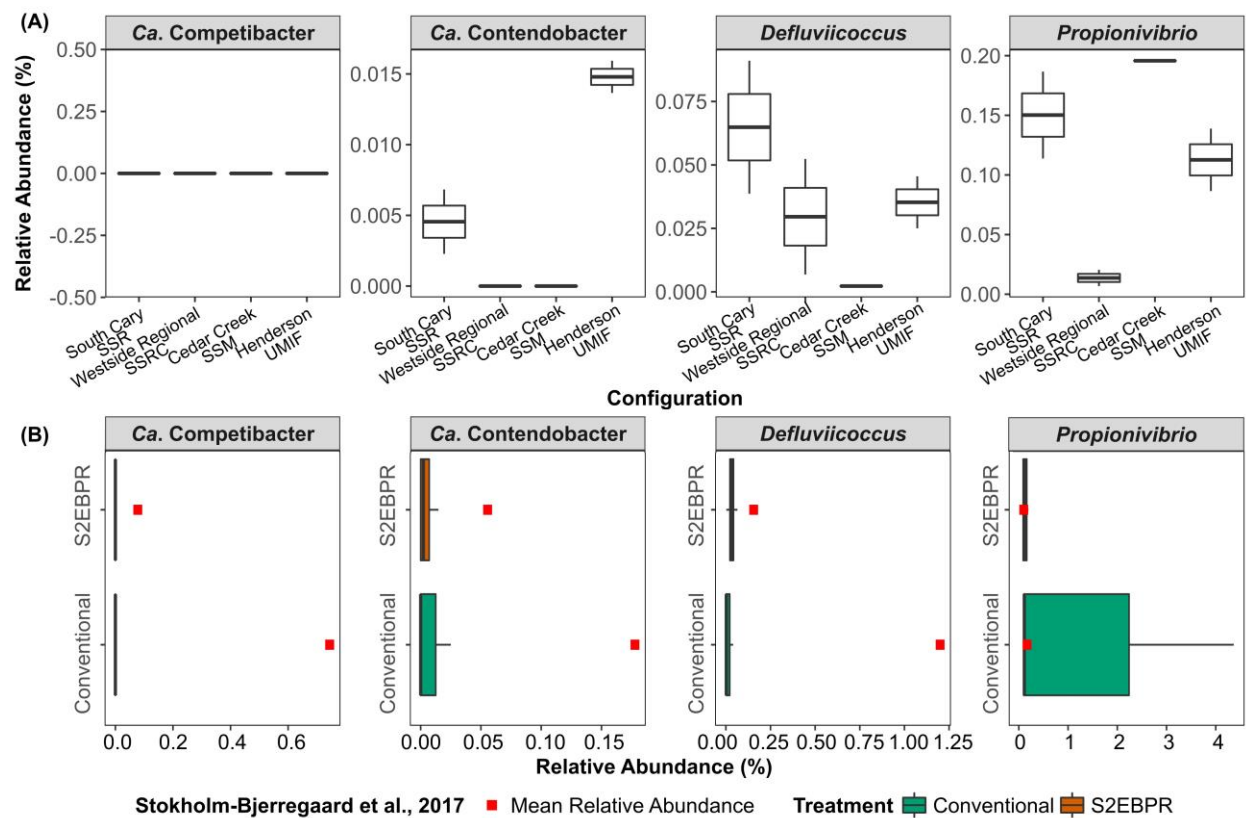


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