# MICROBIAL STRINGENT RESPONSE AND PHOSPHORUS CYCLING IN WASTEWATER

By

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# ABSTRACT

The excess discharge of soluble reactive phosphorus into water streams often enhances the growth of algae, which can lead to eutrophication. Enhanced biological phosphorus removal (EBPR) unit processes have been widely and successfully used in full-scale wastewater treatment plants (WWTP) to remove phosphorus from wastewater. Facilities use EBPR systems because of their lower capital and sludge handling costs, relative to chemical or physical processes. The EBPR process is believed to be facilitated by polyphosphate-accumulating organisms (PAOs).

EBPR inherently imposes stressful environmental conditions on bacteria, and the microbial stringent response (MSR) is a microbial response to stress that also involves phosphorus cycling. Thus, the goal of the study reported herein was to evaluate the role of the MSR in biological phosphorus cycling by activated sludge fed synthetic wastewater. To our knowledge, such a study has not been reported elsewhere. By expanding the current focus on PAOs to also include the MSR, findings from this research may lead to further optimization of existing treatment technologies and perhaps the development of new treatment methods for the biological removal of phosphorus from wastewater.

Major findings included:

(1) Phosphorus removal to undetectable levels was achieved in the fully oxic and conventional anoxic-oxic EBPR processes, with an increase in the levels of MSR indicator, ppGpp.

(2) Phosphorus cycling was associated with the MSR in four reactors.

(3) The presence of *Rhodocyclus* related PAOs or *Accumulator Phosphatis* in four reactors was insignificant, suggesting the MSR is likely a central element in the biological cycling of phosphorus within wastewater.

(4) The genetic diversity of microorganisms varied considerably between reactors. The extent of microbial diversity may suggest that engineering a system to elicit a specific structure of a mixed microbial consortium may not be as important as a particular function, namely the MSR, for the biological removal of phosphorus from wastewater. In conclusion, the MSR associated with the biological phosphorus cycling and is, most likely, responsible for phosphorus removal in a mixed culture EBPR system.

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# Chapter 1: Introduction and Motivation

## 1.1 Problem Statement

Anthropogenic activities can result in the release of nutrients into aquatic environments that lead to advanced surface water body eutrophication, which in turn can incur significant ecological and social damage (Pretty et al., 2003). In many cases, phosphorus is often the limiting macronutrient for excess algal growth, with threshold in-stream concentrations as low as 0.01-0.02 mg P $L^{-1}$ (Heathwaite et al., 1999; Seviour et al., 2003). Phosphorus is thus of primary concern in wastewater, in that it is commonly measured at effluent concentrations that could lead to advanced eutrophication of surface water bodies ( Mainstone et al., 2002; Lesjean et al., 2003; Oehmen et al., 2007). In wastewater treatment, phosphorus is commonly removed through a process referred to as enhanced biological phosphorus removal (EBPR).

In EBPR, return activated sludge is mixed with primary effluent and then subjected cyclically to anaerobic and oxic environments. The theory of EBPR stipulates that the imposed environmental conditions: (i) enrich for polyphosphate accumulating organisms (PAOs) (Mino et al., 1987; Seviour et al., 2007), and (ii) promote the biological cycling of aqueous phosphorus (Comeau et al., 1986; Mino et al,, 1987; Seviour et al., 2007). Anaerobically, PAOs (i) hydrolyze intracellular polyP and release phosphate into wastewater, and (ii) assimilate and store carbon from wastewater as polyhydroxyalkanoates (PHA). Aerobically, PAOs (i) assimilate aqueous phosphate and store it as intracellular polyP, and (ii) utilize PHA for energy, growth, and maintenance.

The quantity of aqueous phosphate assimilated oxically exceeds that released by PAOs anaerobically, thereby reducing the bulk solution phosphorus concentration. A portion of the activated sludge is wasted from the EBPR process at the end of the oxic phase to achieve net phosphorus removal.

Further refinement of our knowledge of metabolic control mechanisms in EBPR is a critical step, among many, necessary in the possible redesign of EBPR systems to meet these stringent discharge limits on a consistent basis.

### 1.2 Research Objectives and Description

The overall goal of the research reported herein was to evaluate the role of the MSR in EBPR wastewater treatment. The specific objectives were to demonstrate whether the MSR is the main mechanism responsible for phosphorus removal in EBPR systems, and whether *Accumulator Phosphates* is a dominant organism in EBPR. Four sequencing batch reactors (SBRs) seeded with a non-EBPR activated sludge obtained from a municipal wastewater treatment plant (i.e., a mixed microbial culture) were fed with synthetic wastewater. The SBRs were operated under either fully oxic or anoxic/oxic cycling conditions (i.e., to stimulate MSR) and fed either acetate or peptone. A culture-independent method, 16S rRNA gene analysis using universal bacterial primers (i.e., to reduce experimental bias) was used to determine the microbial diversity. The function of each reactor was assessed through the measure of (i) soluble chemical oxygen demand, orthophosphate, ammonia, and nitrate; and (ii) biomass concentrations of

phosphorus, polyhydroxyalkanoate, guanosine tetraphosphate, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate

### 1.4 Dissertation Plan

The results obtained in this work are organized in two chapters, as follows. Chapter 2 presents the manuscript titled *Microbial Stringent Response and Phosphorus Cycling in Wastewater Treatment*. The overall goal of the research was the investigation of the role of the MSR in the EBPR wastewater treatment process, using SBRs fed with synthetic wastewater and seeded with a non-EBPR activated sludge derived from an activated sludge wastewater treatment plant. The specific objectives were the assessment of the impact of i) nitrogen limitation, and ii) electron acceptor cycling on the MSR indicator, ppGpp, and on phosphate removal.

Finally Chapter 3 summarizes the contributions of this study, provides the final conclusions of this dissertation and presents guidelines for future work.

# Chapter 2: Microbial Stringent Response and Phosphorus Cycling in Wastewater Treatment

**Microbial Stringent Response and Phosphorus Cycling in Wastewater Treatment**

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## ABSTRACT

Four bench-scale sequencing batch reactors (SBRs) seeded with activated sludge were operated under either fully oxic or anoxic/oxic conditions and fed synthetic wastewater containing either peptone or acetate. A culture-independent method was used to determine community structure. The function of each reactor was assessed through the measure of (i) soluble chemical oxygen demand, orthophosphate, ammonia, and nitrate; and (ii) biomass concentrations of phosphorus, polyhydroxyalkanoate, guanosine tetraphosphate, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate. The mixed microbial consortium in each of the four reactors cycled phosphorus. Phosphorus cycling was associated with the MSR, suggesting the MSR is likely a central element in the biological cycling of phosphorus within wastewater. With the recognition of the role of MSR on phosphorus cycling within wastewater, additional research may lead to further optimization of existing treatment technologies and the development of new treatment systems for the biological removal of phosphorus from wastewater. The genetic diversity of microorganisms varied considerably between reactors. The extent of microbial diversity may suggest that engineering a system to elicit a specific structure of a mixed microbial consortium may not be as important as a particular function, namely the MSR, for the biological removal of phosphorus from wastewater.


**Keywords:** Enhanced biological phosphorus removal, wastewater treatment, phosphate accumulating organisms, polyhydroxyalkanoate, guanosine tetraphosphate.

## 1. Introduction

In pure microbial cultures, the biological storage of phosphorus has been shown to involve the transfer of the terminal phosphate of adenosine triphosphate (ATP) to a short-chain polyphosphate (polyP) using polyP kinase 1 (PPK1) or polyP kinase 2 (PPK2) (Rao et al., 2009). PPK can also catalyze the reverse reaction, resulting in polyP hydrolysis. PolyP hydrolysis to soluble orthophosphate has also been attributed to exopolyphosphatase (PPX1 and PPX2) (Ault-Riché et al., 1998). Control of intracellular polyP levels is linked with the inactivation of PPX or stimulation of PPK, with the general metabolism referred to as the microbial stringent response (MSR) ( Kuroda et al., 1997; Ault-Riché et al., 1998). The MSR is a global regulatory system that functions as an important cellular survival mechanism under conditions of stress (Chatterji et al., 2001). The MSR is characterized, in part, by accumulation of the alarmones guanosine tetra- (ppGpp) and penta-phosphate (pppGpp) (Kuroda et al., 1997), and the synthesis of (p)ppGpp is regulated through expression of the RelA and/or SpoT (Potrykus et al., 2008). The MSR is stimulated, in part, by unbalanced growth conditions, and can be elicited by subjecting cells to nutritional or osmotic stress (Ault-Riché et al., 1998), amino acid starvation (Kuroda et al., 1997), a downshift in aqueous pH (Wells et al., 2006), or changes in terminal electron acceptor ( Glass et al., 1979; Mouery et al., 2006).

Anthropogenic activities can result in the release of nutrients into aquatic environments that lead to advanced surface water body eutrophication, which in turn can incur significant ecological and social damage (Pretty et al., 2003). In many cases,

phosphorus is often the limiting macronutrient for excess algal growth, with threshold in-stream concentrations as low as 0.01-0.02 mg P $L^{-1}$ (Heathwaite et al., 1999; Seviour et al., 2003). Phosphorus is thus of primary concern in wastewater, in that it is commonly measured at effluent concentrations that could lead to advanced eutrophication of surface water bodies ( Mainstone et al., 2002; Lesjean et al., 2003; Oehmen et al., 2007). In wastewater treatment, phosphorus is commonly removed through a process referred to as enhanced biological phosphorus removal (EBPR). In EBPR, return activated sludge is mixed with primary effluent and then subjected cyclically to anaerobic and oxic environments. The theory of EBPR stipulates that the imposed environmental conditions: (i) enrich for polyphosphate accumulating organisms (PAOs) (Mino et al., 1987; Seviour et al., 2007), and (ii) promote the biological cycling of aqueous phosphorus (Comeau et al., 1986; Mino et al,, 1987; Seviour et al., 2007). Anaerobically, PAOs (i) hydrolyze intracellular polyP and release phosphate into wastewater, and (ii) assimilate and store carbon from wastewater as polyhydroxyalkanoates (PHA). Aerobically, PAOs (i) assimilate aqueous phosphate and store it as intracellular polyP, and (ii) utilize PHA for energy, growth, and maintenance. The quantity of aqueous phosphate assimilated oxically exceeds that released by PAOs anaerobically, thereby reducing the bulk solution phosphorus concentration. A portion of the activated sludge is wasted from the EBPR process at the end of the oxic phase to achieve net phosphorus removal.

With the general acceptance of anaerobic/oxic cycling as a prerequisite to induce EBPR, research has largely focused on identifying PAOs. From the 1970’s to mid 1990’s, *Acinetobacter* was commonly believed to be the principal PAO responsible for

phosphorus removal in EBPR processes (Seviour et al., 2007). Through advancements in molecular techniques, *Rhodocyclus* has been identified as the putative predominate organism in EBPR processes (Seviour et al., 2007), with recognition of the role of other microorganisms in phosphorus removal ( Zilles et al., 2002; Kawaharasaki et al., 2002; Seviour et al., 2007; Majed et al., 2009). Recent proteomic and metagenomic analyses of mixed microbial consortia have shown that the majority of the microorganisms are *Accumulator phosphatis* (a member of the *Rhodocyclus* genus) (Martin et al., 2006; Wilmes et al., 2008). While research on putative PAOs has certainly been productive toward better understanding the EBPR process, the mechanisms driving phosphorus cycling also must be better understood if biological methods (rather than the less environmentally sustainable chemical methods) are to be more predominantly applied.

EBPR inherently imposes stressful environmental conditions on bacteria, and the MSR is a microbial response to stress that also involves phosphorus cycling. Thus, the goal of the study reported herein was to evaluate the role of the MSR in biological phosphorus cycling by activated sludge fed synthetic wastewater. To our knowledge, such a study has not been reported elsewhere. By expanding the current focus on PAOs to also include the MSR, findings from this research may lead to further optimization of existing treatment technologies and perhaps the development of new treatment methods for the biological removal of phosphorus from wastewater.

# 2. Materials and Methods

## *2.1. Experimental Section*

Bench-scale sequencing batch reactors (SBRs) inoculated with a non-EBPR activated sludge obtained from a municipal wastewater treatment plant were fed synthetic wastewater. The SBRs were operated under either fully oxic or anoxic/oxic conditions and fed either peptone (an undefined media originating from the enzymatic digest of animal protein) or a defined medium containing acetate. A culture-independent molecular method, 16S rRNA gene analysis using universal bacterial primers, was applied to determine community structure. The function of each reactor was assessed through the measure of (i) soluble chemical oxygen demand (sCOD), orthophosphate, ammonia, and nitrate; and (ii) biomass concentrations of phosphorus, PHA, ppGpp, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP). Concentrations of AMP, ADP, and ATP are reported in terms of the adenylate energy charge (EC).

## *2.2. Operation of sequence batch reactors*

Four bench-scale SBRs were fabricated from an acrylic glass column with working volumes of 4 L. The SBRs were fed synthetic wastewater and inoculated with activated sludge derived from the University of California Davis (UCD) wastewater treatment

plant (WWTP), which is operated as an extended aeration activated sludge process with a mean cell residence time (MCRT) of 20 days. Each laboratory reactor was operated with three feed-cycles per day, a hydraulic residence time (HRT) of 1 day and MCRT of 16 days, at a controlled room temperature of 21±1$^{o}$C. Each feed-cycle consisted of 1.5 hours settling, 0.25 hour decanting, 0.25 hour feed addition, and 6 hours reaction. Two SBRs were operated with a fully oxic reaction period, while the other two SBRs were operated with a reaction period consisting of 2.5 hours of pre-anoxic conditions and 3.5 hours of oxic conditions. Air was supplied (1.5 L min$^{-1}$) to provide oxygen and mixing, regulated by an air flow meter (Cole-Parmer Instrument Co, Vernon Hills, USA). Nitrogen gas was provided by a nitrogen tank with a regulator, and was used to facilitate a dissolved oxygen concentration of approximately zero during the anoxic period of the anoxic-oxic SBRs. MasterFlex pumps (L/S 7553-70, Cole-Parmer Instrument Co, Vernon Hills, USA) were used for feeding and decanting. The MasterFlex pumps were connected to digital timers (Model DT 17, Intermatic Incorporated, Spring Grove, USA) to control the feed, reaction, settling, and decant periods.

### *2.3. Synthetic wastewater*

The synthetic wastewater contained per liter either 2 g peptone (Bacto peptone, BD, Franklin Lakes, USA) or 2 g sodium acetate ($CH_3COONa$); 0.8 g $NaHCO_3$; 0.25 g $KH_2PO_4$; 0.30 g $NH_4Cl$; 0.42 g KCl; 0.42 g $CaCl_2 \cdot 2H_2O$; 0.40 g $MgSO_4 \cdot 7H_2O$; 5.5 mg $C_6H_8O_7 \cdot H_2O$; 3.03 mg $FeCl_3 \cdot 6H_2O$;, 0.5 mg $H_3BO_3$; 0.18 mg KI; 0.03 mg $CuSO_4 \cdot 5H_2O$;

0.12 mg $MnCl_2 \cdot 4H_2O$; 0.06 mg $Na_2MoO_4 \cdot 2H_2O$; 0.12 mg $ZnSO_4 \cdot 7H_2O$; 0.15 mg $CoCl_2 \cdot 6H_2O$; and 10 mg EDTA (Goel et al., 2006). All chemicals were greater than 99.2% (w/w) grades and obtained from either Fisher Scientific (Fair Lawn, USA) or EMD Chemicals (Gibbstown, USA).

### *2.4. Analytical methods*

Reactor performance over the acclimation period (first six months of operation) was monitored every two weeks by sampling for sCOD, phosphorus ($PO_4^{3-}$-P), ammonia ($NH_3$-N), and nitrate ($NO_3^-$-N); and mixed liquor suspended solids (MLSS) at the beginning and at end of the entire feed-cycle. Following the acclimation period, sampling was performed over the course of a single feed-cycle once every two months for a total time period of two years. During a typical sampling event for the anoxic/oxic SBRs, seven samples were collected: one each at the beginning and end; two samples during the anoxic period; one sample at the end of the anoxic period; and two samples during the oxic period. A 20 mL sample was recovered from each reactor for MLSS and mixed liquor volatile suspended solids (MLVSS) analyses in accordance with Standard Method 2540 E (American Public Health Association, 2005). Another 12 mL sample was recovered from each reactor, filtered through sterilized 0.22-µm filters (Millipore Corporation, Billerica, USA), and analyzed for soluble sCOD, phosphorus, ammonia, and nitrate. The sCOD test was performed in accordance with Standard Methods 5220-D (American Public Health Association, 2005) using Hach low-range ampules (Hach

Company, Loveland, USA). Ammonia and nitrate concentrations were measured using a continuous-flow inorganic nitrogen analyzer (Timberline Model 383, Timberline Instruments Inc., Boulder, USA). Concentrations of soluble phosphorus were measured using Hach method 8048 (Hach Company, Loveland, USA). The Hach method is equivalent to method 4500-PE of Standard Methods (American Public Health Association, 2005). A 50 mL sample was also collected from each reactor, centrifuged, and stored at -80°C for microbial analyses. Cell dry weight (CDW) was estimated from a 10 mL sample, which was centrifuged for 10 min at 4,000 rpm and then rinsed once with Milli-Q water before drying at 105ºC overnight.

Dry biomass PHA content was determined by gas chromatography/mass spectrometry (GC/MS) as previously described by Braunegg et al. (1978). Briefly, 40 mL biomass samples were collected and inactivated by adding 4 mL of commercial grade bleach (10% sodium hypochlorite) (Clorox, Oakland, USA) and centrifuged at 2500 rpm for 15 min. The pellet was then dried overnight at 60 ºC. The dried biomass samples were digested at 100 ºC in 2 mL each of acidified methanol (3% [v/v] sulfuric acid) (EMD Chemicals, Gibbstown, USA) and chloroform (Fisher Scientific, Fair Lawn, USA). Benzoic acid was added to the chloroform as an internal standard. Following vortexing of the mixture with 1mL deionized water, PHA-rich chloroform was recovered for analysis. The chloroform phase was dehydrated by filtering the PHA-rich solution through sodium sulfate before analysis. GC-MS was performed with split injection under an initial oven temperature of 40°C (2 min) ramped up to 200°C at 5°C $min^{-1}$ using a 30m ZB-624 column (0.25mm i.d., 1.4µm film; Phenomenex, Torrance, USA). The compounds

(methyl ester derivatives) were scanned by comparing the MS spectra in Wiley 275 library to confirm PHA forms. The specified PHA polymers were identified by retention time and mass spectral matching 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV) and quantified based on the external and internal standards. Total cellular PHA content was determined on a dry weight basis (e.g., mass PHA/mass of dry biomass, [w/w]).

Dry biomass phosphorus content was determined through sulfuric acid digestion of the sludge in accordance with Standard Methods 4500-PB (American Public Health Association, 2005), and expressed on a dry weight basis (w/w). Briefly, 25 mL samples were collected from each reactor and centrifuged at 2500 rpm for 15 min (J6-HC, Beckman Coulter Inc., Fullerton, USA). The supernatant was discarded and the pellet stored at 4ºC. The pellet was then dried overnight at 105ºC and placed in a digester vial with the addition of 1 mL of Milli-Q water (Millipore Corp, Billerica, USA), 1 g of glass beads (diameter of 0.40-0.60 mm, Glasperlen, B. Braun Biotech International, Germany), and 10 mL sulfuric acid ($H_2SO_4$; 95.08% w/w) (EMD Chemicals, Gibbstown, USA). The samples were placed on a preheated digester (block digester Model BD-46, Lachat instruments, Hach Company, Loveland, USA) and digested for 2 hours and 15 min at 320ºC. The samples were cooled to room temperature and filtered through sterilized 0.22 µm filters (Millipore Corp, Billerica, USA) into 100 mL beakers and diluted to 100 mL by the addition of Milli-Q water. The soluble reactive phosphorus was then quantified, in accordance with Hach method 8048, using Hach low-range ampules (Hach Company, Loveland, USA).

Dry biomass concentrations of ppGpp, AMP, ADP, and ATP were determined using high performance liquid chromatography (HPLC), as described by Neubauer et al. (Neubauer et al., 1995) with the addition of 0.3 g of glass beads (diameter of 0.40-0.60 mm, Glasperlen, B. Braun Biotech International, Germany) during sonication to improve cell lysis. Briefly, 5 mL samples were collected from the reactor with a 5-mL disposable syringe and placed in 15 mL tubes pre-loaded with 100 µL formaldehyde (38%, w/w) (Sigma-Aldrich, St. Louis, USA) to cease cellular activities (sample handling was performed on ice). The sample was mixed vigorously for 10 sec and centrifuged at 2500 rpm for 15 min (J6-HC, Beckman Coulter Inc., Fullerton, USA). The supernatant was discarded and the pellet stored either at 4 ºC for same day analysis or at -80 ºC after flash freezing (using liquid nitrogen) for later analysis. The pellet was re-suspended in freshly prepared 0.1 N potassium hydroxide (KOH) (100-µL $(mg\ CDW)^{-1}$) and incubated on ice for 30 min. Ultrasonic treatment (Misonix incorporated, Farmingdale, USA) was performed at approximately 8 W for 90 sec. To adjust the pH following extraction, a freshly prepared HPLC buffer containing 125 mM of monopotassium phosphate ($KH_2PO_4$), 10 mM tetrabutyle ammonium dihydrogen phosphate ($(C_4H_9)4N(H_2PO_4)$) (GFS Chemicals Inc., Columbus, USA) , 1.49 M methanol ($CH_4O$) (Fisher Scientific, Fair Lawn, USA), and 75 mM sodium hydroxide (NaOH) (Fisher Scientific, Fair Lawn, USA) was added to the pellet (100 µL $(mg\ CDW)^{-1}$), which was then centrifuged at 12,000 rpm (Eppendorf centrifuge 5417 R, Eppendorf, Germany) for 5 min. The supernatant was filtered through sterilized 0.22-µm filters (Millipore Corp, Billerica, USA) prior to HPLC analysis. The adenylate energy charge (EC), which represents an

estimate of the amount of metabolically available energy stored in the adenylate pool, was calculated per equation (1) (Chapman et al., 1971):

$$EC = \left[\frac{ATP + (0.50 \times ADP)}{(AMP + ADP + ATP)}\right] \qquad (1)$$

HPLC analyses of the extract samples were performed using a HP1100 series HPLC system (Agilent Technologies Inc., Santa Clara, USA) and an Agilent ChemStation system consisting of two pumps, an autosampler, and a diode array detector. Ion-pair reverse-phase chromatography was performed with a Supelcosil™ LC-18-T column (15 cm × 3 mm, 3-µm particle size) connected to a Supelguard LC-18-T guard column (2 cm × 3 mm) (Supelco, Bellefonte, USA). The temperature was set at 25ºC, the flow rate of the eluent (HPLC buffer) at 0.50 mL $min^{-1}$, and absorbance detection at 252 nm. The injection volume was set at 10 µL, with a needle wash after injection. A sample containing only Milli-Q water and methanol was analyzed after every 10 samples as a QA/QC data quality procedure.

### *2.5. DNA extraction, PCR, and cloning*

Following an operational period of approximately one year, a 50 mL sludge sample was collected from each reactor, centrifuged, and stored at -80°C for microbial community analysis. Deoxyribonucleic acid (DNA) was extracted from each sample using the FastDNA Spin for Soil Kit (MP Biomedicals, Cleveland, USA) according to the

manufacturer's instructions. The extracted DNA was used as template for polymerase chain reaction (PCR).

PCR amplification of the bacterial 16S rDNA gene sequence was performed using an Applied Biosystems Model 2400 Thermal Cycler (Foster City, USA) with universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTG TTACGACTT-3′). The 50 μL reaction volume contained a reaction mixture of 5 μL PCR buffer; 4 μL of deoxynucleoside triphosphate (dNTP) (Applied Biosystems, Carlsbad, USA); 4 μL magnesium chloride ($MgCl_2$) (Fisher Scientific, Fair Lawn, USA); 2 μL F-primer (8F); 2 μL R-primer (1492R); 0.2 μL of Taq DNA polymerase; 0.5 μL of BSA; 2 μL of template (DNA); and 30.3 μL of sterilized reagent grade water (Sigma-Aldrich, St. Louis, USA). Amplification was performed with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72 °C for 1.5 min, and final extension at 72°C for 10 min. PCR products were verified visually using 1.5% agarose gel electrophoresis in 1X Tris/Borate/EDTA (TBE) and ethidium bromide Green I staining (SYBR) (Qiagen, Valencia, USA).

The amplified PCR fragments were inserted into TOPO vector containing kanamycin resistance gene and the constructed plasmids were transformed into *E. coli* competent cells (Invitrogen Corporation, Carlsbad, USA). The transformed *E. coli* cells were first grown on an agar plate with 50 μg $mL^{-1}$ kanamycin (Invitrogen Corporation, Carlsbad, USA). Clones were then selected and grown in LB liquid medium (USB Corporation, Cleveland, USA) with 50 μg $mL^{-1}$ kanamycin. Plasmid extraction from

individual clones was achieved by using microcolumns following manufacturer guidelines (Qiagen Sciences Inc., Germantown, USA).

### *2.6. DNA sequencing and phylogenetic analysis*

DNA sequencing was performed at the UC Berkeley Sequencing Facility (Berkeley, USA). A total of 95 microbial clones were randomly obtained for each reactor, with sequences audited using Bioedit (Hall, 1999) to remove indistinct reads. The 16S rRNA gene sequences were aligned with the National Center for Biotechnology Information program Muscle using default settings (Edgar, 2004). Each sequence was evaluated against the nucleotide database Blastn (Camacho et al., 2009), excluding uncultured/environmental sample sequences. The taxonomy of the aligned sequences was confirmed by Ribosomal Database Project, with 95% confidence, using a Bayesian classifier (Wang et al., 2007). Phylogenetic trees were constructed by means of the software tool SplitsTree (Huson et al., 2006) using the neighbor-joining method and bootstrap re-sampling analysis with 1000 iterations.

## 3. Results

### *3.1. Stabilization of SBRs*

Stable operating conditions were reached within six months of operation for Reactors 1 (fully oxic fed peptone) and 2 (fully oxic fed acetate) and within three months for Reactors 3 (anoxic/oxic fed peptone) and 4 (anoxic/oxic fed acetate). MLVSS in the SBRs stabilized at 3,000-3,200 mg $L^{-1}$ for Reactor 1, 3,400-3,800 mg $L^{-1}$ for Reactor 2, 3,300-3,700 mg $L^{-1}$ for Reactor 3, and 4,000-4,300 mg $L^{-1}$ for Reactor 4.

### *3.2. Performance of anoxic/oxic SBRs*

During the anoxic period in the peptone-fed anoxic/oxic reactor (Reactor 3; Fig. 1): (i) sCOD was converted into PHA (Fig. 1a), (ii) biomass phosphorus was cleaved and released into solution (Fig. 1c), (iii) the concentration of $NH_3$-N increased (likely from ammonification associated with hydrolysis of peptone, which is an organic nitrogen rich carbon source), and (iv) the concentration of $NO_3^-$-N was below the detection limit for all but the first 10 min (i.e., the anoxic period was thus largely anaerobic) (Fig. 1b). During the subsequent oxic period: (i) biomass PHA was consumed for microbial growth and cell maintenance (Fig. 1a), (ii) aqueous phosphorus was incorporated into biomass (Fig. 1c), and (iii) the concentration of $NH_3$-N decreased with a concurrent increase in $NO_3^-$-N (indicative of nitrification) (Fig. 1b). Overall, in regard to the cycling of phosphorus, PHA, and sCOD, the mixed microbial consortium performed according to EBPR theory. Of particular note, the MSR, reflected in the temporal biomass concentration of ppGpp, appeared correlated with the cycling of phosphorus. During the anoxic/anaerobic period the biomass concentration of phosphorus and ppGpp decreased from 102 to 88 mg

biomass phosphorus (g CDW)$^{-1}$ (Fig. 1c) and 394 to 177 nMole ppGpp (g CDW)$^{-1}$ (Fig. 1d), respectively. Concentrations subsequently increased to 105 mg biomass phosphorus (g CDW)$^{-1}$ and 859 nMole ppGpp (g CDW)$^{-1}$, respectively, by the end of the oxic period. Applying a mass balance on phosphorus (P), the increase in CDW P aligns with the actual quantity of P removed from bulk solution. Biomass ppGpp was correlated with the metabolically available energy stored in the adenylate pool, as represented by EC. The EC level decreased from 0.98 to 0.81 over the anoxic period, and subsequently increased to a value of 0.93 by the end of the oxic period (Fig. 1d). Generally, the trend in biomass ppGpp concentration (Fig. 1d) was observed to be inversely proportional to the biomass concentration of PHA (Fig. 1a).

The mixed microbial consortium in Reactor 4, which in contrast to Reactor 3 was fed acetate (i.e., idealized carbon substrate for EBPR), generally cycled sCOD, phosphorus, and ammonia during the anoxic period similarly to that observed in Reactor 3 (Figs. 1 vs. 2). Specifically, (i) sCOD was converted into biomass PHA (Fig. 2a), (ii) biomass phosphorus was cleaved and released into solution (Fig. 2c); and (iii) the concentration of $NH_3$-N decreased (Fig. 2b). The concentration of $NO_3^-$-N was below the detection limit (i.e., the anoxic period was anaerobic) (Fig. 2b). In the subsequent oxic period: (i) biomass PHA was consumed (Fig. 2a), (ii) aqueous phosphorus was incorporated into biomass (Fig. 2c), and (iii) the concentration of $NH_3$-N decreased below the detection limit resulting in a nitrogen limitation (i.e., non-detectable concentrations of both $NH_3$-N and $NO_3^-$-N) (Fig. 2b). Similar to that observed in Reactor 3, in regard to the cycling of phosphorus, PHA, and sCOD, the mixed microbial consortium performed

according to EBPR theory. Similar to that observed with Reactor 3, the quantity of P removed from bulk solution aligns well with the increase in CDW P. Again the MSR, reflected in the biomass concentration of ppGpp, appeared correlated with the cycling of phosphorus, with the biomass concentration of phosphorus and ppGpp decreasing in concert anoxically (Figs. 2c and 2d), and then subsequently increasing oxically. In addition, biomass ppGpp again appeared correlated with the metabolically available energy (assessed as EC) stored in the adenylated pool. The EC level decreased from 0.84 to 0.69 over the anoxic period, and subsequently increased to a value of 0.82 by the end of the oxic period (Fig. 2d). The biomass concentration of ppGpp (Fig. 2d) cycled in a pattern inversely to the biomass concentration of PHA (Fig. 2a). The primary observed difference between Reactors 3 and 4 performance was that the latter realized a limitation of inorganic nitrogen during the oxic phase, thereby imposing additional stress on the microbes.

### *3.3. Performance of fully oxic SBRs*

As a contrast to the cyclically anoxic/oxic Reactors 3 and 4, Reactors 1 and 2 were operated under fully oxic conditions; substrate conditions, however, were identical. For Reactor 1 (peptone fed), immediately after receiving fresh substrate the mixed microbial consortium exhibited a feast response (i.e., rapid sCOD depletion concurrent with PHA synthesis); this response is consistent with feast-famine PHA synthesis (Dionisi et al., 2004). The feast period, which occurred within the first 10 min of the cycle, was characterized by a: (i) rapid decline in sCOD (Fig. 3a), (ii) rapid increase in biomass

PHA (Fig. 3a), (iii) release of biomass phosphorus into solution (Figure 3c), and (iv) increase in $NH_3$-N (likely from ammonification) and a statistically insignificant decrease in $NO_3^-$-N (Fig. 3b). During the famine period (herein defined as occurring immediately following peak PHA synthesis): (i) biomass PHA was consumed (Fig. 3a), (ii) aqueous phosphorus was incorporated into biomass (Fig. 3c), and (iii) the concentration of $NH_3$-N decreased with a concurrent increase in $NO_3^-$-N (indicative of nitrification) (Fig. 3b). Of note, PHA degradation and residual sCOD utilization occurred concurrently in the famine period, suggesting either that some of the sCOD could not be converted to PHA or that non-PHA synthesizing microbes were also present in the consortium; sCOD was depleted within the first three hours of operation. Overall, the mixed microbial consortium in Reactor 1 did not perform according to EBPR theory because of a lack of anaerobic/oxic cycling, yet phosphorus was nonetheless cycled in the reactor. Phosphorus cycling under fully oxic conditions has been observed by others (Pijuan et al., 2005; Vargas et al., 2009). The MSR, reflected in the biomass concentration of ppGpp, again appeared correlated with phosphorus cycling. The biomass concentration of phosphorus and ppGpp decreased from 47.1 to 46.4 mg biomass phosphorus (g CDW)$^{-1}$ and 373 to 343 nMole ppGpp (g CDW)$^{-1}$, respectively, during the feast period, and subsequently increased to a value of 49.3 mg biomass phosphorus (g CDW)$^{-1}$ and 506 nMole ppGpp (g CDW)$^{-1}$, respectively, by the end of the famine period. A mass balance analysis revealed that the quantity of P removed from bulk solution accounted for the increase in CDW P. Again, biomass ppGpp appeared to correlate with the metabolically available energy stored in the adenylate pool; the microbial EC increased from a value of 0.51 to 0.62 by the end of

the famine period (Fig. 3d). Finally, the temporal biomass concentration of ppGpp (Fig. 3d) was observed to be inversely proportional to the biomass concentration of PHA (Fig. 3a).

The mixed microbial consortium in Reactor 2 – which was fed acetate – generally cycled nutrients similar to that observed in Reactor 1, which was fed peptone. The feast period, which lasted approximately the first 5 min of the cycle, was characterized by: (i) a rapid decline in sCOD (Fig. 4a), (ii) an increase in biomass PHA (Fig. 4a), (iii) a release of biomass phosphorus into solution (Fig. 4c), (iv) a decrease in $NH_3$-N, and (v) a non-detectable concentration of $NO_3^-$-N (Fig. 3b). Of note, PHA accumulation by the Reactor 2 mixed microbial consortium was most significant of all reactors tested; such a response could be expected, given that operational conditions and carbon source were ideal for feast/famine PHA synthesis (Coats et al., 2007; Coats et al., 2010). During the subsequent famine period: (i) biomass PHA was consumed (Fig. 4a), (ii) aqueous phosphorus was incorporated into biomass (Fig. 4c), and (iii) the concentration of $NH_3$-N decreased below the detection limit, ultimately resulting in a nitrogen limitation (e.g., non-detectable concentrations of both $NH_3$-N and $NO_3^-$-N) (Fig. 2b). Similar to Reactor 1, the mixed microbial consortium in Reactor 2 did not perform according to EBPR theory because of a lack of anaerobic/oxic cycling, however, significant phosphorus removal was nonetheless observed. Further, the MSR, reflected in the biomass concentration of ppGpp, again appeared correlated with phosphorus cycling. The biomass concentration of phosphorus and ppGpp decreased from 73 to 72 mg biomass phosphorus (g CDW)$^{-1}$ and 834 to 630 nMole ppGpp (g CDW)$^{-1}$, respectively, during the feast period, and

subsequently increased to a value of 76 mg biomass phosphorus (g CDW)$^{-1}$ and 1190 nMole ppGpp (g CDW)$^{-1}$, respectively, by the end of the famine period. While the relative changes in CDW P are certainly small, similar to that observed with the other three reactors, the quantity of P removed from bulk solution aligns well with the increase in CDW P. The biomass concentration of ppGpp (Fig. 4d) was also inversely proportional to the biomass concentration of PHA (Fig. 4a). In addition, biomass ppGpp was observed to be correlated with the metabolically available energy stored in the adenylated pool; the microbial EC increased from a value of 0.73 to 0.78 by the end of the famine period (Fig. 4d).

### ***3.4. Microbial community composition in the SBRs***

The 56 clones obtained from the fully oxic reactor fed peptone (Reactor 1) were categorized into the following phylotypes: *Proteobacteria* (64%), *Bacteroidetes* (21%), *Verrucomicrobia* (11%), and *Gemmatimonadetes* (4%). The primary genera within these phylotypes (Fig. 5a) included: an unknown *Alphaproteobacteria* (32%), an unknown *Bacteroidetes* (17%), and an unknown *Burkholderiales* (9%). The 80 clones obtained from the fully oxic reactor fed acetate (Reactor 2) were categorized into the following phylotypes: *Proteobacteria* (84%), *Bacteroidetes* (11%), and *Verrucomicrobia* (5%). The primary genera within these phylotypes (Fig. 5b) included: *Paracoccus* (28%), an unknown *Xanthomonadales* (17%), and *Pedobacter* (11%). The 75 clones obtained from the anoxic/oxic reactor fed peptone (Reactor 3) were categorized into the following

phylotypes: *Proteobacteria* (49%), *Bacteriodetes* (47%), and *TM7* (4%). The primary genera within these phylotypes (Fig. 6a) included: *Haliscomenobacter* (40%) and an unknown *Alphaproteobacteria* (20%). Finally, the 70 clones obtained from the anoxic/oxic reactor fed acetate (Reactor 4) were categorized into the following phylotypes: *Proteobacteria* (39%), *Bacteriodetes* (47%), *Verrucomicrobia* (6%), *Firmicutes* (4%), *Acidobacteria* (1%), and *Clostridia* (3%). The primary genera within these phylotypes (Fig. 6b) included: *Haliscomenobacter* (41%) and *Hydrogenophaga* (18%).

## 4. Discussion

### *4.1. Phosphorus cycling and the microbial stringent response*

The primary objective of this research was to investigate the potential linkage between the microbial stringent response and phosphorus cycling by mixed microbial consortia. In all tested reactors, the consortia were subjected to environmental stress (both imposed and realized), and the MSR was quantified by measuring intracellular ppGpp concentrations and by calculating the EC. As shown, (Figs. 1-4), the respective consortium in each of the four reactors did in fact cycle phosphorus, and with the exception of Reactor 1 (peptone-fed fully oxic), excellent phosphorus removal was observed. Based on ppGpp measurements, phosphorus cycling appears to have been associated with the MSR. For the reactors subjected to anoxic/oxic cycling (Reactors 3

and 4; Figs. 1 and 2), the MSR may have been induced individually or by a combination of the following factors: the ultimate lack of a terminal electron acceptor during the anoxic period, nutritional stress generated by the feast-famine cycling of organic carbon over the course of a given cycle, and/or a cyclical limitation in inorganic phosphorus. In addition to these factors, the consortium in Reactor 4 further realized stress associated with a nitrogen limitation (Fig. 2b). For the fully oxic reactor fed peptone (Reactor 1; Fig. 3), the MSR appeared to be triggered by the feast-famine cycling of carbon over the course of a given feed-cycle, although ammonia depletion may have played a contributing role (since the use of nitrate as a nitrogen source (for amino acid synthesis, etc.) is not energetically favorable). Similarly, in the fully oxic reactor fed only acetate as the organic carbon source (Reactor 2; Fig. 4), the MSR was likely triggered individually or a combination of carbon limitation, nitrogen limitation, and/or phosphorus limitation. In particular, for both Reactors 1 and 2, note that the ppGpp concentration also increased significantly following the depletion of ammonia.

In pure culture, the accumulation of phosphorus has been demonstrated to involve the inhibition of exopolyphosphatase (PPX) and activation of polyphosphate kinase (PPK) by, in part, the RelA- and SpoT-dependent pathways (Chatterji et al., 2001). ppGpp likely works at the transcription level in response to environmental stress (Potrykus et al., 2008) and has been shown to inhibit PPX but not PPK (Ault-Riché et al., 1998). Hence, the MSR may be associated with phosphorus cycling in the respective mixed microbial consortium investigated in this study through transcriptional regulation of *ppx*. However, regardless of the specific mechanism for the association between the

MSR and phosphorus cycling, the MSR appears to be an important metabolic response associated with the biological cycling of phosphorus. Anaerobic/oxic cycling is one of many environmental conditions that can be engineered to elicit the MSR, and hence, phosphorus removal within wastewater treatment. With the recognition of the role of MSR on phosphorus cycling within wastewater, additional research may lead to further optimization of existing treatment technologies and the development of new treatment systems for the biological removal of phosphorus from wastewater.

As described, the genetic diversity of microorganisms varied considerably between reactors. Although the specific role that each organism played in the cycling of phosphorus is unclear, all the phylotypes identified in the respective reactors aligned with species capable of producing PHA and accumulating phosphorus (with the exception of *Haliscomenobacter*, which store carbon as polysaccharide (i.e., not PHA)) (Dworkin et al., 2006). In particular, the number of sequences belonging to *Rhodocyclus*, a group of organisms commonly believed to predominate in EBPR systems (Martin et al., 2006; Wilmes et al., 2008), were 0%, 9%, 3%, and 9% for Reactors 1, 2, 3, and 4, respectively. Note that Reactor 1, which contained negligible *Rhodocyclus spp.*, removed the least phosphorus. These values are significantly below the values reported by Wilmes et al. (2008). The discrepancy in the abundance of *Rhodocyclus* may result from differences between the two studies in media compositions, operating conditions, and/or potentially PCR primers. Other researches have observed varying abundance of *Rhodocyclus* in reactors that effectively cycled phosphorous. As two examples, Coats et al. (2010-accepted) observed relatively significant variability in abundance of *Candidatus*

*Accumulibacter Phosphatis* (a member of the *Rhodocyclus* family) in EBPR reactors fed real wastewater; He et al. (2010) similarly observed a shift in abundance of *Candidatus Accumulibacter Phosphatis* clades in two laboratory-scale EBPR reactors fed synthetic wastewater. The extent of microbial diversity observed in this study – and similarly by that of Coats et al. (2010-accepted) and He et al. (2010) – suggests that engineering a system to elicit a specific structure of a mixed microbial consortium may not be as important as a particular function, namely the MSR, for the biological removal of phosphorus from wastewater.

## 5. Conclusions

Phosphorous was cycled by mixed microbial consortia in SBRs that were operated under either fully oxic or anoxic/oxic conditions and fed a synthetic wastewater containing either peptone or acetate. Phosphorus cycling was associated with the MSR, suggesting the MSR is likely a central element in the biological cycling of phosphorus within wastewater. The genetic diversity of microorganisms varied considerably between reactors. The extent of microbial diversity suggests that engineering a system to elicit a specific structure of a mixed microbial consortium may not be as important as a particular function, specifically the MSR. With the recognition of the role of MSR on phosphorus cycling within wastewater, additional research may lead to further optimization of

existing treatment technologies and the development of new treatment systems for the biological removal of phosphorus from wastewater.

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**LIST OF FIGURES**


| FIGURE | TITLE |
|---|---|
| 1 | Concentrations of (a) sCOD and PHA, (b) ammonia and nitrate, (c) aqueous and biomass phosphorus, (d) ppGpp and EC over the course of one feed-cycle in the peptone fed anoxic/oxic SBR (Reactor 3). |
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| 6 | Maximum likelihood 16S rRNA tree showing the anoxic/oxic SBRs sequences with 95% identity clustered into phylotypes. Circle sizes indicate relative abundance. Each bar represents 1% sequence divergence. |

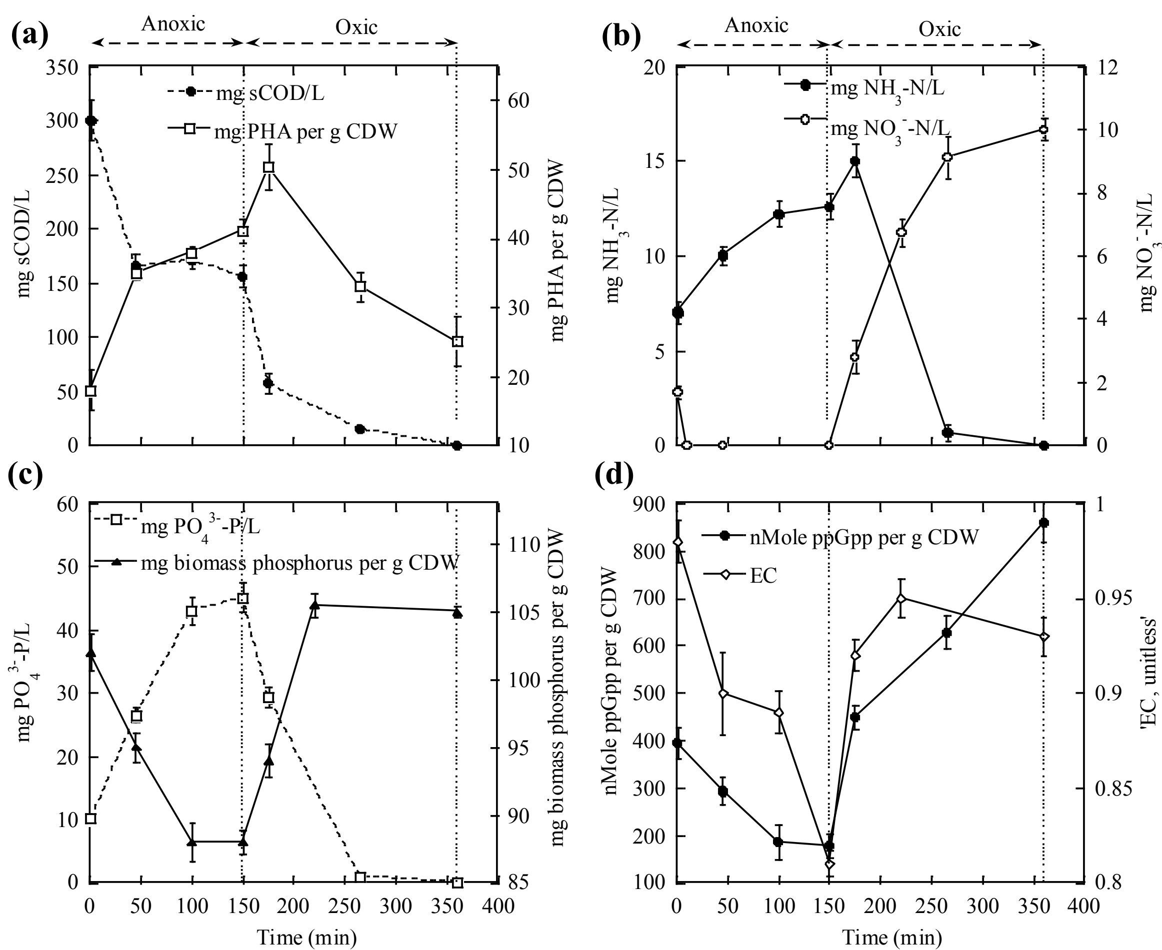



**Fig. 1**

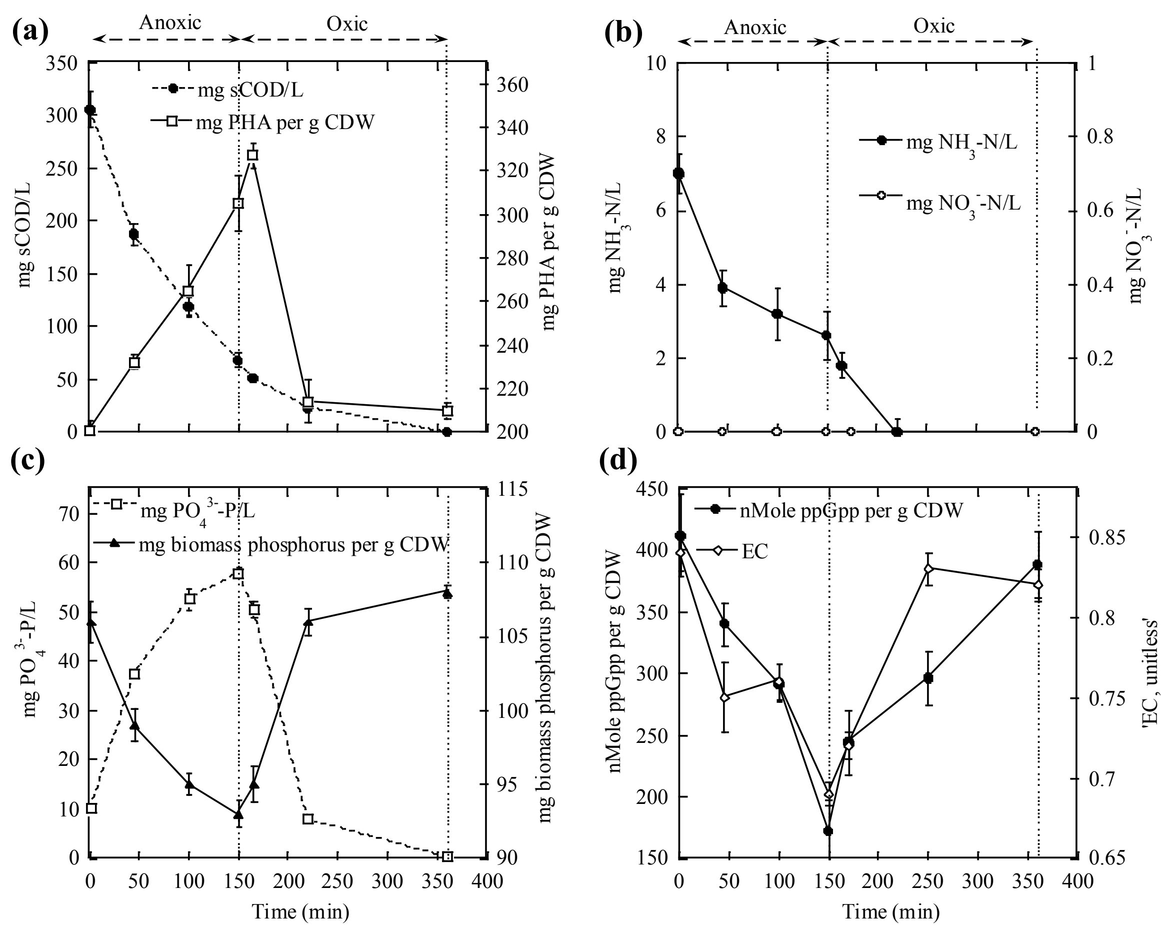
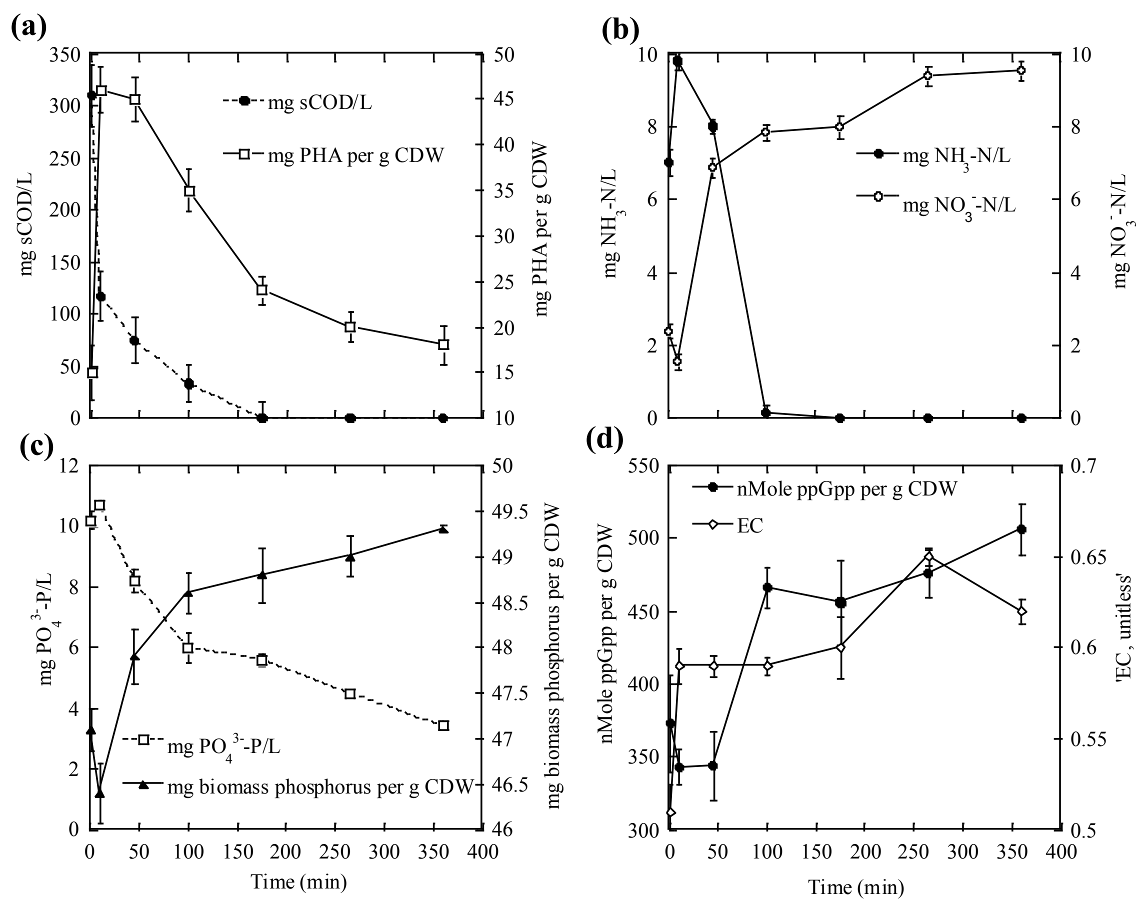



**Fig. 2**

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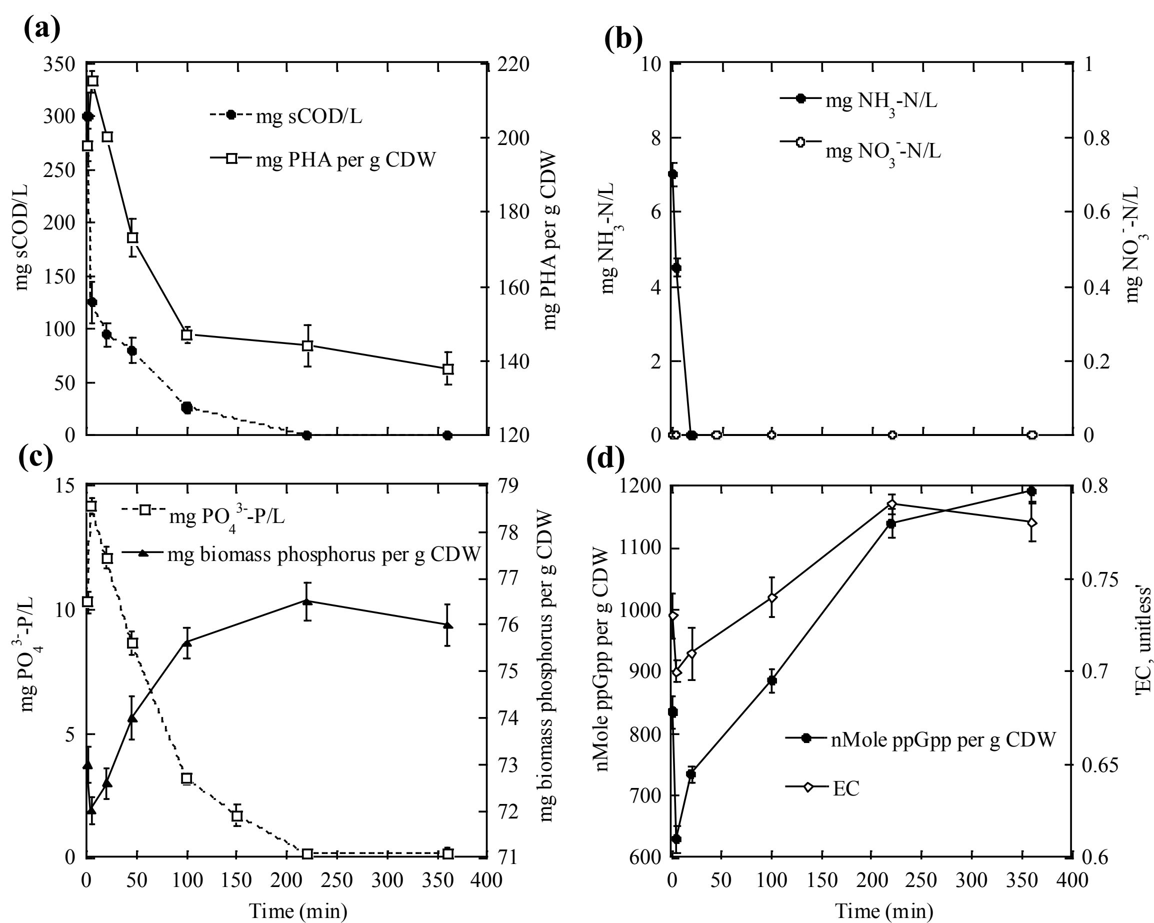


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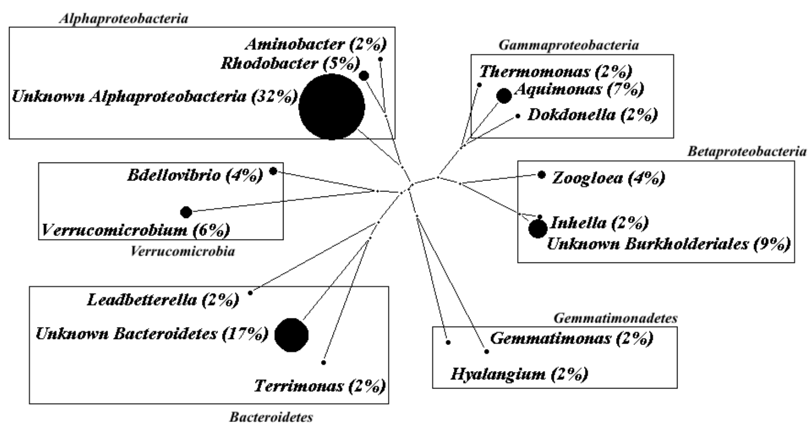
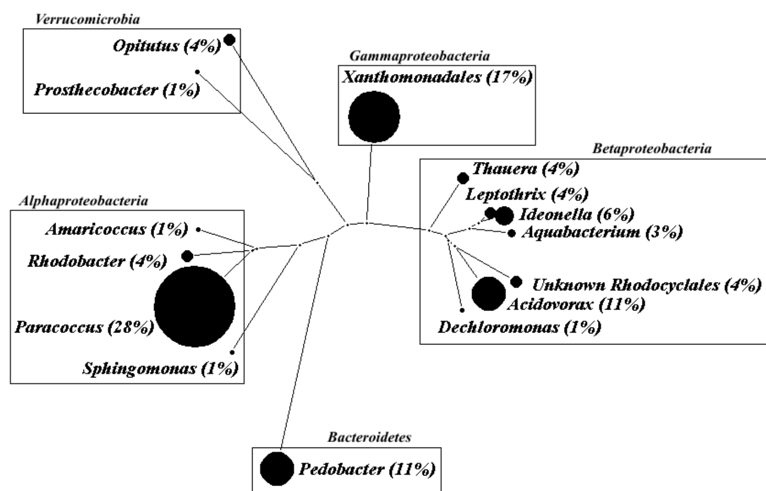
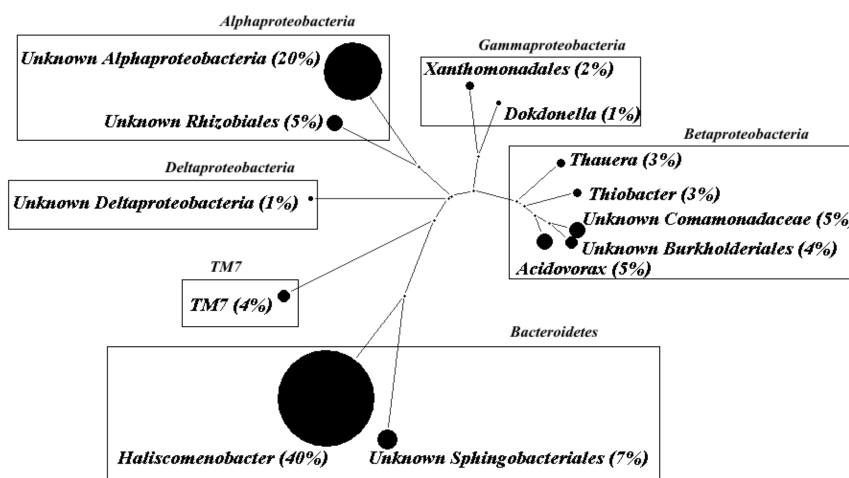



**(a) Reactor 1 (peptone fully oxic)**

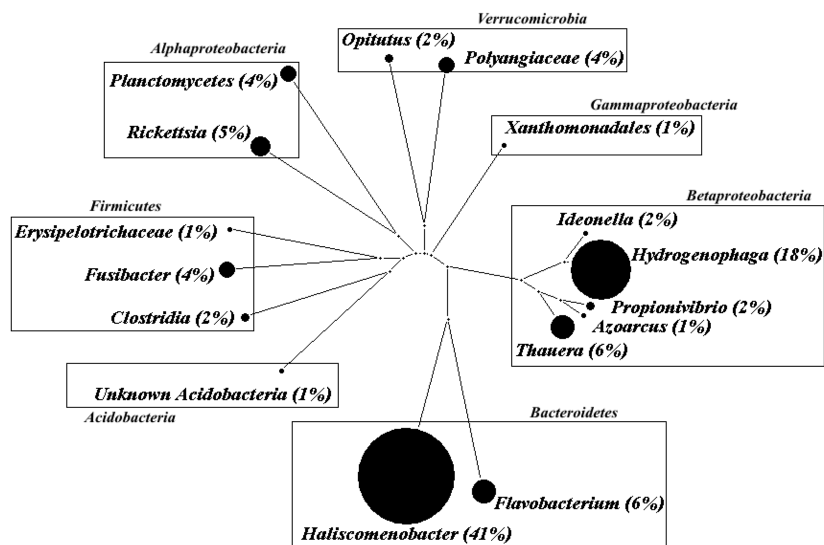



**(b) Reactor 2 (acetate fully oxic)**

**Fig. 5**




**(a) Reactor 3 (peptone anoxic/oxic)**




**(b) Reactor 4 (acetate anoxic/oxic)**

**Fig. 6**

# Chapter 3: Summary and Conclusions

A two-year comparative study was conducted, with fully oxic and conventional anoxic/oxic EBPR processes, operated in parallel under similar conditions using bench-scale SBRs, to evaluate whether the microbial stringent response (MSR) is the primary mechanism responsible for phosphorus removal. Phosphorus removal to undetectable levels was achieved in both systems, with an increase in the MSR indicator, an alarmone ppGpp, levels. These results suggest that the MSR is the primary mechanism responsible for phosphorus removal in wastewater. The behavior of a mixed culture with respect to phosphorus cycling appears to coincide with that of a pure culture. This study demonstrates that it is possible to enrich inocula for EBPR under fully oxic conditions to achieve near complete phosphorus removal.