SUSTAINABLE PRODUCTION OF BIODEGRADABLE THERMOPLASTICS THROUGH WASTEWATER TREATMENT, AND A NEW THEORY ON BIOLOGICAL PHOSPHORUS REMOVAL

By

ERIK ROBERT COATS

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of ERIK ROBERT COATS find it satisfactory and recommend that it be accepted.

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Abstract

By Erik Robert Coats, Ph.D.
Washington State University
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Chairs: Michael P. Wolcott, Frank J. Loge

Biologically-derived polyesters known as polyhydroxyalkanoates (PHAs) represent a potentially sustainable replacement to fossil fuel-based thermoplastics. However, current production practices do not achieve this objective. Herein we report on the production of PHA with a mixed microbial consortium indigenous to a wastewater treatment process on carbon present in wastewaters in a unit-operation configuration congruent with wastewater treatment. PHA production averaged 85, 10, and 53% (w/w) on pulp-and-paper mill foul condensate, biodiesel, and municipal wastewaters, respectively. Given the proposed polymer production process eliminates the energy and costs associated with feedstock production and bioreactor operation (two of the three major energy and cost sinks), and further that wastewater treatment is mandated in most countries, results presented suggest environmentally benign (e.g., sustainable) PHA production is feasible. As a further enhancement, PHA-rich microbial cell mass was utilized to produce a natural fiber reinforced thermoplastic composite (NFRTC). Specifically, polyhydroxybutyrate-rich biomass was synthesized with the mutant *Azotobacter vinelandii* UWD and utilized in unpurified form to manufacture NFRTCs with wood flour. Resulting

composites exhibited statistically similar material properties despite relatively different PHB contents. Further, the microbial cell debris allowed for NFRTC processing at significantly reduced polymer content. By integrating the unpurified polyhydroxybutyrate in NFRTC construction, the third principal energy and cost sink is eliminated while yielding a fully biologically based commodity. Finally, through investigations on integrating PHA production into wastewater treatment, a new theory was elucidated for microbial phosphorus removal. Enhanced biological phosphorus removal, which is currently an empirical process predicated on creating a sequential anaerobic-aerobic operational pattern to elicit polyphosphate and PHA cycling, is considered unstable and unreliable due to a limited understanding of the process mechanistics. As a contrast to past research, we investigated the process utilizing real wastewater. Results clearly demonstrate enhanced microbial phosphorus removal is associated with the bacterial stringent response. Specifically, environmental feast-famine conditions elicit a response for the synthesis of ppGpp, ultimately leading to excess phosphorus removal. Feast-famine on oxygen, coupled with an unbalanced nutrient condition, results in reduction of bulk aqueous orthophosphate conditions to negligible levels. We further show that the cycling of polyphosphate and PHA are not intrinsically linked.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Dedication

I dedicate this dissertation to my wife, Kristine, to whom I will forever owe a debt of gratitude for reluctantly, yet willingly, uprooting and embarking on this journey with me. Thank you for always being there with overwhelming support and encouragement. I also want to dedicate this dissertation to my son, Nathan, who is the light of my life, and who was always so deeply concerned about the general health and welfare of the "vacteria" associated with my research activities. I hope the blessing of your wonderful imagination grows with you forever.

CHAPTER ONE

INTRODUCTION

In our interest to move toward a more sustainable society, we strive to make human activities environmentally benign and manufacturing processes 'green'. We focus not only on the production of chemical commodities from renewable feedstocks, but life-cycle analyses of products and associated manufacturing processes [1] to ensure that while modification of certain elements within a manufacturing process may satisfy one or more of the guiding principles of green engineering [2], the environmental impacts have not simply shifted within the product life-cycle [2]. The fields of composites, polymers, and wastewater management converge within this context.

Synthetic-based composites, while commercially appealing due to the ability to engineer materials with well defined and controlled properties to meet a variety of diverse applications, represent an environmental liability both implicit in the raw materials production and upon disposal [3]. However, while the environmental element alone may be academically viewed as a driver to change, ultimately cost is the principal controlling factor, and at a commercial level synthetic-based composites are cost competitive. Natural fiber reinforced thermoplastic composites (NFRTCs) partially respond to the environmental impacts by integrating biodegradable filler material, such as flax, hemp, and wood flour, in lieu of synthetic fillers, such as glass, carbon, or steel [3], yet more importantly do so with a concomitant cost reduction. Specifically, the cost of biodegradable filler material is better than an order of magnitude less than synthetic reinforcing fibers [3]; moreover, NFRTCs can be engineered for a variety of applications wherein synthetic-based composites would be over-designed [3]. Ultimately, though, integration of biodegradable material, even if derived as a waste product, does not completely address the environmental liabilities; these commodities remain bio-recalcitrant upon

disposal [4] based on the use of synthetic thermoplastics as the principal binding material, and moreover the manufacture of synthetic thermoplastics is not without environmental ramifications [5].

Biologically-derived polyesters known as polyhydroxyalkanoates (PHAs) represent a potentially 'sustainable' replacement to fossil fuel-based thermoplastics. PHAs are natural thermoplastic polyesters - essentially carbon storage reserves - that over 300 different bacterial species are known to synthesize and accumulate in the form of cytoplasmic granules [6]. Certain plants, including corn, have also been genetically modified with bacterial PHA synthetic genes, such that they will accumulate PHA [7, 8]. Poly-3-hydroxybutyrate (PHB or P3HB) was the first PHA discovered (over 75 years ago), and hence is the most extensively characterized type [9, 10]. Since this initial discovery, many forms of hydroxyalkanoic monomer units have been identified [9]. Common precursors to PHA synthesis include simple sugars such as glucose and fructose, and organic acids such as acetic and propionic acid. The carbon substrate form dictates the polymeric structure of the PHA [9], with some of the most commonly studied forms including PHB, poly-hydroxyvalerate (PHV), and poly-4-hydroxybutyrate (P4HB). In turn, each form of PHA yields different polymer properties. PHB exhibits similar characteristics to polypropylene, including melting temperature and crystallinity, but the polymer is brittle upon crystallization and thus exhibits little stress resistance [9]. Polymer improvements are accomplished through copolymerization with PHV to yield a less brittle, less crystalline thermoplastic with a lower processing temperature [9]. PHA biosynthesis is stimulated by either excess soluble carbon with a concurrent macronutrient limitation (typically lacking either nitrogen or phosphorus), a limitation in a terminal electron acceptor (with oxygen as the most common), or a so-called feast/famine environment wherein microorganisms realize a transient excess of soluble carbon without any nutrient limitations [11].

Use of PHAs in NFRTC construction has been presented as an avenue to address the environmental liability associated with the use of synthetic plastic [3, 12-15]. However, while these polymers might appear to present an ideal opportunity to produce an entirely biologically based, biodegradable, green product, PHAs are not currently cost competitive with synthetic plastics. Further, and more critically, current commercial production practices, which fermentatively produce PHA with pure microbial cultures grown on renewable, but refined, feedstocks (e.g. glucose) under sterile conditions [16], exhibit higher fossil fuel demands and generate more carbon emissions, largely associated with feedstock production and the fermentation process, than fossil fuel-based plastics [17]. Similar conclusions have been made for plant-based PHA production methods [18, 19]. Hence, biologically derived PHA currently is not environmentally benign, nor would PHA-based NFRTCs represent an environmentally benign product.

With current biological PHA production practices [16], the feedstock is estimated to account for ca. 30% to 50% of the total production cost and ca. 12% to 33% of the total embodied energy [17, 20, 21]. These estimates vary somewhat with the microbial species utilized, carbon source, PHA yield, and PHA production capacity [17, 20, 21]. Bioreactor operations used to produce PHA consume an additional ca. 30% to 40% in energy [17, 21], with a concomitant share of the product cost. Research on minimizing these energy and cost elements has focused on either the use of pure cultures with waste streams rich in precursor carbohydrates and organic acids [22-25], pure cultures with waste streams derived from refined waste feedstocks [26], or mixed microbial consortia grown on synthetic feedstocks [11, 27-29]. The concept of producing commercial quantities of PHA with indigenous microbial consortia utilizing as feedstock refined solid or liquid wastes has been proposed [30, 31], but never implemented. Although these alternative PHA production approaches incrementally address the

energy requirements associated with current commercial practices, as a whole, production of this polymer remains unsustainable.

Considering the propensity for microorganisms to synthesize PHA, commercial polymer production would theoretically appear to be a natural extension of the biological treatment regime, and in fact PHA synthesis is associated with the empirical wastewater treatment process known as Enhanced Biological Phosphorus Removal (EBPR) [32, 33]. However, biological synthesis of PHA in full-scale EBPR facilities, estimated at upwards of 4% (w/w) (data not shown), falls short of quantities necessary for commercial exploitation. Nevertheless, with process modifications, the potential clearly does exist, although integrating commodity production with wastewater treatment could yield secondary effects related to achieving the principle goals of wastewater treatment.

The goal of this research was to elucidate and advance concepts relative to the development of an environmentally benign PHA and NFRTC production process, while concurrently investigating the impacts on the wastewater treatment process. The specific objectives were to i) demonstrate that NFRTCs could be produced utilizing unpurified PHB, ii) advance a process for commercial PHA production that is congruent with wastewater treatment practices, and iii) elucidate the potential impacts of the wastewater-based PHA production process on the overarching wastewater treatment objectives and goals. Each of these objectives is sequentially and independently addressed in Chapters 2, 3, and 4, respectively.

CHAPTER TWO

PRODUCTION OF NATURAL FIBER REINFORCED THERMOPLASTIC COMPOSITES THROUGH THE USE OF PHB-RICH BIOMASS

INTRODUCTION

As a first step towards reducing NFRTC product cost while concurrently addressing environmental impacts associated with using PHA in the manufacture of NFRTCs, we focused on the polymer purification step. As noted, polymer processing represents ca. 30%-40% of the energy and cost associated with the biological production of PHA [17, 21]. In addition, the process of extracting bacterial PHA involves the use of various solvents, which generates waste streams that must be managed and potentially compromises the end product purity [20]. Recognizing that the non-PHA material (e.g., cellular biomass) associated with microorganisms is organic, similar to the organic filler material in NFRTCs, the biopolymer arguably does not need to be purified in order to be utilized. The goal of this research was therefore to demonstrate that unpurified bacterial PHB (e.g., PHB-rich biomass) could be utilized in the manufacture of NFRTCs. The specific objectives were to: (i) design and operate a biological reactor for the purpose of mass producing PHB-rich biomass; (ii) harvest and process PHB-rich biomass for use in designing and testing composite products; and (iii) produce and evaluate PHB-rich-biomass and pure PHB based NFRTCs following a factorial design to identify influencing factors and the potential significance of microbial biomass on composite material properties.

EXPERIMENTAL SECTION

Materials. The bacterial mutant *Azotobacter vinelandii* UWD (ATCC 53799), derived from strain UW (ATCC 13705), was selected for this research. PHA production by many *Azotobacter* species is well documented [9], and this particular mutant, created by Page and

Knosp [34], has the demonstrated ability to hyper-produce PHB without any nutrient limitations [34, 35]. More specifically, unlike wild-type microorganisms that require a limitation in an electron acceptor to produce PHB, *A. vinelandii* UWD will synthesize the biopolymer under fully aerobic conditions due to a mutation associated with its respiratory NADH oxidase [34]. This mechanistic advantage simplified large-scale production of PHB-rich biomass, specifically in the operation of the bioreactors.

Reactor Operations and Culture Media. *A. vinelandii* UWD was cultured in 10-L reactors aerated through a 9-inch diameter Sanitaire® Silver Series II membrane fine bubble disc diffuser (Brown Deer, Wisconsin, USA). Reactors were operated continuously in two 9-month periods for the sole purpose of producing large quantities of PHB-rich biomass. The reactors were operated as sequencing batch reactors, batch decanted and fed once daily at a dilution rate ranging from 0.5 d^{-1} to 0.9 d^{-1}. The mixed liquor suspended solids (MLSS) concentration stabilized at ca. 5 g L^{-1} in all reactors. The reactors were operated in environmentally controlled rooms with the temperature maintained at 30°C. The growth medium consisted of the following, expressed in terms of g L^{-1}: KH_2PO_4, 0.16; K_2HPO_4, 0.64; $MgSO_4 \cdot 7H_2O$, 0.16; $CaSO_4 \cdot 2H_2O$, 0.08; $FeCl_3$, 0.04; $Na_2MoO_4 \cdot 2H_2O$, 0.008; Glucose, 20; Sucrose, 20. The medium was autoclaved prior to addition to the reactors.

PHB-rich Biomass Recovery and Processing. Daily reactor decants of PHB-rich biomass were centrifuged at ca. 10,000xg. The biomass pellet was chlorinated with 12.5% sodium hypochlorite for 60 minutes in accordance with Berger *et. al.* [36] to lyse the cells, re-suspended and re-centrifuged, and then dried at 60°C. Chlorination was utilized to arrest the bacterial metabolic activity during the drying phase, thus preventing PHB bio-degradation.

Analytical Procedures. Utilization of soluble organic carbon in the bioreactors was assessed as chemical oxygen demand (COD) in accordance with Standard Methods 5220-D.

Hach high range ampules (Hach Company, Loveland, Colorado, USA) were utilized, with a Hach COD reactor and a Spectronic® 20 Genesys™ spectrophotometer. MLSS was determined gravimetrically in accordance with Standard Methods 2540-D, with Millipore 1.2 micron glass/fiber filters (Millipore Corp, Billerica, MA, USA).

Biomass PHA content was determined by GC-MS, following the method of Braunegg *et. al.* [37]. Briefly, dried PHA-rich biomass samples were digested at 100°C in 2 mL each of acidified methanol (3% v/v sulfuric acid) and chloroform. Benzoic acid was added to the chloroform as an internal standard. Following vigorous vortexing of the mixture with 1 mL deionized water, PHA-rich chloroform was recovered for analysis. The chloroform phase was dried with sodium sulfate prior to analysis. GC-MS was performed on a Thermofinnigan PolarisQ iontrap GC-MS instrument in positive ei mode. The sample was introduced using split injection. Separation was achieved on a ZB1 (15m, 0.25mm ID) capillary column with helium as the carrier gas (1.2 ml min^{-1}) using a temperature program of 40°C (2 min) ramped to 200°C at 5°C min^{-1}. Data was analyzed on the software program Xcalibur (Thermo Electron Corporation). The identity of the compounds was confirmed by retention time and mass spectral matching with known standards (as methyl ester derivatives), and quantified based on internal standards.

Composite Processing. Natural fiber reinforced thermoplastic composites were manufactured with the PHB-rich biomass and a purified PHB obtained from PHB Industrial S/A (Biocycle 1000®). Sixty-mesh wood (pine) flour was obtained from American Wood Fibers (Wisconsin, USA) and added to the composite formulation as the natural fiber reinforcement. Prior to injection molding (IM), the dry materials (below 2% moisture content) were melt-blended in a torque rheometer for 5 minutes at 170°C. The mixed materials were then fed into a capillary rheometer with a barrel temperature of 170°C. The capillary rheometer was used to

inject the composite into a heated die creating two solid rectangular bars (5 x 12 x 94mm). Once the bars were removed from the die, they were allowed to equilibrate for 1 week at a 22°C and 50% relative humidity condition.

The conditioned specimens were tested in flexure according to the ASTM D790 procedures. The stiffness (MOE), bending strength (MOR), and strain at break were calculated and reported. Statistically significant differences in material properties of the biomass-based and pure PHB NFRTCs were assessed with an analysis of variance, using density as a covariate, utilizing SAS software.

RESULTS AND DISCUSSION

Polyhydroxybutyrate-Rich Biomass. Chlorination of the PHB-rich biomass, also referred to as alkaline hydrolysis, was utilized principally as a mechanism to arrest bacterial metabolic activity and prevent depolymerization of cellular PHB. However, the treatment is also a common first step in commercial purification of PHB [38]. Therefore, selected analyses were undertaken to confirm that minimal polymer purification occurred. *A. vinelandii* UWD is a gram negative organism; hence we believe much of the non-PHB biomass would consist of constituents associated with the cell membrane (e.g. various forms of lipids esterified to other constituents such as glycerol phosphate derivatives). Saponification, which is the alkaline hydrolysis of fatty acid ester bonds, and subsequent centrifugation of the biomass material would be expected to remove some of the solublized biomass constituents. Gas chromatographic analysis, which is a recognized method for the detection of lipids [39], was applied to determine the potential treatment effects on cellular lipids (Figures 1 and 2). For unchlorinated biomass (Figure 1), PHB methyl ester was eluted at 2.71 minutes, while the esterified internal standard, benzoic acid, eluted at 8.06 minutes; the remaining peaks (24, 27.48, 28.01, and 31.3 minutes) consist principally of esterified cellular lipids. Chlorinated biomass yielded similar results for

PHB and benzoic acid, however, the lipid constituents were significantly reduced (Figure 2). Based on these results, a quantitative PHB assessment was applied to more completely elucidate the extent of cellular material removed from the polymer-biomass matrix. Average PHB content for the chlorinated biomass was determined to be ca. 23.8% (w/w), while non-chlorinated PHB content was estimated at 16.8%, yielding ca. 29% loss of cellular material through alkaline lysis and centrifugation. Therefore, although the applied cell disruption technique did indirectly result in some biomass removal, the residual matrix clearly remained in an unpurified form.

As indicated on the GC plots (Figures 1 and 2), PHB in the biomass samples was readily detected and validated. Mass spectrometry analysis of the relative PHB peaks revealed a molecular weight pattern consistent with that of pure PHB samples (Figures 3-5). Variability in the relative peaks is associated with the mass of the sample analyzed.

While the unique metabolic capabilities of this mutant microorganism satisfied our research objective of producing a sufficient quantity to manufacture NFRTCs, the PHB yield (on a w/w basis) was significantly less than published for this and other PHB-producing microorganisms on glucose/sucrose. For example, Page and Knosp [34] obtained PHB yields in excess of 65% (w/w) from *A. vinelandii* UWD. One potential explanation for the reduced yield lies in the reactor operations. Analysis of a typical reactor operational cycle suggests that PHB yield peaked prior to harvest (Figure 6). Moreover, the peak at 8 hours was similar in magnitude to that determined by the Page and Knosp [34]. Hence, a shorter operating cycle, and thus a shorter hydraulic residence time, may have yielded higher PHB quantities. The form of the mutation may also have contributed to reduced yield. As noted, *A. vinelandii* UWD is mutated in its ability to oxidize NADH [34]. Hence, the microorganism cannot efficiently oxidize NADH and transfer electrons to oxygen during respiration, thereby inhibiting a critical aerobic metabolic activity that serves as a primary source of energy. As contrasted with the mutated species, Page

and Knosp [34] found that the parent species produced PHB at ca. 22%, which is more consistent with our yield. Recognizing that over time microorganisms maintain the ability to repair or circumvent mutated functions [40], *A. vinelandii* UWD may have developed capabilities to utilize the readily available oxygen, thereby reducing our yield. Regardless of the lower polymer yields, the material served effectively in the production of NFRTCs.

PHB-Rich Biomass Composites. Molding of PHB-rich biomass composites was hindered with increased wood flour content incorporated into the formulation, principally due to the relatively low polymer content in the biomass and concomitant higher viscosity relative to a pure polymer. Above the 30% wood content level, the increased viscosity associated with the biomass-based polymer resulted in the inability for the material to flow efficiently to the outer extension of the mold. Therefore, the experimental design matrix included composite formulations (%PHB-rich biomass:%wood) of 100:0, 90:10, 80:20, and 70:30 which yielded actual PHB contents (total composite weight basis) of ca. 24%, 21%, 19%, and 17%, respectively. Table 1 summarizes the experimental design composite formulations used in these investigations. Table 1 also includes results from a design development formulation and composite analysis that utilized a PHB-rich biomass with 43% PHB content (microbial w/w). The resulting 60:40 formulation yielded ca. 26% PHB content (composite w/w).

Factorial analysis on the data in Table 1 revealed that the influence of density on composite mechanical properties was significant for the MOE, but not for the MOR. In order to independently consider the MOE and MOR, a covariate analysis was applied to remove the influence of density. A Tukey-Kramer pairwise comparison was then applied to the least square means for MOE and MOR. For the biomass-based composites, wood content was found to have an insignificant effect on the elastic properties (MOE) between the 100% and 90% formulations; similar results were determined between the 80% and 70% formulations (Table 2). The two

lower wood content formulations yielded significantly different, and lower, MOEs (Table 2). However, there was no significant difference in bending strength (e.g., MOR) between all biomass-based formulations (Table 2). This latter finding is of particular interest, considering the relatively significant difference in PHB content between the formulations (e.g., from 24% at 100:0 to 17% at 70:30). Foremost, these results demonstrate that unpurified PHB can be utilized in the manufacture of NFRTCs. Further, these results indicate that wood flour compensates for decreased polymer and increased cell debris content to maintain composite strength, although concurrently yielding a stiffer composite, and that improved composite material properties can be obtained through the use of PHB-rich biomass with higher cellular polymer content. A final interesting point is that pure PHB-based NFRTCs could not be produced to match the biomass-based formulations (for instance, 17% PHB and 83% wood flour), revealing that the presence of cell debris acts as some form of "lubricant" that allows for NFRTC formulations at significantly reduced polymer contents.

A contrast between the 60:40 PHB-rich biomass formulation, which was produced using a higher PHB content biomass, and the 100:0 PHB-rich biomass formulation provides an indication regarding the effects of increased microbial PHB content and decreased cell debris content on composite material properties. The formulations (% PHB:biomass:wood) were 26:34:40 and 24:76:0, respectively. The 60:40 formulation yielded the highest MOR of all biomass-based composites (ca. 29% higher than the 100:0 formulation - Table 1). Furthermore, the strain at break increased 100%. Considering that the PHB content between the 100:0 and 60:40 formulations was essentially the same, these results confirm that 1) increased PHB content within the microbial cell, and thus decreased cellular debris content, would measurably improve composite material properties, and 2) the non-PHB cellular material does not offer comparable structural properties to wood flour.

Pure PHB-based Composites. In order to ascertain the potential to improve composite material properties through increased microbial PHB content, pure PHB-based NFRTCs were produced and analyzed. These composites, which contained greater than 70% polymer on a total composite weight basis, yielded significantly higher bending strengths as compared to the biomass-based composites (Table 1). However, there was no significant difference in bending strength between the three pure PHB samples containing wood-flour (data not shown). The MOEs among all pure PHB formulations were generally consistent; statistical analysis of the pure PHB composites revealed no significant difference in elasticity between all samples (data not shown). Further, contrasting pure and biomass-based PHB formulations on MOE revealed generally no significant differences; only the two biomass-based formulations with the least wood content exhibited statistically different MOE's from all other formulations (data not shown). The presented findings are consistent with previous PHA NFRTC investigations [14, 41], and demonstrate that the wood flour did not influence overall composite material properties for the formulations analyzed. These results further suggest that there is a threshold maximum polymer content (w/w), estimated at 70% based on the results presented herein, above which diminishing returns occur. In terms of utilizing unpurified PHB to produce this threshold composite formulation, commercial production of PHB-rich biomass can readily achieve this target cellular content [9].

NFRTC Densities. An interesting observation between the biomass and pure PHB composites was that density increased with wood loadings only in the pure PHB system. The biomass-rich PHB composites showed a decrease in density. Generally, the density of wood-thermoplastic composites will increase with higher wood fiber loadings due to either the polymer filling the wood voids and/or the wood cell wall collapsing. The wood cell wall is estimated to have a density around 1,500 kg/m^3 [42], whereas pure PHB utilized in this study had a density

around 1,150-1,200 kg/m^3. The decrease in density of the PHB-rich biomass composites with the addition of wood flour would indicate insufficient dispersion of the polymer-based matrix material within the wood. Alternatively, the density decrease could be partially attributed to the density of the PHB-rich biomass relative to wood flour; as shown in Table 1, the PHB-rich biomass (100:0 formulation) was of relatively high density.

CONCLUDING REMARKS

While minimizing or mitigating environmental impacts is often cited as a driving factor in improving commodity manufacturing, ultimately cost competitiveness controls practice. Only through addressing the two factors together will we make significant strides toward minimizing anthropogenic impacts of manufacturing processes on the natural environment. Herein we have advanced such a concept by integrating unpurified PHB in NFRTC manufacturing to yield a product that is entirely biologically derived and biodegradable. The direct integration of cellular biomass (e.g. unpurified polymer) addresses a significant energy and cost sink [17, 21] in the polymer production process. Although the PHB-rich biomass formulations yielded brittle composites with relatively low strain at break, the presented results strongly indicate that increased microbial PHB content could significantly improve material properties. Recognizing that typical commercial PHB production yields significantly higher polymer content [43], clearly there are opportunities to address this issue. Moreover, again recognizing that PHB is a very brittle polymer [9], utilizing polymer-rich biomass with certain other PHA co-monomers would be expected to yield significantly improved material properties. Nevertheless, considering the apparent processing advantages conveyed by the cell debris in that lower polymer content composites could be produced, arguably there are applications for the produced PHB-rich biomass formulations.

CHAPTER THREE

POOP TO PLASTIC: COMMERCIAL PRODUCTION OF POLYHYDROXYALKANOATES IN MUNICIPAL AND INDUSTRIAL WASTEWATER TREATMENT

INTRODUCTION

Integration with and interconnectivity to raw material flow [2], wherein commercial production of PHA occurs in bioreactors (e.g., activated sludge processes) employed in wastewater treatment (for the removal of organic carbon commonly measured as Biological Oxygen Demand (BOD)) using wastewater as the feedstock, has neither been proposed nor implemented. Such a technology would arguably eliminate the costs associated with both feedstock production and bioreactor operations given that wastewater treatment is mandated in most countries. Recognizing that many waste streams are rich in PHA precursors, the potential for complete process integration exists. Further, as noted previously, PHB synthesis does occur within the EBPR wastewater treatment process. The goal of this research was therefore to demonstrate that PHA production could readily be integrated into various waste treatment schemes, and that conditions could be established to concurrently achieve treatment objectives and generate high PHA yields. The specific objectives were to: (i) identify suitable waste streams for the production of PHA; and (ii) design and operate laboratory-based biological reactors, congruent with full-scale configurations, for coupled PHA production and wastewater treatment.

MATERIALS AND METHODS

Source of Microorganisms. The mixed microbial seed was obtained from the Moscow, Idaho, EBPR wastewater treatment facility, which had been determined, through a series of

facility screening studies, to be capable of synthesizing PHA. *Azotobacter vinelandii* UWD was purchased from the American Type Culture Collection (ATCC 53799).

Culture Conditions, Growth Media, and Harvesting Procedures. *A. vinelandii* UWD was cultured in 10-L reactors aerated through a 9-inch diameter Sanitaire® Silver Series II membrane fine bubble disc diffuser (Brown Deer, Wisconsin, USA). Reactors were operated continuously in two 9-month periods for the sole purpose of producing quantities of PHB-rich biomass. The reactors were operated as sequencing batch reactors (SBR), with a dilution rate ranging from 0.5 d^{-1} to 0.9 d^{-1}, and were batch decanted and fed once daily. The mixed liquor suspended solids (MLSS) concentration stabilized at ca. 5 g L^{-1} in all reactors. The reactors were operated within environmentally controlled rooms, and the temperature was maintained at 30°C. The medium for PHB production consisted of the following, g/L (reagent grade): KH_2PO_4, 0.16; K_2HPO_4, 0.64; $MgSO_4 \cdot 7H_2O$, 0.16; $CaSO_4 \cdot 2H_2O$, 0.08; $FeCl_3$, 0.04; $Na_2MoO_4 \cdot 2H_2O$, 0.008; Glucose, 20; Sucrose, 20. The medium was autoclaved prior to addition to the reactors. Daily reactor decants of PHB-rich biomass were centrifuged at ca. 10,000xg; the biomass pellet was chlorinated with 12.5% sodium hypochlorite for 60 minutes, re-suspended in de-ionized water and re-centrifuged, and dried at 60°F.

PHB production on pulp-and-paper mill foul condensate wastewater was accomplished in two 4-L completely mixed reactors. Both reactors were operated as SBRs on a 24-hour cycle, with a dilution rate of 0.25 d^{-1}. One of the reactors was continually aerated resulting in a measurable dissolved oxygen concentration; the other reactor was cycled every 12-hours between oxic and anoxic environments. Anoxic conditions were established by bubbling nitrogen gas continuously into the reactor. Nitrogen gas and air were supplied through a 9-inch diameter Sanitaire® Silver Series II membrane fine bubble disc diffuser (Brown Deer,

Wisconsin, USA) installed at the base of the reactor. Foul condensate wastewater was provided by the Lewiston, Idaho, Potlatch Corporation pulp-and-paper mill.

PHB production on biodiesel wastewater was accomplished in 500 mL flasks incubated by shaking at 250 rpm for 4 days at 30°C. Biodiesel-derived wastewater was provided by the University of Idaho Department of Biological and Agricultural Engineering. Two batches of wastewater were obtained, one with residual ethanol and one without, and each batch was fed into two reactors, one at 1% (v/v) and the second at 5% (v/v).

Wastewater biosolids fermentate was produced in a 10-L completely-mixed primary solids fermenter operated as a SBR, with a dilution rate of 0.25 d^{-1}. The daily decant was centrifuged at ca. 10,000xg, and the supernatant (e.g. fermentate) recovered. Primary solids utilized to produce fermentate were obtained from the Pullman, Washington, wastewater treatment facility. The fermentate-fed wastewater treatment reactor consisted of a 2-L vessel continuously operated on a 24-hour cycle (anoxically for six hours following feeding, then oxically for 18 hours). Anoxic conditions were accomplished through the continuous supply of nitrogen gas. Nitrogen gas and air were supplied through a 9-inch diameter Sanitaire® Silver Series II membrane fine bubble disc diffuser installed at the base of the reactor. Side-stream PHA production was accomplished in a 2-L flask seeded with 400 mL from the treatment reactor and fed 600 mL fermentate. The reactor was operated for 7 hours, continuously aerated and mixed. PHA-rich biomass from all wastewater-based processes was centrifuged at ca. 10,000xg; the biomass pellet was chlorinated with 12.5% sodium hypochlorite for ca. 5 minutes, re-suspended in de-ionized water and re-centrifuged, and dried at 60°C.

Analytical Techniques. Chemical oxygen demand (COD) tests were performed in accordance with Standard Methods 5220-D. Hach high-range ampules (Hach Company, Loveland, Colorado, USA) were utilized, with a Hach COD reactor and a Spectronic® 20

Genesys™ spectrophotometer. Soluble orthophosphate was determined in accordance with Hach method 8048 (equivalent to Standard Methods 4500-PE), with samples filtered through sterilized 0.22 μm filters prior to analysis. MLSS was determined gravimetrically in accordance with Standard Methods 2540-D, with Millipore 1.2 micron glass/fiber filters (Millipore Corp, Billerica, MA, USA). Biomass PHA content was determined by GC-MS, following the method of Braunegg *et. al.* [37]. Briefly, dried PHA-rich biomass samples were digested at 100°C in 2 mL each of acidified methanol (3% v/v sulfuric acid) and chloroform. Benzoic acid was added to the chloroform as an internal standard. Following vigorous vortexing of the mixture with 1-mL deionized water, PHA-rich chloroform was recovered for analysis. GC-MS was performed on a Thermofinnigan PolarisQ iontrap GC-MS instrument (Thermo Electron Corporation) in positive ei mode. The sample was introduced using a split injection. Separation was achieved on a ZB1 (15m, 0.25mm ID) capillary column (Phenomenex, Torrance, California, USA) with helium as the carrier gas (1.2 mL min^{-1}) using a temperature program 40°C (2 min) ramped to 200°C at 5°C min^{-1}. The Xcalibur software program (Thermo Electron Corporation) was used to analyze the data. The identity of the compounds was confirmed by retention time and mass spectral matching with known standards (as methyl ester derivatives), and quantified based on the internal standard.

RESULTS AND DISCUSSION

Production of PHA in Industrial Wastewater Treatment. Commercial quantities of PHA were successfully produced within a wastewater treatment context utilizing industrial wastewaters. Recognizing that industries are largely driven by profit, this type of process integration is not only fundamental to green engineering principles [2], but produces a commercial commodity that can be sold to offset the cost of wastewater treatment.

PHA Production in Foul Condensate Wastewater. Pulp-and-paper mill foul condensate wastewater is highly enriched with methanol, yielding a chemical oxygen demand (COD) in excess of 10,000 mg L^{-1}. The concentration of soluble organic carbon is generally reduced with biological processes; the addition of nitrogen, phosphorous, and other macro- and micro-nutrients is often necessary to achieve adequate removal of COD to meet permitted discharge requirements. While the nutrient limitations are often viewed as troublesome from a conventional wastewater treatment perspective, the coupled high carbon-low macronutrient environment is potentially ideal for stimulating PHA synthesis. In addition, methanol is a quality carbon source for PHA synthesis [44, 45].

The concept of coupled foul condensate wastewater treatment and PHA production was investigated with laboratory sequencing batch reactors. Utilizing a mixed-microbial seed obtained from an EBPR wastewater treatment facility, a fully aerated sequencing batch reactor fed with foul condensate concurrently yielded PHB at ca. 35% to 85% (w/w) while reducing COD levels by ca. 20%. GC analysis on biomass samples repeatedly verified both the presence and quantity of PHB in the biomass samples (Figure 7). PHB yield was significantly less (ca. 1% to 16%) under alternating anoxic/oxic reactor conditions. The removal of oxygen appears to have created antagonistic conditions for PHB synthesis associated with nearly complete inhibition of microbial activity.

Methylotrophic bacteria are the principal microbial species associated with PHB synthesis on methanol [46, 47]. Certain species are capable of producing upwards of 80% PHB [46], which is consistent with our peak yield. The mechanism for stimulating polymer synthesis appears to be one of macronutrient limitation [46, 48], which is also consistent with our operations. However, previous research suggests higher carbon-to-macronutrient ratios may be necessary [46, 48], and our reactors did exhibit variable yields. In addition, excess methanol has

also been shown to have an inhibitory effect on overall biomass production and PHB synthesis [48]. Hence, in terms of process scale-up, site-specific investigations will be needed to optimize nutrient conditions. However, clearly there is potential to integrate polymer production into this industry as a value-added process.

PHA Production in Biodiesel Wastewater. Biodiesel is a potential replacement or supplement to petroleum-based diesel fuels [24]. However, within the context of green engineering [2], the 'green' label is arguably a misnomer because the high strength wastewater stream generated as a byproduct [24] has simply shifted the environmental impacts within the overall life-cycle. Biodiesel waste, which exhibits a COD in excess of 10,000,000 mg L^{-1}, principally consists of residual ethanol, glycerol, fatty acid ethyl (or methyl) esters, and residual fatty acids [24]. Glycerol, ethanol, and fatty acids are direct precursors to PHA synthesis [44, 45]. Production of PHA on biodiesel waste is not presented here as the exclusive method of making this manufacturing process 'green', but rather as an example of how the production of commodities concurrent with the flow of waste streams (viewed here simply as raw materials) can be used to mitigate the shift in environmental impacts within the overall life-cycle of a manufacturing process.

Utilizing biodiesel waste and a microbial seed derived from an EBPR facility, PHB yield ranged from ca. 6% (w/w) on the ethanol-enriched biodiesel to ca. 10% (w/w) on wastewater that contained no ethanol. Somewhat surprisingly, the yield on the ethanol-enriched biodiesel, which most accurately represents biodiesel wastewater and also represents a more diverse carbon substrate for PHA synthesis, was lower than the waste stream that contained no ethanol. Concurrent COD reduction was ca. 67 and 60%, respectively. Considering the COD strength of biodiesel, this level of treatment is quite significant. While PHB yield was low, reactor optimization would likely result in improved productivity concurrent with additional COD

reduction. In fact, previous research on pure cultures has yielded upwards of 42% PHB (w/w) [24].

Production of PHA in Municipal Wastewater Treatment. While the industrial applications validated the proposed concept of commercial production of PHA concurrent with the flow of waste streams, further process refinement resulted in stable and consistent PHA production in a municipal wastewater treatment environment. Utilizing a PHA-producing mixed-microbial seed obtained from an EBPR wastewater treatment facility, a fermentate-fed sequencing batch reactor (SBR) consistently maintained a microbial consortium capable of producing PHA at a rate of ca. 10% to 22%, while concurrently reducing COD by ca. 62% and orthophosphate by ca. 83% (Figure 8). Unlike industrial waste streams, copolymerization of both PHB and PHV was achieved in municipal wastewaters (Figure 9).

To further increase the concentration of PHA, fermentate was fed to a side-stream batch reactor seeded from the SBR. Within 3.5 hours, PHA production peaked at ca. 53% (w/w) (Figure 10). Importantly, PHA production occurred rapidly, compared to conventional pure culture-based operations [21] that exceed typical hydraulic residence times employed in wastewater treatment. In addition, the PHB:PHV ratio could be maintained at approximately 1:1 (Figure 10). Considering that the carbon loading was relatively low (less than 1,200 mg L^{-1} COD) and that PHA synthesis peaked with the COD plateau, these results suggest that saturation of PHA synthesis did not occur, and furthermore that higher PHA yields would be achievable. The continuously operated SBR yielded lower amounts of PHA and more non-specific material (e.g., lipids) than the side-stream batch reactor (Figures 9 versus 11). This lipid reduction is likely associated with the preferential selection of organisms capable of producing PHA, and hence, a shift in the relative abundance or distribution of organisms in the mixed consortium. The higher lipid content in the continuously operated SBR could also be associated with the

relatively higher quantities of phosphorus sequestered by the consortium and a commensurate increase in lipid synthesis associated with fatty acid metabolism.

Unlike production of PHA in industrial wastewaters, wherein a macronutrient deficiency appeared to be the PHA stimulus, PHA production in municipal wastewaters followed a feast-famine cycle (Figures 8 and 10). Maximum PHA production occurred at a defined period after feeding concurrent with maximum reduction in soluble carbon. The occurrence of this feast-famine condition is consistent with previous investigations [11, 28, 49] that focused on environmental matrices other than wild microbial consortia and wastewater.

Based on these results, the following novel concept for coupled PHA production and wastewater treatment in municipal environments is proposed. Treatment of raw wastewater would occur in a biological treatment train consisting of reactors in series designed to create selective anoxic-oxic environmental pressures. This treatment train would principally be designed to remove carbon, nitrogen, and phosphorus, but the selective environmental pressures coupled with the wastewater feed would concurrently yield a PHA-producing microbial consortium. This proposed treatment scheme is consistent with the Phoredox process (e.g., a reactor with alternating anoxic-oxic conditions) originally proposed by Barnard [50], and represents the basic biological scheme for phosphorus removal. Additional selective growth environments, such as conditions for nitrate removal, could also be integrated into the process as long as the principal objective of maintaining a PHA-producing consortium was satisfied. Mass production of PHA would occur in a separate biological reactor receiving biomass routinely wasted from the wastewater treatment reactor. Primary solids fermentate (e.g. a waste stream highly enriched in soluble carbon), derived from a primary solids fermentation reactor, would be supplied to both the wastewater treatment reactor and PHA production reactor. The supply of fermentate is critical to the success of this process scheme, as it provides the quantity and quality

of necessary carbon precursors for PHA synthesis. The proposed process, in addition to producing a commodity, would result in reduced requirements for biosolids handling. Current practices are to separately digest wasted biomass, then either land apply or landfill the stabilized solids. Hence the proposed scheme both reduces treatment costs and creates commercial opportunities.

Elements of this proposed concept to produce commercial quantities of PHA on wastewater are either already practiced in full-scale wastewater treatment facilities, or easily adaptable with no exogenous inputs of feedstock outside of what is already present or generated on-site. For example, side-stream fermenters are currently used as a method to increase phosphate removal in EBPR facilities [51]; feast-famine production is conducive to selector activated sludge [52] with PHA rich solids removed at a stage intermediate to that required for removal of biological oxygen demand (BOD); and operation of a side-stream reactor for hyper-production of PHA is compatible to concepts employed in contact stabilization activated sludge [52]. Hence, the laboratory results and proposed treatment scheme are viewed as representative of what can be achieved in a full-scale facility.

Mixed Microbial Consortia for the Production of PHA in Wastewaters. While the simple fact that such copious amounts of PHA could be produced with an indigenous microbial consortium in real wastewater certainly is significant, another observation carries similar weight. Quantitative PHA analysis on the original microbial seed indicated ca. 0.2% (w/w) PHB and insignificant amounts of PHV, suggesting either limited numbers of PHA-producing organisms, or a limited production capacity. In either case, when exposed to more optimum PHA-producing conditions, this same microbial seed flourished.

In a cursory survey conducted as part of this study, the concentration of PHA in the mixed liquor suspended solids collected from 10 EBPR wastewater treatment facilities ranged

from 0.1 to 4% (data not shown). Given that we produced commercial quantities of PHA from a seed culture that exhibited measurable, yet minimal, PHA, we are assuming the concepts presented in this paper would thus extend to all EBPR wastewater treatment facilities with no modification to the existing microbial consortium. Further, given that all EBPR facilities evaluated as part of this study exhibited microbial PHA synthesis, which is consistent with the current mechanistic view of EBPR [32, 33], we believe that modification of all suspended growth processes to simulate the operational conditions of EBPR would naturally select for a consortium capable of producing limited quantities of PHA that could be further enhanced with operational modifications similar to the ones implemented in this study.

CONCLUDING REMARKS

The PHA production technology demonstrated and proposed herein eliminates costs associated with feedstock production; recycles waste carbon that would otherwise be landfilled, land applied, or combusted; and has negligible energy requirements given that wastewater treatment is mandated in most countries. Recognizing that secondary commodities associated with waste treatment processes, such as reclaimed water for non-potable uses and biosolids utilized as agro-fertilizers, are gaining favor, the extension to PHA production is a natural progression in the evolution beyond simply managing waste.

CHAPTER FOUR

TOWARD A UNIFIED THEORY ON BIOLOGICAL PHOSPHORUS REMOVAL: BACTERIAL STRINGENT RESPONSE

INTRODUCTION

Anthropogenic activities can result in the release of nutrients into the aquatic environment that create a nutrient imbalance leading to advanced surface water body eutrophication which in turn can incur significant ecological and social damage [53, 54] including impacts on raw water intakes and water treatment, and reduced recreational value. Within this context, nitrogen and phosphorus are viewed most critically, as these inorganic compounds are critical nutrients associated with the proliferation of algae, which is a primary indicator of impaired water quality and accelerated eutrophication. Phosphorus, however, is often the limiting macronutrient, with threshold bulk aqueous concentrations as low as 0.01 to 0.02 mg P L^{-1} [55, 56]. Although non-point source discharges arguably contribute the largest load of these critical nutrients, point source discharges such as wastewater treatment facilities nonetheless receive the most attention due to their obtrusiveness and ease with which to regulate. Moreover, removal of phosphorus from wastewater effluent is often viewed as a panacea in the mitigation of eutrophication. In fact, for the first time the U.S. Environmental Protection Agency is proposing national nutrient criteria that in some regions would establish in-stream phosphorus concentrations at this threshold level (Federal Register: January 6, 2003 (Volume 68, Number 3)).

Phosphorus removal from wastewater is accomplished through either biological or chemical means. The former process, commonly referred to as enhanced biological phosphorus removal (EBPR), is applied quite extensively and with certain field successes [56-58], although

the process is often unstable and unreliable [56]. We conducted a survey of some of the most efficient EBPR facilities currently operated in the United States, which revealed that average effluent phosphorus concentrations generally were within 0.3 to 1.0 mg L^{-1}; Kalispell, Montana arguably operates one of the most proficient EBPR facilities, accomplishing an average effluent phosphorus concentration of 0.11 mg L^{-1} for the year 2003, but in our survey this facility was an exception and not the rule. Hence, even the most "efficient" full-scale EBPR systems cannot achieve complete phosphorus removal, which effectively becomes the treatment objective when permit limits are established at the level prescribed above. Considering these process limitations, many facilities also incorporate chemical removal regimes to reliably meet stringent effluent requirements [59]. However, while EBPR processes are integrated into the principal biological wastewater treatment train and arguably enhance the biosolids quality through increased cellular nutrient content, chemical phosphorus removal requires a separate series of treatment units (most commonly chemical coagulation, flocculation, and filtration) and introduces synthetically derived chemicals that often include metals (often iron) that can pose long term problems with biosolids utilization. Further, chemical treatment requires more capital and operational investment. EBPR process failure, and the inability to strategically troubleshoot failed operations, is principally associated with our limited understanding of the highly complex microbial metabolisms involved in microbial utilization and management of phosphorus within the wastewater treatment environment [51, 56]. Therefore, although in concept biological phosphorus removal arguably remains the most environmentally benign method of phosphorus treatment, until these mechanisms are better understood, the use of chemical treatment systems to mitigate these anthropogenic impacts will continue.

The empirical EBPR process, which was initially proposed by Fuhs and Chen [60] based on investigations with the bacterium *Acinetobacter* cultured on synthetic substrate, theoretically

requires the cycling of a microbial consortia between anaerobic (e.g., no exogenous electron acceptor such as oxygen or nitrate) and aerobic (e.g., oxygen or nitrate as the terminal electron acceptor) environments in order to elicit the required metabolic responses. Figure 12 presents a general metabolic diagram highlighting the key empirical process elements, as presented by Grady *et. al.* [52]. Within the anaerobic zone certain microorganisms are postulated to concurrently uptake and store carbon (most often assumed to be acetate) in the form of polyhydroxybutyrate (PHB - the electron acceptor) and hydrolyze internally stored polyphosphate for energy. The resulting energy is proposed to be utilized for carbon transport and metabolism to PHB precursors, as well as for basal microbial maintenance. The hydrolyzed phosphate molecules are cleaved from the cell, yielding a temporal increase in bulk aqueous phosphate concentrations. In the subsequent aerobic zone these same microorganisms are postulated to utilize the stored carbon for growth and maintenance with concurrent uptake and storage of excess phosphate in the form of polyphosphate (upwards of 15% [56]), yielding a net removal of phosphate from the bulk solution.

With theory driving practice, full-scale EBPR facilities are designed to incorporate sequential anaerobic and aerobic zones to elicit the necessary bacterial responses. Further, full-scale EBPR facilities often incorporate primary solids fermentation to generate the prerequisite waste stream rich in soluble short chain organic acids (principally acetate) [59]. In accordance with the above described model, the empirical premise is that the resulting acid-enriched waste stream will be sufficient to maintain a minimum critical influent carbon:phosphorus ratio necessary to drive the prerequisite enhanced metabolic activities [52]. While the recommended ratio varies with nutrient removal goals and treatment reactor configurations, the minimum recommended ratio is 26 mg COD per mg P [52]. Specific to organic acids, the minimum recommended ratio is 7 to 10 mg organic acid per mg phosphorus [61]. However, although the

addition of fermentation products does appear to enhance EBPR, as implied by its incorporation as a design criterion, the explanation for process success is likely not as current theory would suggest.

While the proposed concept of integrated polymer cycling (e.g., PHA and polyphosphate) has been demonstrated in the laboratory with mixed microbial consortia cultured under optimal microbial growth conditions utilizing synthetic substrates and single carbon sources [62-64], the combined metabolic activities have not been shown to occur within a single microorganism within a mixed consortia, nor have any pure cultures been isolated and cultured that accomplish both these metabolic activities [51]. More significantly, negligible field validation has been performed in order to correlate the optimized laboratory conditions with the non-ideal environmental conditions that predominate within a municipal wastewater treatment plant wherein waste streams are often relatively carbon-limited and mixed carbon types prevail [65]. Further, EBPR research has predominantly been conducted utilizing synthetic wastewaters rather than real wastewater. In addition to the seminal research, the two current empirical models proposed to generally explain the broader EBPR mechanistic processes [33, 66-68] were based on mixed microbial consortia cultured on synthetic substrate under optimal growth conditions. Subsequent efforts to better elucidate the complex process mechanistics have been conducted in a similar manner [63, 64, 69-73]. Full-scale wastewater treatment systems operate under dynamic and metabolically unbalanced substrate conditions that very likely elicit different microbial responses than those observed in the laboratory utilizing synthetic, metabolically-balanced wastewaters.

Beyond the implicit variabilities between laboratory and actual field conditions that likely yield different metabolic responses and thus effect how we interpret EBPR metabolism, certain elements of the empirical model are also not necessarily well supported from a microbiological

perspective. Concerning the microbial use of polyphosphate as an energy reserve (in lieu of ATP), even elevated microbial polyphosphate concentrations could only supply ATP-equivalent energy for a few seconds [74, 75]. Further, cellular polyphosphate levels are closely regulated within the cell, resulting in relatively rapid polymer turnover [76]. In fact, two critical genes associated with polyphosphate synthesis and hydrolysis (e.g., polyphosphate kinase and polyphosphatase) are located within the same operon which leads to a coordinated regulation [77]. Hence it is unlikely that hydrolysis of polyphosphate reserves could supply the necessary energy to sustain the mechanistic processes proposed to occur within the EBPR anaerobic zone, even if microorganisms could overcome the implicit genetic controls on polymer concentrations.

Another aspect of the proposed EBPR metabolic model is also not exclusive to, nor highly favorable to occur within, the prescriptive EBPR environments. Specifically, synthesis of PHB, which is but one monomer within the general class of biological polyesters known as polyhydroxyalkanoates (PHAs), readily occurs under fully aerobic conditions [11, 27, 49] and without excess phosphate removal. More importantly, oxygen-deficient conditions are not the dominant driving mechanism for microbial PHA synthesis [78]. Moreover, the limited cellular energetic conditions within the anaerobic environment would not be highly favorable for the prerequisite activities associated with the synthesis of PHB precursors.

An alternative EBPR theory that is congruent with the current model within the context of establishing the prerequisite reactor environmental conditions (e.g., the addition of acid-rich wastewater to elicit a carbon storage response; alternating anaerobic-aerobic environment) but diverges in regard to the postulated microbial metabolic responses is one associated with the bacterial stringent response. Specifically, phosphorus removal is more likely driven by the microbial response to feast-famine environmental conditions (e.g., the transient availability of necessary nutrients for growth, such as exogenous carbon and an exogenous electron acceptor).

While microbes utilize phosphorus in the form of phosphate for energetic purposes, phosphate-based molecules are also critical in the microbial response to stressful environmental conditions (e.g., a famine condition). In fact, the two elements (that is, overall internal cellular phosphorus management activities) may be inextricably linked. Synthesis and accumulation of guanosine tetra- and penta-phosphate ((p)ppGpp) is associated with any exogenous nutritional limitation (e.g., a stressful microbial growth environment) that slows the bacterial growth rate [79]. More specifically, (p)ppGpp is synthesized principally to inhibit ribosome activity and ribosome (e.g., ribosomal RNA) synthesis, among other functions, and since the synthesizing protein resides on the ribosome the magnitude of its synthesis is specifically a function of the microbial growth rate [80]. Furthermore, accumulation of (p)ppGpp has been shown to inhibit the polyphosphate (polyP) hydrolyzing enzyme exopolyphosphatase, allowing for excess accumulation of polyP [74]; thus excess polyP accumulation can occur following synthesis of excess quantities of (p)ppGpp [79]. Finally, considering that PHA is essentially a microbial carbon and energy storage polymer associated with bacterial survival, under certain substrate conditions the bacterial stringent response could arguably also be associated with PHA synthesis and subsequent depletion. Therefore, EBPR may very well be driven by the microbial stringent response.

The goal of this research was to elucidate the relationship between environmental feast-famine conditions that could elicit a microbial stringent response and the EBPR process within the context of an environment that utilizes real wastewater. The specific objectives of the research presented herein were to i) establish an EBPR (e.g., alternating anaerobic-aerobic) reactor that would accomplish phosphorus removal on raw wastewater, ii) establish in the laboratory a municipal primary solids fermenter reactor for the purpose of generating a representative municipal fermenter liquor wastewater rich in soluble carbon, iii) compare and

contrast phosphorus cycling between the raw wastewater-fed EBPR reactor and a fermenter liquor-fed EBPR reactor, iv) compare and contrast phosphorus cycling between the EBPR-based reactors and reactors operated in a non-EBPR mode, v) investigate the extent of a bacterial stringent response occurring in the EBPR-based environments, and vi) present an updated, physiologically-based model to explain the process outcomes.

MATERIALS AND METHODS

Source of Microorganisms. The microbial seed for the EBPR reactors was obtained from the Moscow, Idaho EBPR wastewater treatment facility. The microbial seed for the primary solids fermenter was obtained from the anaerobic digester at the Pullman, Washington wastewater treatment plant.

Source of Wastewater. Raw wastewater was obtained from the Moscow, Idaho EBPR wastewater treatment facility. Thickened primary solids were obtained from the Pullman, Washington wastewater treatment facility.

Description of Source Wastewater Treatment Facilities. The City of Moscow, Idaho operates an EBPR-configured wastewater treatment system. Moscow receives wastewater from both year-round residents and that population associated with the University of Idaho. The City of Pullman, Washington operates a basic complete mix activated sludge treatment system for carbon and ammonia removal. Similar to Moscow, Pullman receives wastewater from both year-round residents and that population associated with Washington State University. With the transient university-based populations, the respective wastewater flows tend to vary quite dramatically with the academic periods.

Culture Conditions. Wastewater biosolids fermentate was produced in a 10-L completely-mixed primary solids fermenter operated as a sequencing batch reactor (SBR), with a dilution rate of 0.25 d^{-1}. The daily decant was centrifuged at ca. 10,000xg, and the supernatant

(e.g. fermentate) recovered. The fermentate-fed wastewater treatment reactor consisted of a 4-L vessel continuously operated on a 24-hour cycle (anaerobically for six hours following feeding, then aerobically for 18 hours). The reactor received undiluted fermentate daily. The raw wastewater reactor consisted of a 4-L vessel continuously operated on a 24-hour cycle (anaerobically for six hours following feeding, then aerobically for 18 hours). Anaerobic conditions were accomplished through the continuous supply of nitrogen gas, and were verified utilizing a dissolved oxygen probe. The reactor received undiluted raw wastewater daily, plus 5 mL of methanol to yield an initial concentration of ca. 0.125% (v/v). Nitrogen gas and air were supplied through a 9-inch diameter Sanitaire® Silver Series II membrane fine bubble disc diffuser (Brown Deer, Wisconsin, USA) installed at the base of the reactor.

Sample Preservation Procedures. Samples for soluble chemical oxygen demand (COD) and soluble orthophosphate were collected and filtered through a sterile 0.22 μm filter. Sodium hypochlorite (5.25%) was added to the PHA samples to inhibit biomass metabolic activity, while formaldehyde (100% purity) was added to the biomass samples assayed for total phosphorus and guanosine tetraphosphate (ppGpp). Samples for ppGpp were further flash-frozen in an ethanol-dry ice bath in order to preserve the nucleotides, and then stored at -80˚C.

Analytical Techniques. COD tests were performed in accordance with Standard Methods 5220-D. Hach high-range ampules (Hach Company, Loveland, Colorado, USA) were utilized, with a Hach COD reactor and a Spectronic® 20 Genesys™ spectrophotometer. Bulk aqueous solution soluble orthophosphate within the treatment bioreactors was determined in accordance with Hach method 8048 (equivalent to Standard Methods 4500-PE). For total cellular phosphorus, dried biomass samples were first digested in 10 mL nitric acid at 150°C, consistent with EPA method 3050, in order to hydrolyze all phosphate-based molecules to orthophosphate. The resulting orthophosphate was determined utilizing a flow injection analyzer

(OI Analytical Flow Solution 3000, College Station, Texas, USA) in accordance with EPA method 365.1. Guanosine tetraphosphate was determined through ion-paired reverse phase high performance liquid chromatography (HPLC) utilizing a Phenomenex Aqua C-18 column (Phenomenex, Torrance, California, USA), in accordance with Neubauer et. al. [81]. Samples were processed on a Hewlett Packard model 1090 series II HPLC, and separations were carried out at room temperature with a flow rate of 1.0 mL min^{-1}. Organic carbon constituents in the fermentate were determined through ion exclusion HPLC analysis utilizing a Rezex™ ROA-Organic acid column (Phenomenex, Torrance, California, USA) and a Waters HPLC (Milford, Massachusetts, USA) equipped with both UV (210 nm) and differential refractive index detectors. Aqueous sulfuric acid (0.005 N) was utilized as the mobile phase, and separations were carried out at 65˚C and at a flow rate of 0.5 mL min^{-1}. Biomass PHA content was determined by GC-MS, following the method of Braunegg *et. al.* [37]. Briefly, dried PHA-rich biomass samples were digested for four hours at 100°C in 2 mL each of acidified methanol (3% v/v sulfuric acid) and chloroform. Benzoic acid was added to the chloroform as an internal standard at 0.50 mg mL^{-1}. Following vigorous vortexing of the mixture with one mL deionized water, the PHA-rich chloroform was recovered for analysis. The chloroform phase was dried with sodium sulfate prior to analysis. GC-MS was performed on a Thermofinnigan PolarisQ iontrap GC-MS instrument (Thermo Electron Corporation) in positive ei mode. The sample was introduced using a split injection. Separation was achieved on a ZB1 (15m, 0.25mm ID) capillary column (Phenomenex, Torrance, California, USA) with helium as the carrier gas (1.2 mL min^{-1}) using a temperature program 40°C (2 min) ramped to 200°C at 5°C min^{-1}. The Xcalibur software program (Thermo Electron Corporation) was used to analyze the data. The identity of the compounds was confirmed by retention time and mass spectral matching with known standards (as methyl ester derivatives), and quantified based on the internal standard.

RESULTS AND DISCUSSION

Phosphorus Removal in a Raw Wastewater-fed EBPR Reactor. Utilizing a mixed microbial seed obtained from an EBPR wastewater treatment facility, a reactor was fed raw wastewater supplemented with methanol (identified as reactor RW-1) and operated in a conventional EBPR scheme. Raw wastewater was also derived from the same facility. Initially, parallel reactors were operated, one with methanol addition and one without. However, while the wastewater-only reactor generally managed phosphorus consistent with current theory, ultimately, the subject reactor was shut down, as the carbon concentration was insufficient (ca. 100 mg L^{-1} as soluble COD) to maintain adequate metabolic activities for the purpose of investigating EBPR. Methanol was added in order to increase the carbon concentration. Methanol was chosen because 1) the carbon concentration could be directly increased without altering the other raw wastewater nutrient concentrations, 2) this form of carbon would provide a contrast with the addition of carboxylic acids, and 3) methanol is utilized at full scale wastewater treatment facilities to enhance denitrification, hence it is a carbon source associated with full-scale wastewater treatment [61].

The cycling of phosphorus in reactor RW-1 effectively followed that response predicted by the current EBPR theory (Figure 13). More specifically, during the six hour anaerobic period the microbial consortia cleaved phosphorus, resulting in an increase in bulk solution concentration of 41%. The increase in bulk solution phosphorus does not appear to be associated with microbial cell lysis, as the biomass concentration essentially remained constant. Phosphorus was then rapidly removed during the aerobic period, with complete removal in 3 hours. Total cellular phosphorus reserves effectively remained constant (Figure 13), which could be predicted considering that the low amount of phosphorus sequestered by the consortia (ca. 1.9 mg L^{-1}) was only ca. 0.2% (w/w) of the average mixed liquor concentration of 930 mg L^{-1}.

With the microbial phosphorus cycling pattern consistent with the current EBPR theory, it was expected that carbon cycling would similarly follow the current model. However, while the consortia generally utilized carbon at a constant rate over time regardless of the exogenous electron acceptor environment, no appreciable PHA synthesis occurred (Figure 13). The only form of PHA detected was PHB; considering that the wastewater carbon was principally methanol, this result was not unexpected, as methanol is a direct precursor to PHB synthesis [44, 45]. Total carbon removal during the nine hour time period was ca. 29%, with ca. 61% carbon removal occurring over a full 24 hour cycle.

The nutrient cycling patterns for described above for reactor RW-1 were found to be consistent over time. More specifically, reactor RW-1 was independently established and operated three separate times within a 9 month period, and the operational patterns were consistent throughout. This repeatability and consistency across independent reactors demonstrates a robust and stable operating condition.

Primary Solids Fermentation. To contrast operations with the raw wastewater reactor and to establish consistency with current EBPR design theory, a lab-based primary solids fermentation reactor was established to generate the prerequisite wastewater to investigate the relationship between fermentation byproducts and phosphorus removal. To maximize the production of fermentation byproducts and minimize the potential for methanogenic microorganisms and thus oxidation of the desired organic carbon, the fermenter was operated at a dilution rate of 0.25 d^{-1} (e.g., a solids retention time of 4 days), which is consistent with recommended design guidelines [52]. Pullman's primary solids concentration generally ranged from 3% to 4% (w/v). The resulting fermentate exhibited a soluble chemical oxygen demand (COD) ranging from ca. 1,500 mg L^{-1} to 1,700 mg L^{-1}, with soluble orthophosphate concentrations at 20 to 25 mg P L^{-1}. Although the fermentate was derived through a

centrifugation process, not all suspended matter was removed; the unfiltered fermentate COD was ca. 3,000 mg L^{-1}. Thus the fermenter operation well emulated full-scale wastewater treatment operations wherein fermentate would always contain some particulate matter that would likely consist of microorganisms as well as some residual primary solids.

Fermentation products can include a variety of reduced organic compounds such as alcohols and other short chain organic acids in addition to acetate. Moreover, most fermentation byproducts are common precursors to PHA synthesis; in fact, the carbon substrate form controls the polymeric structure of the PHA [9]. Hence, the fermentate was evaluated to understand the predominant carbon types. HPLC analyses on the fermentate revealed the primary carbon constituents as acetic acid and propionic acid, at concentrations of ca. 825 and 250 mg L^{-1}. The fermentate was evaluated against standards for acetic, propionic, butyric, and pentanoic acid, as well as ethanol and methanol. A nominal amount of the larger chain carboxylic acids also appeared to be synthesized, with no significant alcohol products. Using typical stoichiometric ratios for acetic and propionic acid (e.g., mg COD:mg acid), these two constituents were determined to be the predominant forms of carbon in the fermentate.

Phosphorus Removal in a Fermenter-fed EBPR Reactor. Utilizing a mixed microbial seed obtained from an EBPR wastewater treatment facility, a fermentate-fed SBR (reactor FE-1) operated following an EBPR scheme consistently maintained a microbial consortium capable of producing PHA while concurrently removing excess phosphorus. While the reactor produced copious amounts of PHA and removed a significant mass of phosphorus, the microbial consortia did not cycle carbon and phosphorus consistent with current theory. More specifically, phosphorus removal was initiated immediately within the anaerobic cycle, and continued at a first-order rate through the aerobic cycle (Figure 14). Total phosphorus removal was ca. 86% within the anaerobic period, and approached a steady state bulk aqueous condition. An

additional 5% removal of phosphorus occurred within the aerobic period. The consortia did not remove any carbon during the anaerobic period, but rapidly consumed the available organic acids immediately upon transitioning into an aerobic cycle with concurrent PHA synthesis (Figure 14). A total aerobic soluble carbon removal of ca. 66% occurred within 3.5 hours, again following first order kinetics. Interestingly, little carbon removal occurred after 3.5 hours; rather, the consortia subsequently depleted the PHA storage reserves. The resulting carbon-to-phosphorus ratio immediately upon reactor feeding was ca. 81:1 (mg COD L^{-1}:mg P L^{-1}).

Accumulation of internal cellular carbon storage reserves mirrored the consortia's management of bulk solution carbon. Total cellular PHA content remained at minimal levels throughout the anaerobic period. Immediately within the aerobic period, PHA synthesis occurred rapidly, reaching a peak of ca. 25% (w/w) (Figure 14). PHA synthesis appeared to follow first order kinetics, similar to the kinetic pattern for bulk solution carbon removal. Further, the form of PHA was generally a 50:50 split between PHB and PHV. Considering that the fermentate was a mixture of short chain carboxylic acids, and that the form of PHA is a function of the carbon source, the resulting different forms of PHA was not unexpected. This pattern of carbon cycling is consistent with the theory of microbial PHA synthesis in a feast-famine environment [11, 49].

Total phosphorus analyses revealed that although relatively significant quantities of phosphorus were removed from bulk solution, total cellular phosphorus remained essentially constant at 3.06%±0.20% (mg P mg^{-1} cell dry weight (CDW) – Figure 14). During the nine hour period, ca. 10.6 milligrams of phosphorus were removed from bulk solution. A mass balance analysis on bulk aqueous and soluble phosphorus revealed that all the phosphorus ended up as biomass-based material. To a certain extent this outcome of minimal change in cellular phosphorus content could be predicted, considering that the amount of phosphorus removed

relative to the reactor biomass concentration was relatively low. More critically, considering that the average cellular phosphorus content was only ca. 50% higher than basal microbial phosphorus requirements of 2% [61], which is much less than the upwards of 15% cited by Seviour *et. al.* [56] for a polyP accumulating EBPR consortia, these results suggest that more members of the microbial consortia were involved in phosphorus removal.

The described operating conditions were replicated in three distinct reactors separately started and operated within a nine month period. Each of these reactors was established with a new microbial seed obtained from the Moscow, Idaho facility, with the fermenter operated with similarly new (e.g., "fresh") material. Further, the reactor microbial seed and primary solids were obtained under different seasonal conditions (e.g., fall, winter, spring) and under varying City wastewater conditions (e.g., with and without the large university student populations in Pullman and Moscow). Thus, in our view, the presented results represent a robust and stable operating condition.

Effects of Switching Reactor Feedstock. To evaluate how each established microbial consortia would function under dynamic nutrient conditions, the feedstock to the respective reactors was switched. More specifically, the feedstock to reactor FE-1 was converted to raw wastewater and methanol (reactor identified as RW-2), and the feedstock to reactor RW-1 was converted to fermentate (reactor identified as FE-2). All other operating parameters remained the same. Three interesting mechanistic responses were observed. First, each reactor microbial consortia ultimately switched metabolic responses. More specifically, the fermenter-fed reactor that was switched to raw wastewater/methanol ultimately stabilized to cycle phosphorus and carbon consistent with the results shown in Figure 13 (data not shown), and vice versa. These results strongly indicate that the microbial consortia is quite robust in that it is capable of adapting well to a changed nutrient condition, and can continue to remove significant quantities

of phosphorus. Second, relative to RW-1 converted to FE-2, the microbial consortia adapted to a significant increase in initial reactor phosphorus concentration to ultimately remove, and store, much higher quantities of phosphorus, and also adapted to synthesize both PHB and PHV, wherein the consortia was previously only producing PHB. Further, the consortia appeared to be more efficient at phosphorus removal, as contrasted with reactor FE-1. Specifically, the ratio of phosphorus removed to biomass concentration increased by a factor of approximately two in reactor FE-2, suggesting that the switch in feedstock elicited a selection for phosphorus removing microbes. Third, reactor FE-1 (switched to RW-2) was originally acclimated to operate at quite high and more balanced feedstock conditions, yet the consortia stabilized to function efficiently under the reduced and more imbalanced nutrient conditions (as reactor RW-2).

As would be expected with the altered, and implicitly different, nutrient conditions, the relative biomass concentrations also adjusted, with the FE-1 reducing from ca. 1,870 mg MLSS L^{-1} to ca. 930 mg L^{-1} in RW-2, and RW-1 increasing from ca. 600 mg L^{-1} to ca. 1,300 mg L^{-1} in FE-2. Significantly altering the substrate nutrient condition appears to have yielded a more robust and efficient microbial population in both reactors, and also possibly a less diverse, more specialized microbial population. Hence, the respective reactor performances were less a function of the number of microbes and more a function of the number of microbes that could most efficiently function in the individual environments. Also of critical interest relative to full scale EBPR operations was the resulting operational condition during the period of time that the consortia acclimated to the new feedstock conditions. During this period of approximately 10 days, a process upset condition prevailed, wherein minimal, if any, phosphorus removal occurred.

Phosphorus Removal in a non-EBPR Reactor. As noted, conventional EBPR theory requires both an anaerobic zone and a fermentate-supplemented (e.g., organic acid-rich) feedstock. To investigate the relative effects of these parameters and whether the two factors are exclusively related in the biological removal of excess phosphorus, a fully aerated, fermentate-fed bioreactor (reactor FE-3) was operated to establish baseline microbial phosphorus removal and PHA synthesis capabilities. This reactor was effectively operated as a carbon removal activated sludge wastewater treatment facility [61], with a solids retention time of 5 days.

Utilizing a PHA-producing mixed microbial seed from the Moscow, Idaho EBPR wastewater treatment facility, a fermentate-fed SBR concurrently achieved phosphorus removal, carbon removal, and PHA synthesis (Figure 15). Following a small initial increase in phosphorus and COD, both COD and phosphorus concentrations diminished simultaneously according to first order reaction rate kinetics. Steady state concentrations were effectively achieved within 8 hours, with 31% phosphorus removal and 67% COD removal; over 24 hours nearly 40% phosphorus and 71% COD removal was achieved. PHA synthesis occurred concurrently with phosphate and COD depletion, peaking with depletion of substrate at a concentration of ca. 12% (w/w). Complete PHA depletion occurred within the 24 hour cycle.

Contrasting reactors FE-1 and FE-3 reveals some critical insights toward a better understanding on EBPR. First, while the percent removal and quantity of phosphorus removed within the EBPR-based reactor configuration were significantly greater in reactor FE-1 (91% versus 40%; 10.7 mg/L versus 2.3 mg/L), on a biomass basis (e.g., mg P removed per mg reactor biomass) phosphorus removal was comparable. The difference in removal was thus associated with a lower biomass concentration in reactor FE-3, which can be attributed to the longer operating aerobic cycle (24 hours versus 18 hours) and resulting endogenous decay, and also less initial carbon. The similar stoichiometric ratios suggest that the anaerobic condition is not the

exclusive driving environmental famine condition, and further that a famine condition on carbon (e.g., complete depletion of available carbon reserves) could elicit a microbial stringent response that leads to additional phosphorus uptake. These results also suggest that the anaerobic period does not necessarily select for a significantly different microbial population with regard to phosphorus removal. Second, the carbon removal pattern was similar between reactors FE-1 and FE-3, both in terms of environmental response (e.g., carbon removal exclusive to the aerobic time period), quantity of carbon removed (ca. 60%), and the initial fate of carbon (e.g., PHA synthesis). The total PHA accumulated in reactor FE-3 was approximately half that of reactor FE-1, however, the initial COD concentration in FE-1 was twice that of FE-3. Based on this reactor FE-3 operation, it can be concluded that 1) in addition to a famine condition on oxygen, a famine condition associated with carbon depletion could elicit the necessary microbial response to accomplish excess phosphorus removal, and 2) the carbon cycling pattern does not appear to be influenced by the anaerobic conditions.

Mechanism for PHA Synthesis on Fermentate. While the PHA response determined in the EBPR and non-EBPR fermentate-fed reactors certainly provides strong evidence to support an aerobic feast-famine PHA synthesis condition, we further investigated the phenomenon under elevated substrate conditions (e.g., higher F:M ratio). Fermentate was fed to a fully aerated batch reactor that was seeded with the daily decant from reactor FE-1. The volumetric feed ratio was 0.6:1 (fermentate volume:total reactor volume), as contrasted with a ratio of 0.2:1 in the parent reactor, thus providing significantly more carbon to the microbes (e.g., higher F:M). Within 3.5 hours, PHA production peaked at ca. 53% (w/w), concurrent with apparent depletion of readily utilized carbon, with a PHB:PHV ratio of approximately 1:1 (Figure 16). As shown, the reactor PHA concentration at t=0 was negligible. These results clearly demonstrate that PHA synthesis is not coupled to the presence of an anaerobic cycle

preceding the aerobic period. Rather, the mechanism for PHA synthesis undoubtedly follows the feast-famine PHA synthesis model [61]. More critically, these results confirm that PHA synthesis in all of the fermenter-based reactors follows the feast-famine response.

An Advanced Theory on Enhanced Biological Phosphorus Removal – Microbial Stringent Response. The two feedstock-contrasted EBPR configurations reviewed herein both imposed stressful (e.g., feast-famine) growth environments, although in dissimilar manners and at different magnitudes. Under fermentate-fed conditions, the microbes were presented with a relatively nutrient balanced and rich feedstock condition such that all microbial growth requirements were initially met. However, with the relative excess of readily utilizable carbon substrate eliciting a feast-famine PHA synthesis response, ultimately carbon became a limiting nutrient as the consortia transitioned from a "feast" environment with the microbes likely in a hyper-log growth phase to a "famine" environment wherein the microbes likely transitioned into a stationary phase of growth. Further, the alternating anaerobic-aerobic condition yielded a feast-famine on oxygen (or more generally, on the exogenous electron acceptor). Hence the fermentate-fed microbial consortia were presented with two environmentally stressful conditions. As a contrast, the raw wastewater/methanol-fed reactor did not realize the same concentrated and well-balanced nutrient conditions, and thus the alternating anaerobic-aerobic condition was the dominant stressful (e.g., feast-famine) environment. Ultimately each microbial consortium managed phosphorus in a different manner, which is believed to be directly a function of the different stressful conditions imposed. More specifically, it is believed that the different feast-famine conditions elicited different magnitudes of a microbial stringent response, and that the magnitude of the stringent response is the controlling factor in the microbial ability to sequester additional, or excess, phosphorus. To elucidate the extent and relative magnitude of this

microbial stringent response and the implications on biological phosphorus removal, biomass samples were assayed to determine the concentration of ppGpp.

EBPR Response No. 1. For reactor FE-1, HPLC results revealed that ppGpp concentration was relatively constant during the anaerobic period, but increasing over time during the aerobic period (Figure 17). Moreover, the anaerobic ppGpp concentration was generally 50% or more higher than the background level of ca. 100 nmol per gram cell dry weight found in pure microbial cultures [81]. Considering that this consortia had experienced hyper-log growth during the carbon "feast" phase, the ribosome concentration would have been relatively high upon transition both into the carbon famine period and the anaerobic (e.g., oxygen famine) period, thus increasing the potential for a higher ppGpp concentration. Thus, not only could ppGpp serve as a sink for phosphate, the ppGpp appears to have been at a sufficient concentration to inhibit polyP hydrolysis both during the anaerobic and aerobic periods. Evidence to this fact can be seen both in the total phosphate levels of ca. 3%, which is 50% higher than basal microbial needs [61], and that no apparent polyP cleavage occurred as indicated by bulk solution phosphorus monitoring. Regarding the elevated total phosphate levels, this number also suggests that potentially a smaller fraction of the population was accumulating excess phosphorus.

EBPR Response No. 2. As a contrast to reactor FE-1, ppGpp concentrations in the reactor RW-1 biomass decreased during the anaerobic period when phosphorus was cleaved from the cell; during the aerobic period when phosphorus uptake occurred, ppGpp concentrations increased significantly (Figure 17). There does appear to be a lag effect between cellular ppGpp concentration and phosphate cleavage from the cell, as evidenced in the first two anaerobic hours when the ppGpp concentration remained relatively constant (and at the same level determined for reactor FE-1 wherein no phosphate hydrolysis occurred). During this brief period the

consortia likely experienced a moderate stringent response due to the imposition of the oxygen famine condition that would clearly inhibit growth, which generated some ppGpp. However, this reactor did not experience a similar hyper growth phase nor a feast-famine carbon condition (e.g., PHA synthesis and depletion), hence the cellular ribosome concentration would have been significantly reduced as contrasted with reactor FE-1, thus appreciably reducing the ability to sustain ppGpp synthesis (e.g., the stringent response) throughout the anaerobic period. The general trend of ppGpp decreasing anaerobically, and ultimately reaching zero, supports this theory, and further confirms that the molecule was not present in sufficiently high concentrations to block polyP hydrolysis, nor balance polyP hydrolysis with synthesis, resulting in phosphorus cleavage. During the subsequent aerobic period the increasing ppGpp concentration coincides with the consortia's uptake of phosphorus; further, the microbes would be in need of restoring the internal polyP reserves that had been significantly depleted anaerobically. Within this aerobic period note that 1) ppGpp concentration peaked concurrent with phosphorus depletion, and 2) ppGpp concentration was at a level comparable to that experienced in reactor FE-1 wherein excess phosphorus accumulation occurred. The relative unbalanced growth conditions (e.g., high carbon concentration relative to the other macronutrients) within the aerobic period likely elicited a moderate stringent response, leading to ppGpp synthesis. Hence ppGpp synthesized within this aerobic environment could be facilitating polyP synthesis and storage. In regards to the potential presence of a sub-population of microbes that sequestered phosphorus above basal microbial levels, although the average cellular content was within the typical 1% to 2% range, it is possible that certain microbes did accomplish above-average accumulation. As evidence, again consider the test of switching feedstock and the outcome of a population that, on average, sequestered phosphorus at above average levels.

The results presented herein demonstrate that 1) the two metabolic processes identified within the current EBPR theory as integrally critical to process success (e.g., PHA synthesis and polyphosphate hydrolysis/phosphate removal) are not metabolically nor intrinsically coupled, and, in fact, excess carbon leading to high rates of PHA synthesis appears to be an environmental condition that may require an additional oxygen famine condition to accomplish complete phosphorus removal; 2) however, the carbon form and concentration, and also the overall relative nutrient balance, does directly effect how the microbial consortia removes phosphorus within the alternating anaerobic-aerobic environment associated with microbial growth and the bacterial stringent response, 3) the addition of an anaerobic environment does yield a necessary stress response that is intrinsically associated with phosphorus removal and the microbial stringent response, and 4) polyphosphate functioning as a source of energy is not a dominant mechanism associated with the microbial management and use of phosphorus; rather, energy production may simply be a secondary outcome.

Full-scale Validation. Some preliminary evidence to support this proposed EBPR *Response No. 2* at a full scale was found with the Kalispell, Montana EBPR facility. This municipal wastewater treatment facility, which is constructed as a Modified UCT process, augments the influent wastewater with fermenter liquor at a fermenter liquor: raw wastewater ratio of 0.05 to 0.1. As noted, this facility accomplishes significant and consistently stable biological phosphorus removal. Assays on bulk solution phosphorus, cellular phosphorus, and PHA within the different environmental zones in the treatment reactor revealed that the microbial consortia responded to the overall environment in a manner quite consistent with that found with the methanol-supplemented raw wastewater (e.g., *Response No. 2*). In addition to providing support for our new model, these results further suggest that the specific form of

carbon is not necessarily critical; rather, the ratio of augmentation to raw wastewater is the critical factor.

Potential Explanation of Full-Scale EBPR Failures. A critical point to be drawn from the results presented herein relates to the overall impact of a dynamic feedstock condition and the microbial response associated with cellular phosphorus management. As shown herein, a dramatic shift in feedstock elicited a significantly different, and arguably competing, phosphorus removal response. Process failure of full-scale EBPR facilities most often goes unexplained due to the overall lack of process understanding [56], however, the cause may be directly related to a dynamic feedstock condition. For facilities that augment their influent waste stream with fermentate, a shift in the relative ratio of fermentate:raw wastewater could elicit opposing metabolic responses as discussed herein that ultimately cause process upset. These results thus suggest that EBPR facilities that augment with fermentate should closely monitor the relative ratio in order to maintain process stability. Alternatively, EBPR facilities could seek opportunities to augment the raw wastewater with a high carbon, low nutrient supplement.

Mixed Microbial Consortia for the Concurrent Removal of Phosphorus and Production of PHA in Wastewaters. As a contrast to the performance of the respective reactors described herein, the original wild microbial seed was estimated to contain ca. 0.2% (w/w) PHB, and insignificant quantities of PHV. However, upon acclimation to the different wastewater conditions, and when subsequently exposed to switched feedstock conditions, the same consortia exhibited notably different metabolic responses. Further, the same consortia exhibited quite robust and flexible metabolic capabilities. These results suggest that it would be reasonable to expect that the results presented herein could be extrapolated to optimize operation of full-scale municipally-based EBPR facilities.

Significance and Technology Advancements. The significance of the findings presented herein relates to the current regulatory climate, and the associated cost of compliance. Within the wastewater treatment community, absolute, or effectively complete, phosphorus removal from wastewater is increasingly becoming a regulatory requirement, which is concurrently requiring alternative, and often expensive, approaches to wastewater management, since, until now, the necessary process understanding required to achieve complete phosphorus removal biologically has been unavailable. For example, in the Pacific Northwest region of the United States, treatment facilities that discharge into the Spokane River system (specifically, the City of Spokane, Washington, and Spokane County) are facing expenses of approximately $760M to add physical/chemical treatment capabilities to meet a proposed new permit limit of 0.01 mg P L^{-1}; the City of McCall, Idaho was forced to completely remove their effluent discharge from the Payette River due to phosphorus treatment requirements; facilities that discharge into the Tualatin River in western Oregon are faced with permit limits of 0.07 mg P L^{-1} that can often be met through their EBPR process, but that demand physical/chemical treatment as backup during process upsets; and the City of Moscow, Idaho is currently planning a $4.0M chemical phosphorus removal upgrade of their EBPR facility in order to meet their permit limit of 0.136 mg P L^{-1}. As noted, on a larger scale, for the first time the U.S. Environmental Protection Agency is proposing national nutrient criteria that in some regions would establish in-stream phosphorus concentrations as low as 0.01 mg L^{-1} (Federal Register: January 6, 2003 (Volume 68, Number 3)).

Herein we have elucidated the fundamental microbial metabolism driving microbial phosphorus removal as the microbial stringent response. Perhaps more critically, implementation of the proposed EBPR model described herein, specifically that targeting *Response No. 2*, can accomplish removal of orthophosphate to negligible bulk solution levels,

thus eliminating the need for additional physical/chemical treatment systems. Critical to process success is the optimization of exogenous wastewater augmentation within an alternating anaerobic-aerobic environment, and the maintenance of conditions that yield a steady state. When implemented correctly, conditions will be established that elicit a stringent response that causes an anaerobic cleavage of phosphorus and a subsequent aerobic uptake of phosphorus. However, there is clearly an exogenous wastewater augmentation threshold that, when crossed, elicits a different microbial phosphorus uptake response. Further, once this threshold is crossed, significant process upset prevails during the resulting stabilization period. The target augmentation ratio must be sufficient to elicit the necessary growth response that results in complete phosphorus removal, while avoiding a hyper-growth response the yields excess PHA synthesis through microbial "feast-famine".

Ultimately the EBPR process can be viewed as a wastewater treatment scheme that functions to subject microorganisms to a bacterial fitness test through the creation of alternating stressful environmental conditions. The concept is exemplified by the molecular synthesis, or lack thereof, of PHA and (p)ppGpp, two molecules that serve as environmental stress mediators for microorganisms. With this fundamental understanding of the principal driving mechanism for biological phosphorus removal, we then gain an improved understanding on how to design and operate these processes to achieve steady state operations.

CHAPTER FIVE

CONCLUSIONS

The research presented herein represents what may initially appear to be a disparate set of concepts, however, within the context of process integration and green engineering [2], the individual pieces become joined into a congruent picture. Integrating polyhydroxyalkanoate (PHA) production into a wastewater management scheme effectively eliminates two of the three major cost and environmental impacts associated with PHA production (e.g., feedstock production and bioreactor operations) [17, 20, 21], and further generates a commodity that could be utilized to offset wastewater treatment costs. By further merging the production of PHA with the NFRTC manufacturing process, and by utilizing PHA-rich microbial material (e.g., not purified), we not only eliminate the cost and energy requirements associated with polymer extraction and purification [17, 21], a specific example of one of the many benefits of process integration in green engineering [2], we generate yet another commercial commodity (NFRTCs) that can be utilized to offset the cost of advanced wastewater treatment.

Regarding coupled PHA production and wastewater treatment, we have shown that conditions conducive for maintenance of a microbial population that can hyper-produce PHA concurrently elicit a microbial response for excess phosphorus removal that is inconsistent with current dogma. More specifically, the feast-famine condition for aerobic PHA production, which ultimately yields a stressful growth environment under the carbon famine condition, coupled with an implicitly stressful anaerobic state (e.g., famine on oxygen), elicits a bacterial stringent response that facilitates guanosine tetraphosphate synthesis and ultimately excess phosphorus removal both anaerobically and aerobically. These results clearly demonstrate that polyphosphate hydrolysis and PHA synthesis are not intrinsically related. Further, the

occurrence and magnitude of the stringent response was shown to be predicated on the form of carbon and the overall wastewater nutrient balance. Under conditions of a nutrient-imbalanced substrate (e.g., methanol-augmented raw wastewater), no carbon feast-famine/PHA synthesis response developed, however, the oxygen famine condition elicited a sufficient stringent response to accomplish effectively complete orthophosphate removal from the wastewater. The elucidated phosphorus removal mechanism is, most critically, consistent with bacterial microbiology and biochemistry, and the response was shown to be the consistent driver across different wastewaters. Hence, this research has generated a new mechanistic description of the critical microbial mechanism associated with microbial phosphorus removal, which will significantly improve the pursuits to design and operate more stable and reliable enhanced biological phosphorus removal treatment facilities.

Clearly further studies are necessary to advance the novel polymer production process, including a detailed life-cycle analysis, development of fundamental relationships between carbon sources present in wastewaters and the type of PHA synthesized, characterization of the mixed-microbial consortia, and process optimization at a full scale. However, given the proposed polymer production process arguably eliminates the energy and costs associated with feedstock production and bioreactor operation, results presented herein suggest environmentally benign production of biodegradable polymers is feasible. With regard to PHA-rich based NFRTCs, further investigations are necessary to better understand the affects of cellular biomass versus conventional filler or fibers on the NFRTC material properties. For example, natural fibers have been shown to increase PHB crystallinity [14, 15], although it is not clear that the composite material properties are thus improved. Biomass may also potentially serve as a plasticizing agent in the composite formulations, and certain plasticizers have been shown to improve NFRTC material properties [13]. To further advance the new biological phosphorus

removal theory, additional investigations are necessary to characterize the effects of different carbon sources and the overall nutrient balance, as well as extend the real wastewater-based investigations to alternative reactor configurations that remove additional nutrients such as nitrogen. Further, molecular studies should be conducted to more specifically elucidate the microbial metabolisms associated with phosphorus utilization and storage under the stringent response condition, such that appropriate design models can be developed for the EBPR process. Specifically, proteomics investigations to determine the relative concentrations of the key proteins associated with the stringent response and cellular polyphosphate maintenance could provide useful kinetic and stoichiometric parameters that could be integrated into process design equations..

FIGURES

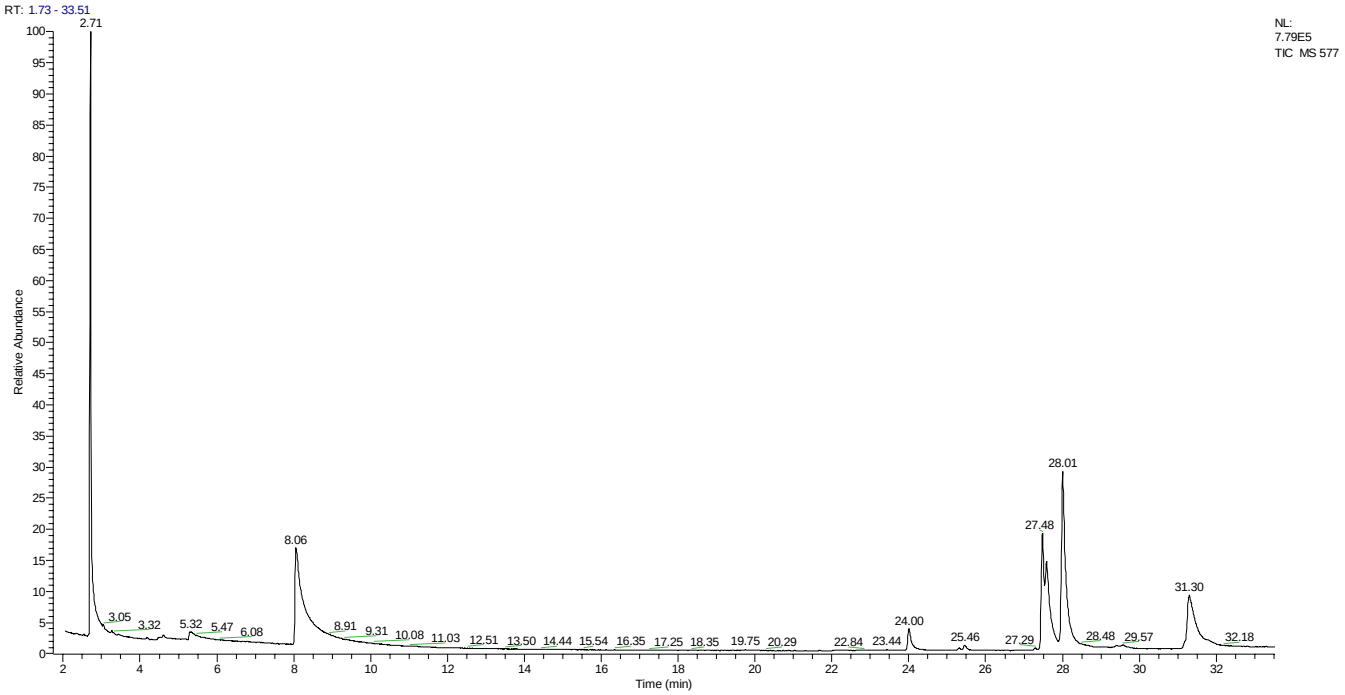

Figure 1. Gas chromatogram of non-chlorinated PHB-rich biomass sample. PHB methyl ester eluted at 2.71 minutes. Other peaks include the benzoic acid methyl esters (8.06 minutes) and esterified cell-wall associated lipids (24, 27.48, 28.01, and 31.3 minutes).

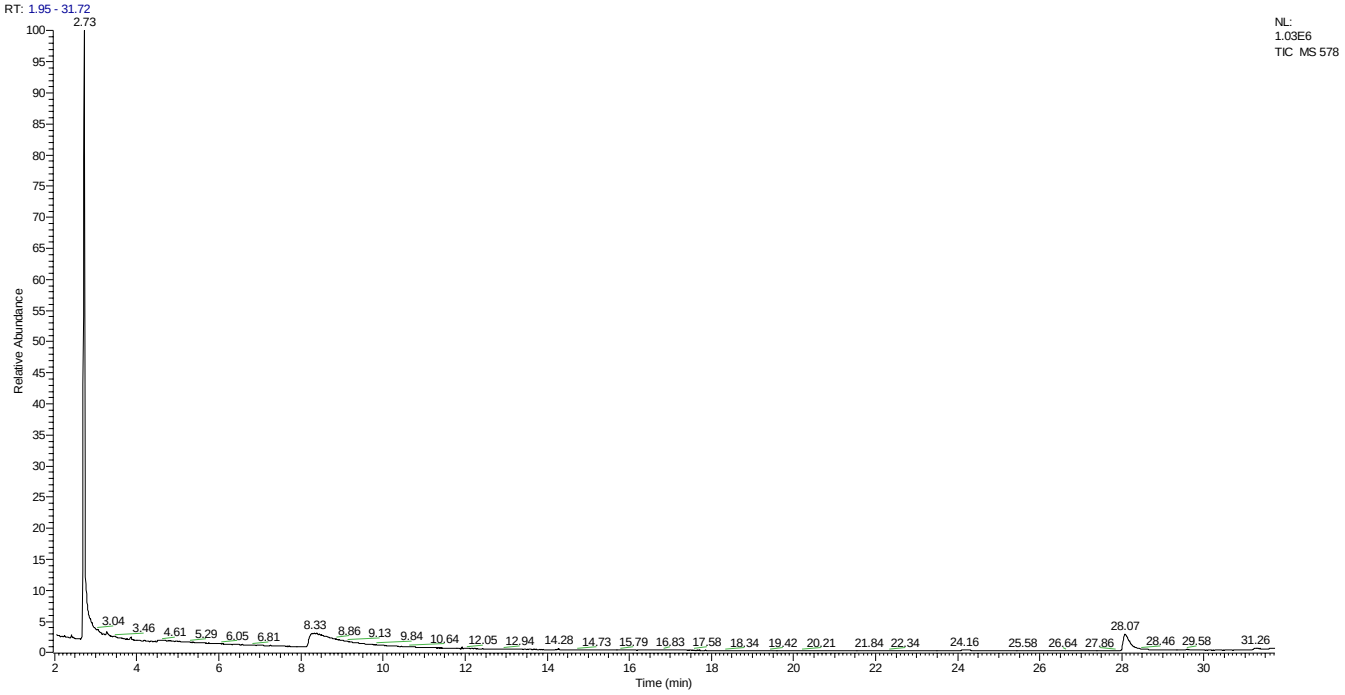

Figure 2. Gas chromatogram of chlorinated PHB-rich biomass sample. PHB methyl ester eluted at 2.73 minutes. Other peaks include the benzoic acid methyl esters (8.33 minutes) and esterified cell-wall associated lipids (28.07 minutes).

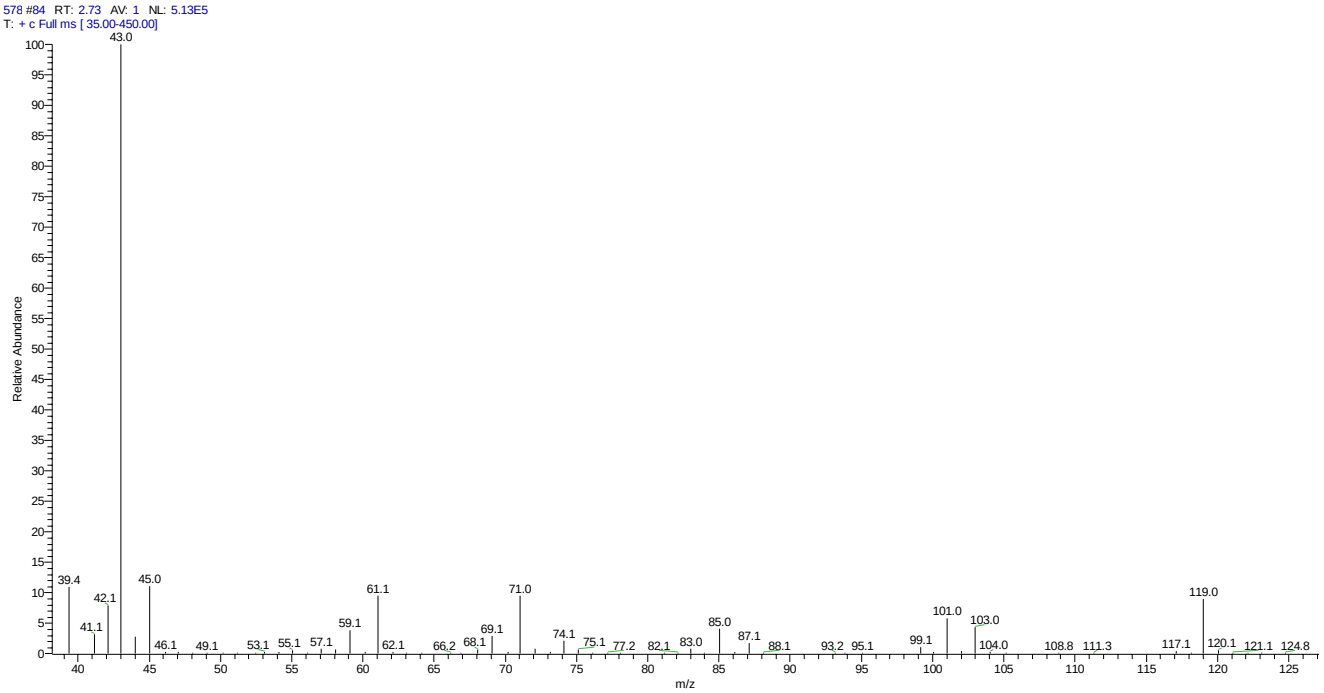

Figure 3. Mass spectrum of methyl 3-hydroxybutyrate, for the gas chromatograph peak of 2.73 minutes from the chlorinated PHB-rich biomass sample (Figure 2).

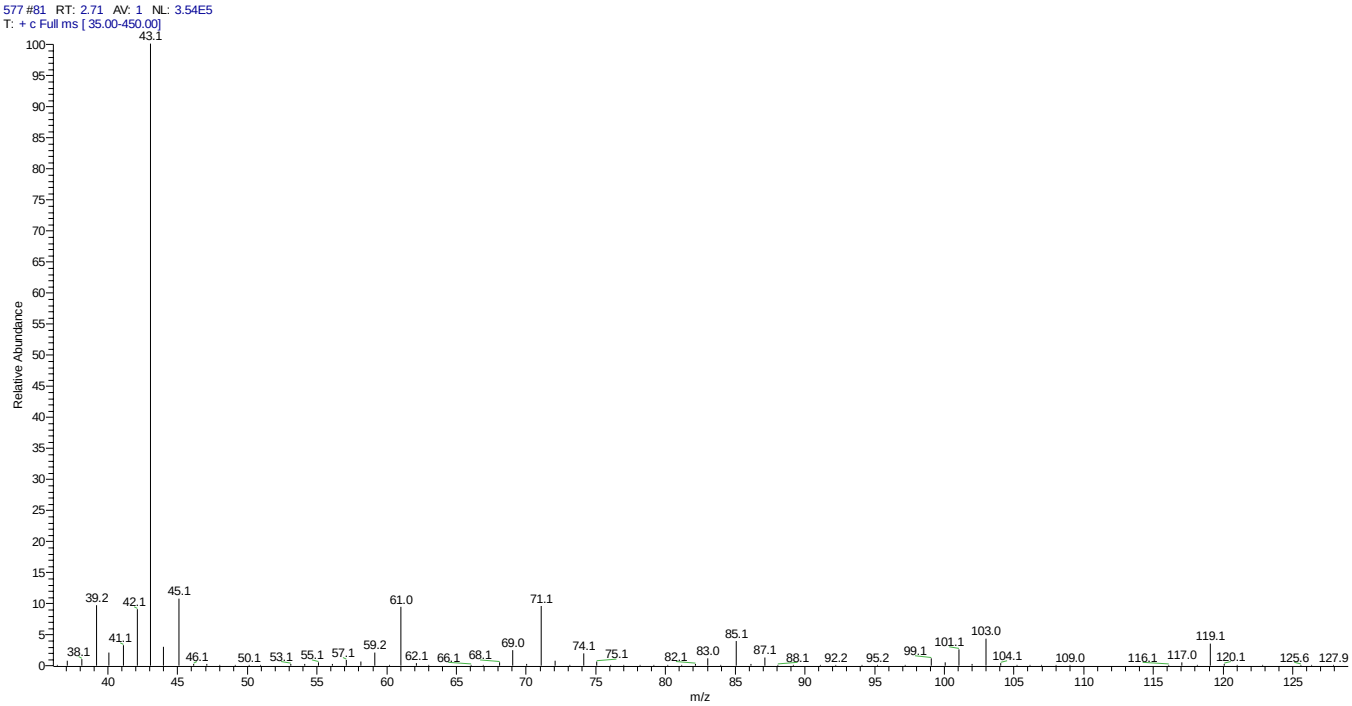

Figure 4. Mass spectrum of methyl 3-hydroxybutyrate, for the gas chromatograph peak of 2.71 minutes from the non chlorinated PHB-rich biomass sample (Figure 1).

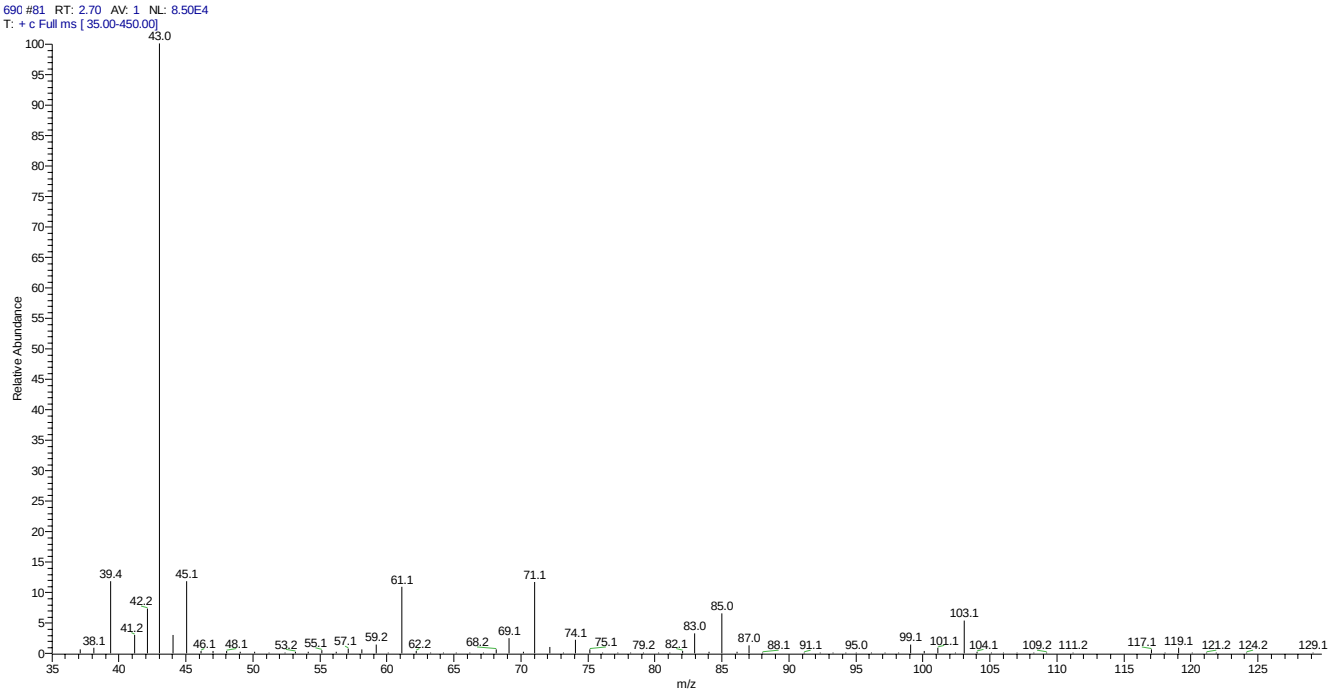

Figure 5. Mass spectrum of methyl 3-hydroxybutyrate, from a pure PHB sample, used as a control.

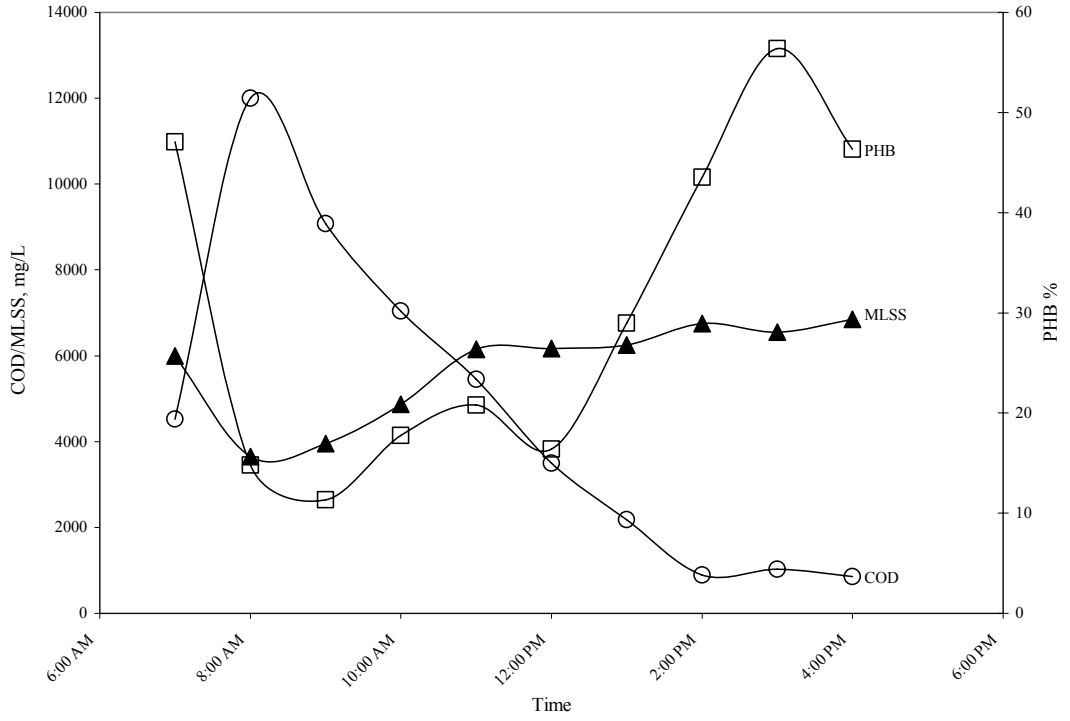

Figure 6. Summary of typical *A. vinelandii* UWD bioreactor operating cycle, highlighting PHB synthesis, carbon utilization (e.g. COD), and bacterial population (e.g. MLSS).

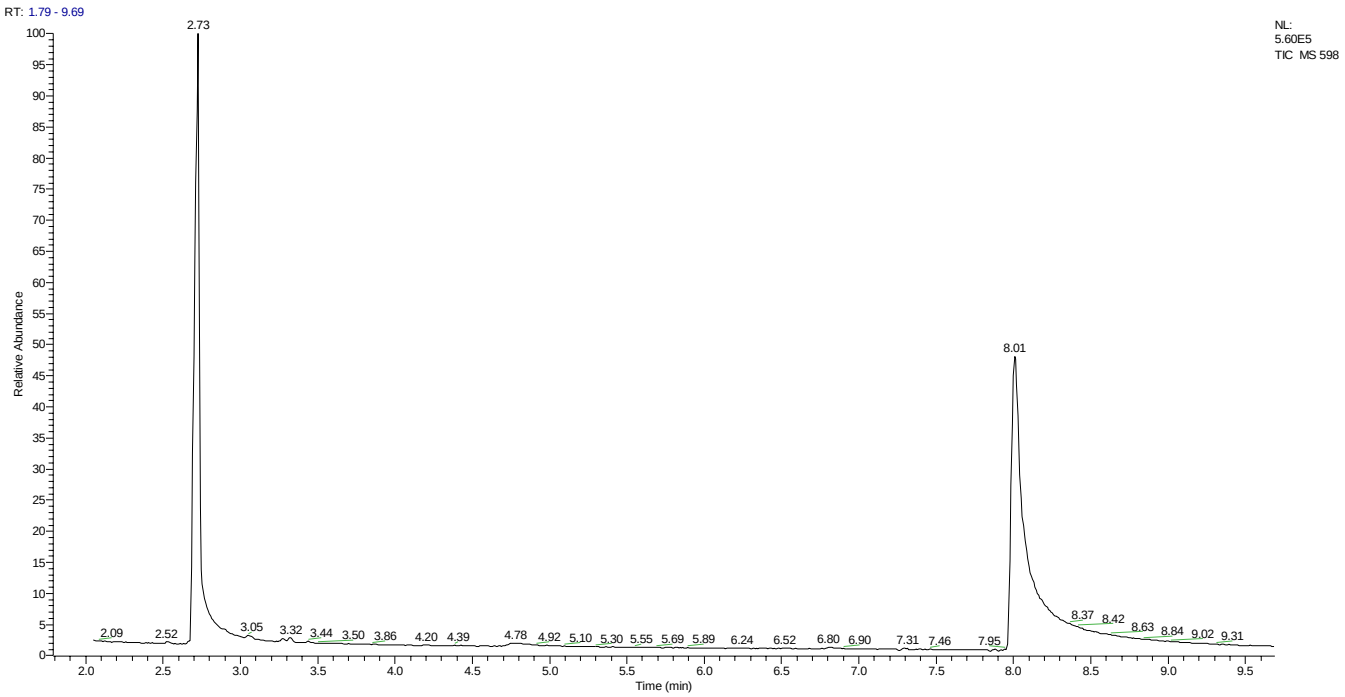

Figure 7. Gas chromatogram of PHB-rich biomass produced on pulp-and-paper mill foul condensate. PHB methyl esters eluted at 2.73 minutes, while benzoic acid methyl esters (e.g. internal standard) eluted at 8.06 minutes. No other significant peaks occurred. PHB yield was estimated at ca. 85% (w/w).

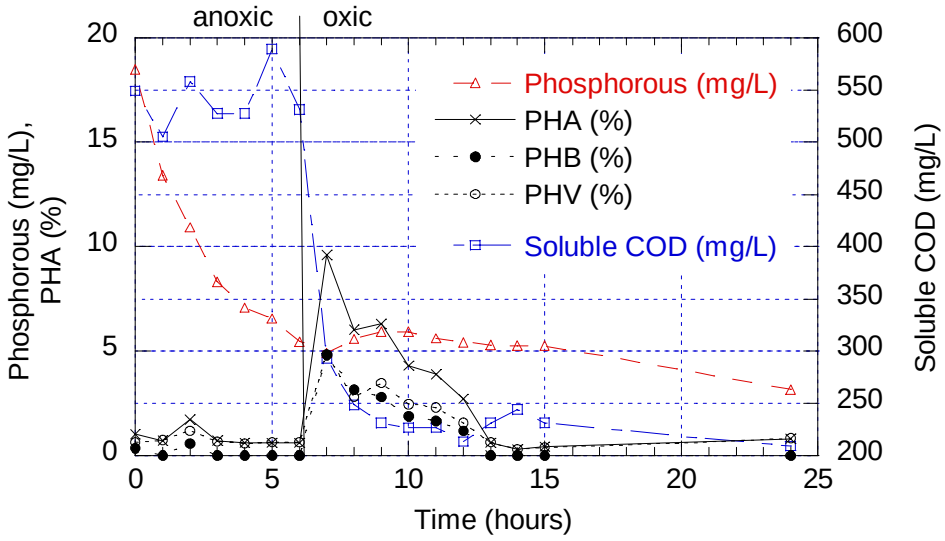

Figure 8. Transient concentrations of phosphorous, soluble chemical oxygen demand (COD), polyhydroxyalkanoates (PHAs), poly-3-hydroxybutyrate (PHB), and poly-hydroxyvalerate (PHV) in a sequencing batch reactor seeded with a mixed microbial consortium and fed fermentate (at t=0) derived from municipal primary solids.

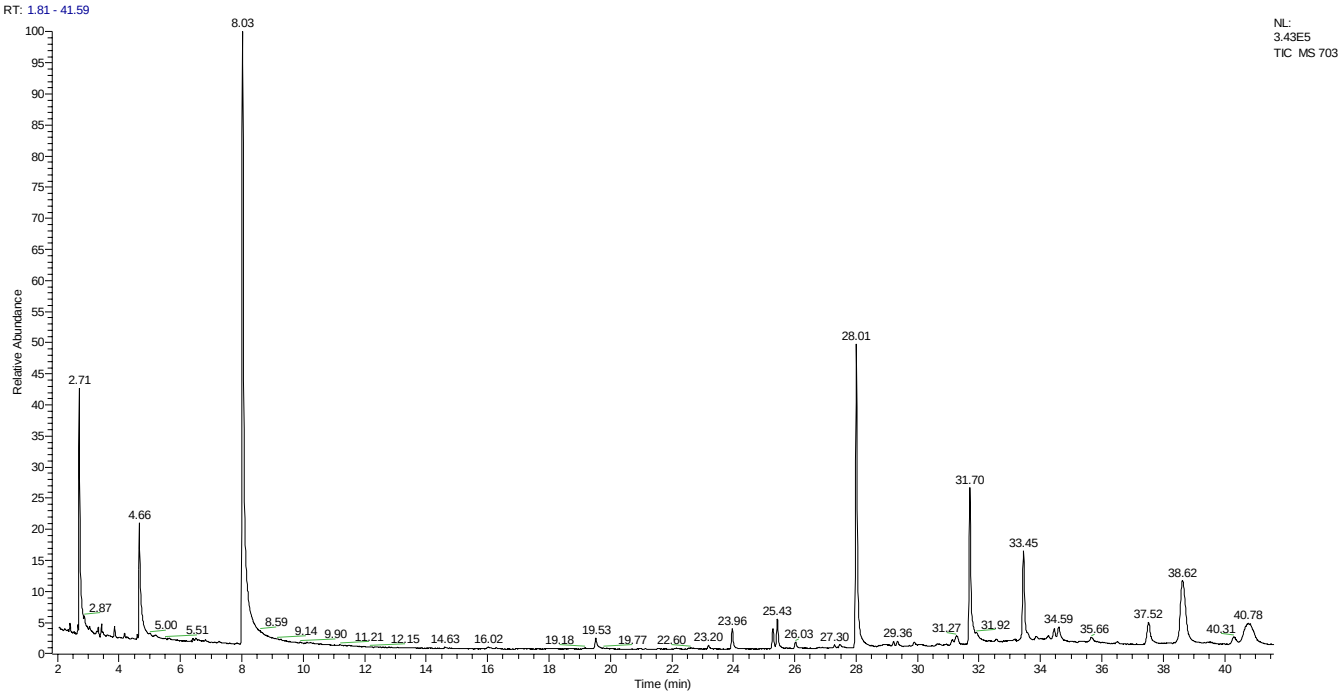

Figure 9. Gas chromatogram of PHA-rich biomass, that contains ca. 22% (w/w) PHB-co-PHV, produced in a fermentate-fed sequencing batch reactor. Peaks originating from the gas chromatograph correspond to: PHB methyl esters (2.71 minutes); PHV methyl esters (4.66 minutes); benzoic acid methyl esters used as an internal standard (8.03 minutes); and esterified cell-wall associated lipids (peaks at times greater than 23.96 minutes).

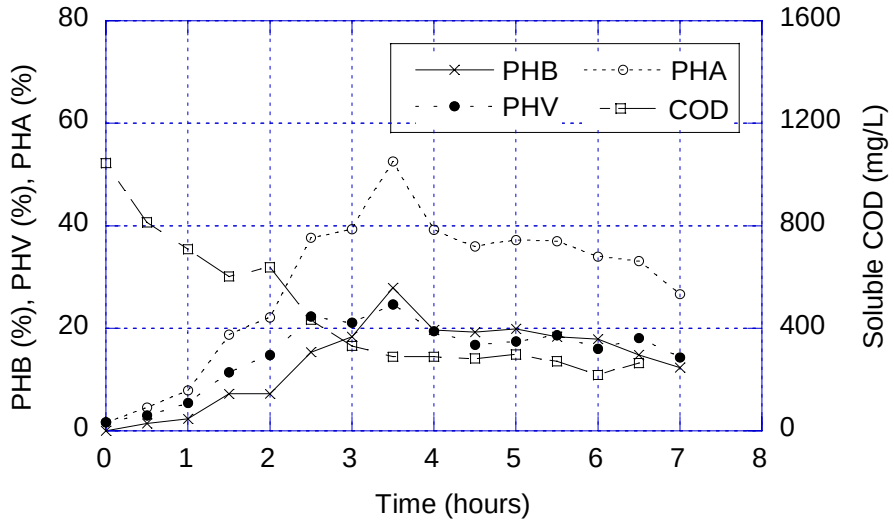

Figure 10. Transient concentrations of soluble chemical oxygen demand (COD), polyhydroxyalkanoates (PHAs), poly-3-hydroxybutyrate (PHB), and poly-hydroxyvalerate (PHV) in a batch reactor seeded with cells obtained from the sequencing batch reactor in Figure 8 at t=24 hours and fed fermentate (at t=0) derived from municipal primary solids.

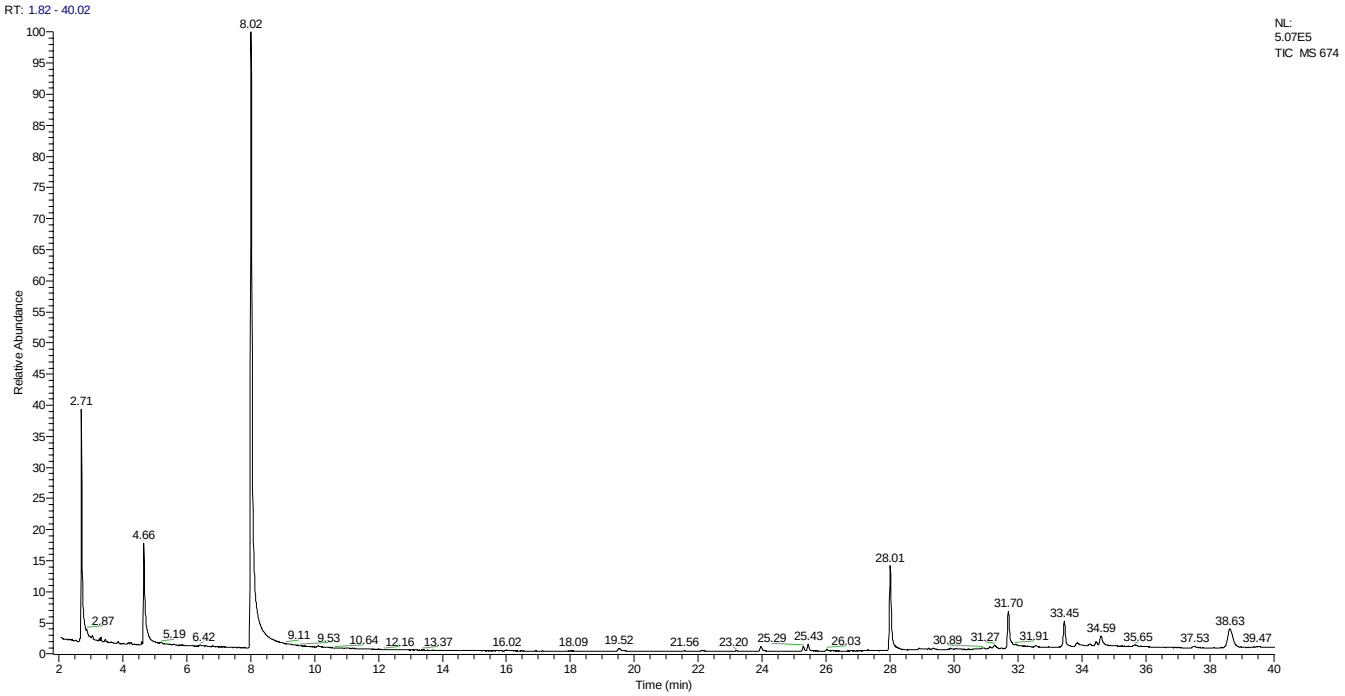

Figure 11. Gas chromatograph plot of PHA-rich biomass produced in fermentate-fed batch reactor seeded from SBR (Figure 10). Peaks originating from the gas chromatograph correspond to: PHB methyl esters (2.71 minutes); PHV methyl esters (4.66 minutes); benzoic acid methyl esters used as an internal standard (8.02 minutes); and esterified cell-wall associated lipids (peaks at times greater than 28.01 minutes).

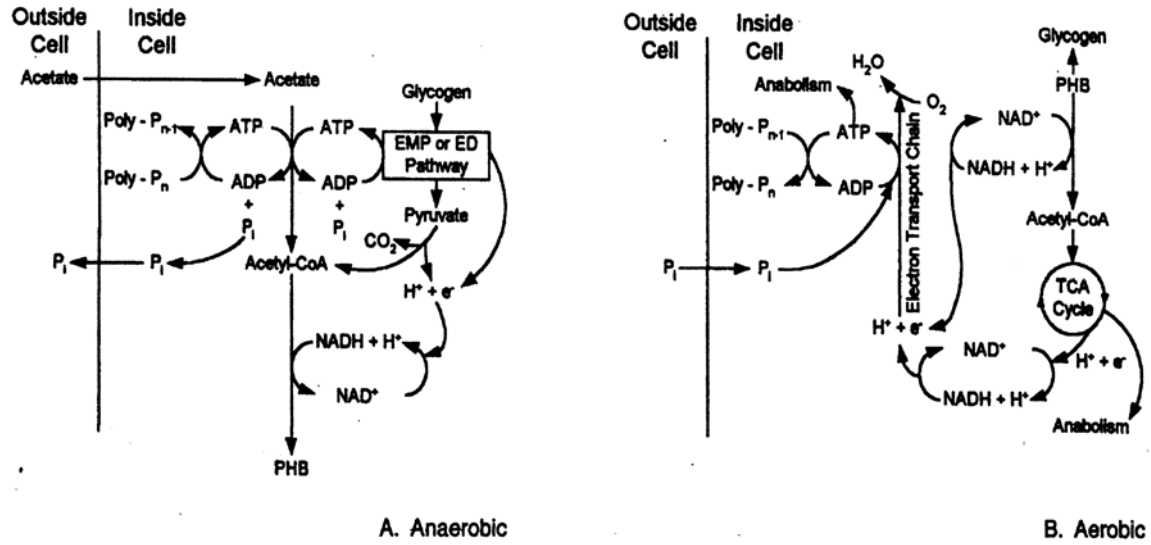

Figure 12. Schematic outline of the current metabolic theory associated with Enhanced Biological Phosphorus Removal. ref: [52].

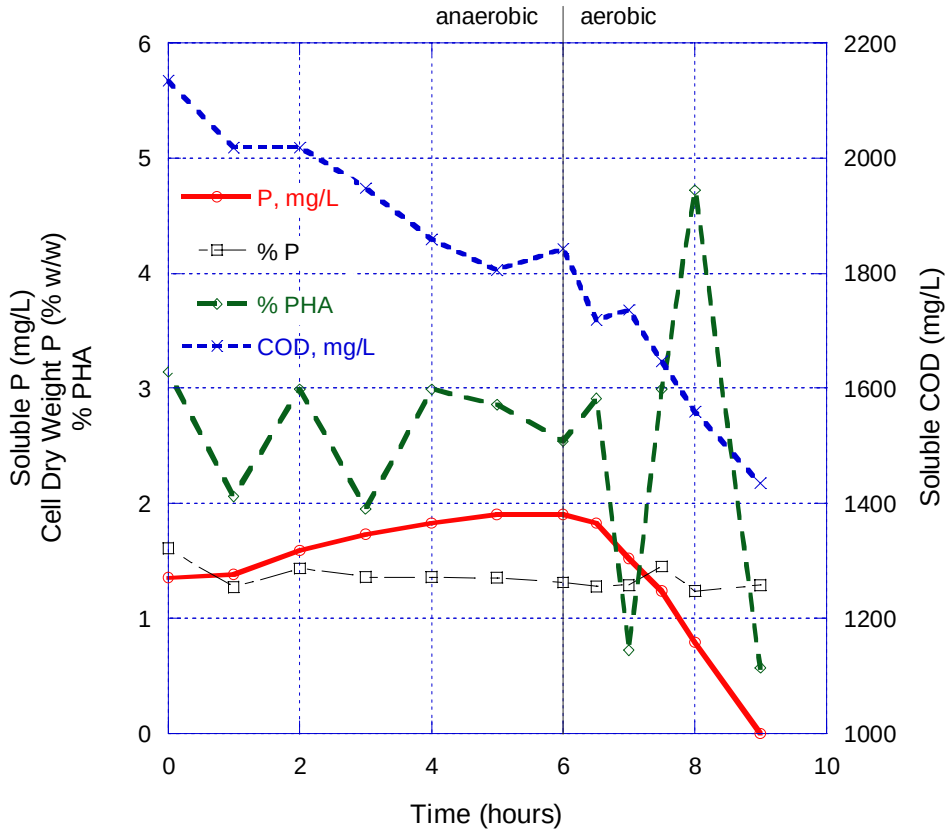

Figure 13. Transient concentrations of bulk solution phosphorous, cellular phosphorus content, soluble chemical oxygen demand (COD), and polyhydroxyalkanoates (PHAs) in a sequencing batch reactor (RW-1) seeded with a mixed microbial consortium and fed raw wastewater and methanol (at t=0).

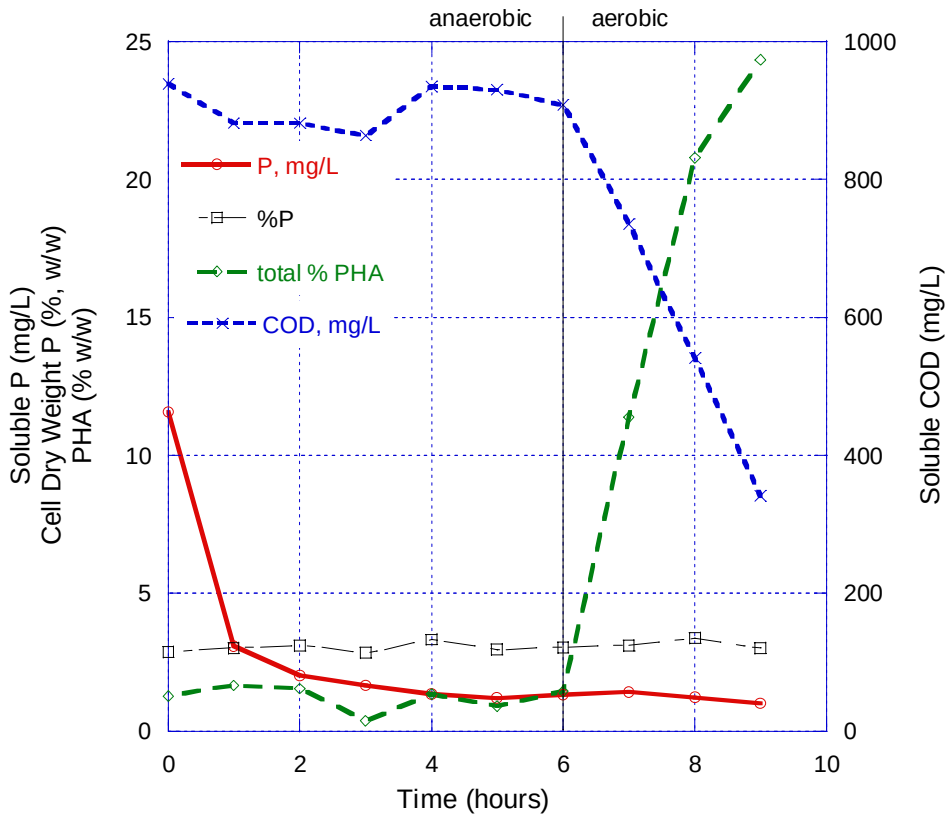

Figure 14. Transient concentrations of bulk solution phosphorous, cellular phosphorus content, soluble chemical oxygen demand (COD), and polyhydroxyalkanoates (PHAs) in a sequencing batch reactor (FE-1) seeded with a mixed microbial consortium and fed fermentate (at t=0) derived from the fermentation of municipal primary solids.

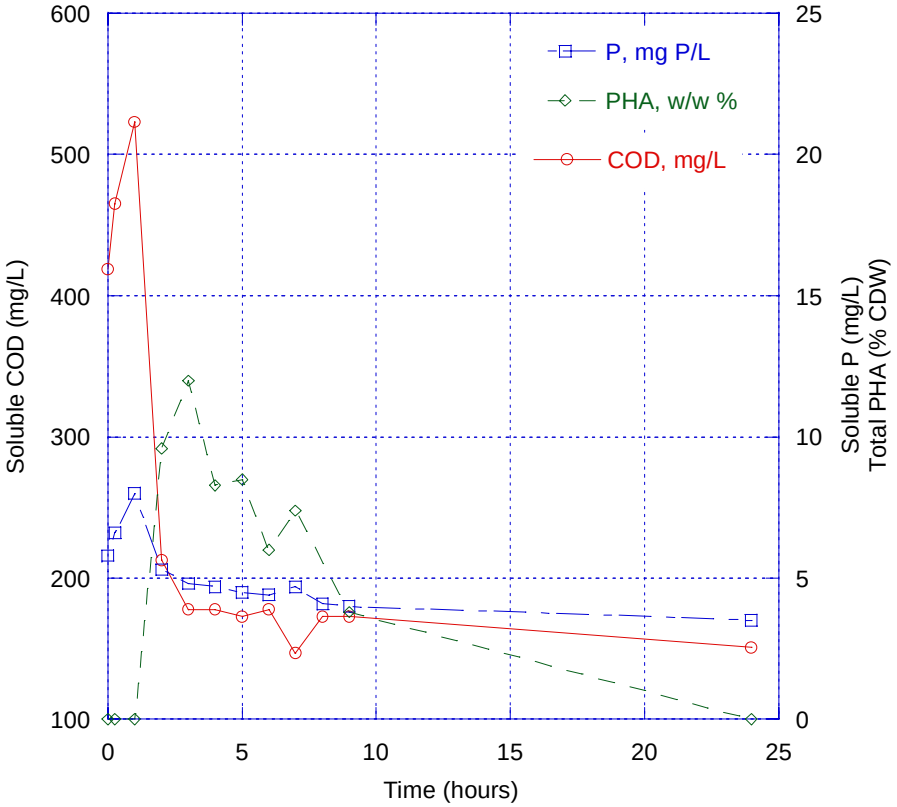

Figure 15. Transient concentrations of bulk solution phosphorous, soluble chemical oxygen demand (COD), and polyhydroxyalkanoates (PHAs) in a sequencing batch reactor (FE-3) seeded with a mixed microbial consortium and fed fermentate (at t=0) derived from the fermentation of municipal primary solids.

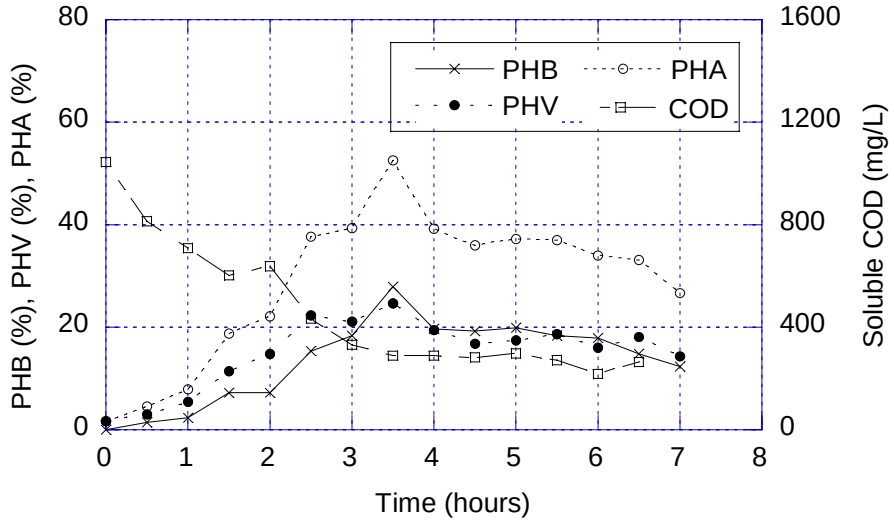

Figure 16. Transient concentrations of soluble chemical oxygen demand (COD), polyhydroxyalkanoates (PHAs), poly-3-hydroxybutyrate (PHB), and poly-hydroxyvalerate (PHV) in a batch reactor seeded with cells obtained from reactor FE-1 at t=24 hours and fed fermentate (at t=0) derived from the fermentation of municipal primary solids.

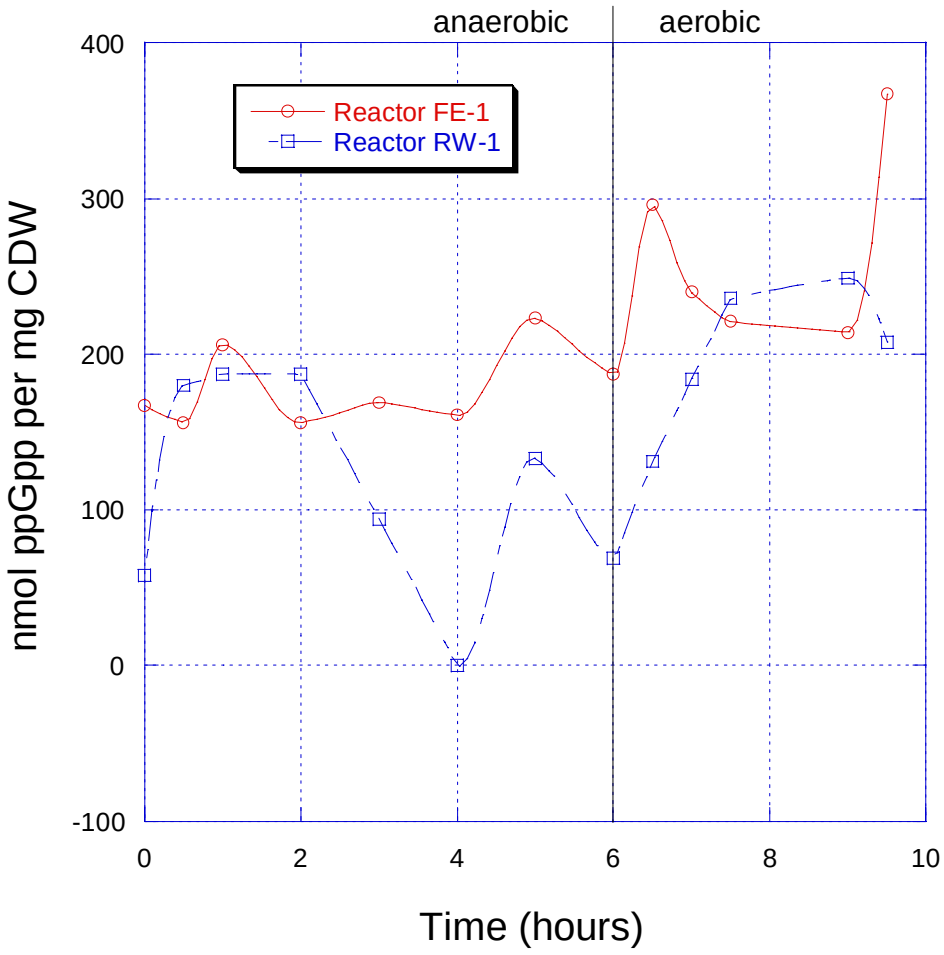

Figure 17. Transient concentrations of guanosine tetraphosphate in reactors FE-1 and RW-1.

TABLES

Table 1. Preliminary material properties of resin-transfer-molded NFRTCs[a]

Thermoplastic Type	Polymer/Wood Ratio	Density		MOE[g]		MOR[h]		Strain @ Break	
		Average (kg/m^3)	COV[f] (%)	Average (GPa)	COV[f] (%)	Average (MPa)	COV[f] (%)	Average (mm/mm)	COV[f] (%)
Purified PHB[c]	*70/30*	1227	1.3	4.69	2.6	49.6	5.0	0.018	6.8
	80/20	1211	0.6	4.50	4.4	52.8	3.7	0.020	3.6
	90/10	1211	0.7	4.31	4.7	49.7	13.9	0.018	16.8
	100/0	1186	0.8	3.93	2.1	57.8	7.4	0.025	14.5
Biomass PHB[d]	*70/30*	1266	4.2	4.76	15.7	19.2	22.5	0.004	15.0
	80/20	1305	1.1	5.00	5.1	18.0	8.4	0.003	5.4
	90/10	1329	0.8	4.62	2.3	16.5	15.4	0.003	19.8
	100/0	1358	1.8	4.51	7.8	20.0	28.0	0.004	33.4
Biomass PHB[e]	*60/40*	1280	3.0	4.24	21.0	25.8	17.5	0.008	10.7
Polypropylene[b]	*60/40*	1003	2.4	3.1	18.7	44	18.0	0.030	17.0

[a] NFRTCs manufactured with 40% pine fiber, with the remaining 60% composed of pure polypropylene, pure P3HB, or biologically derived PHB plus cell debris.
[b] Pure polypropylene obtained from Solvay®.
[c] Pure PHB obtained from PHB Industrial S/A.
[d] PHB produced biologically in Azotobacter vinelandii UWD (ATCC 53799). PHB content on a dry microbial cell basis (not wood fiber basis) was ca. 24%. The 70/30, 80/20, 90/10, and 100/0 formulations thus contained ca. 17%, 19%, 22%, and 24% PHB, respectively.
[e.] PHB produced biologically in Azotobacter vinelandii UWD (ATCC 53799). PHB content on a dry microbial cell basis (not wood fiber basis) was ca. 43%. The 60/40 formulation thus contained ca. 26% PHB.
[f] COV: Coefficient of Variation.
[g] MOE: Modulus of Elasticity.
[h.] MOR: Modulus of Rupture.

Table 2. Tukey-Kramer pairwise comparison summary of composite formulations. Results shown are the least square mean differences and associated 95% confidence intervals.

Formulations Contrasted		MOE (GPa)[a]	MOR (MPa)[b]
Biomass PHB @ 100/0	Biomass PHB @ 90/10	-59189±69814	331±1188
Biomass PHB @ 100/0	Biomass PHB @ 80/20	-147,691±77,312	-21±1,316
Biomass PHB @ 100/0	Biomass PHB @ 70/30	-171,018±96,335	-430±1,639
Biomass PHB @ 90/10	Biomass PHB @ 80/20	-88502±65,376	-353±1,112
Biomass PHB @ 90/10	Biomass PHB @ 70/30	-111829±78,882	-761±1,342
Biomass PHB @ 80/20	Biomass PHB @ 70/30	-23327±69729	-408±1186

[a] MOE: Modulus of Elasticity.
[b] MOR: Modulus of Rupture.

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