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Identification of Functionally Relevant Populations in Enhanced Biological Phosphorus Removal Processes Based On Intracellular Polymers Profiles and Insights into the Metabolic Diversity and Heterogeneity

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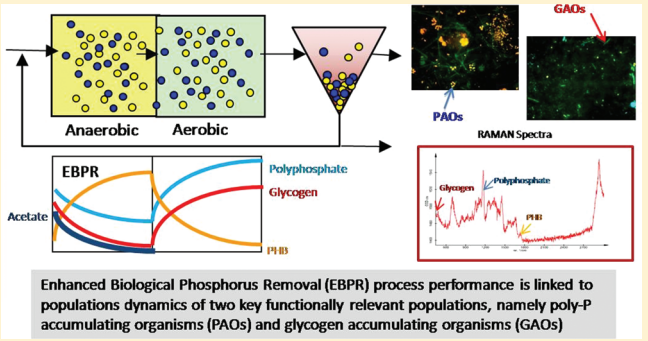
Supporting Information

ABSTRACT: This study proposed and demonstrated the application of a new Raman microscopy-based method for metabolic state-based identification and quantification of functionally relevant populations, namely polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), in enhanced biological phosphorus removal (EBPR) system via simultaneous detection of multiple intracellular polymers including polyphosphate (polyP), glycogen, and polyhydroxybutyrate (PHB). The unique Raman spectrum of different combinations of intracellular polymers within a cell at a given stage of the EBPR cycle allowed for its identification as PAO, GAO, or neither. The abundance of total PAOs and GAOs determined by Raman method were consistent with those obtained with polyP staining and fluorescence in situ hybridization (FISH). Different combinations and quantities of intracellular polymer inclusions observed in single cells revealed the distribution of different sub-PAOs groups among the total PAO populations, which exhibit phenotypic and metabolic heterogeneity and diversity. These results also provided evidence for the hypothesis that different PAOs may employ different extents of combination of glycolysis and TCA cycle pathways for anaerobic reducing power and energy generation and it is possible that some PAOs may rely on TCA cycle solely without glycolysis. Sum of cellular level quantification of the internal polymers associated with different population groups showed differentiated and distributed trends of glycogen and PHB level between PAOs and GAOs, which could not be elucidated before with conventional bulk measurements of EBPR mixed cultures.



Enhanced Biological Phosphorus Removal (EBPR) process performance is linked to populations dynamics of two key functionally relevant populations, namely poly-P accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs)

INTRODUCTION

Enhanced biological phosphorus removal (EBPR) process has been widely applied to remove phosphorus from wastewaters for control of eutrophication in the receiving water bodies. Alternating anaerobic and aerobic phases in the EBPR process enrich for two important populations groups that affect the function of the system, namely polyphosphate (polyP) accumulating organisms or PAOs and glycogen accumulating organisms or GAOs. PAOs are able to uptake volatile fatty acids (VFA) and store as intracellular polyhydroxyalkanoates (PHAs, either as poly ß-hydroxybutyrate (PHB) or as poly hydroxyvalerate (PHV)) during anaerobic phase, using the energy and reducing power generated from the hydrolysis of intracellular polyP and internally stored glycogen.[1,2] In the subsequent aerobic or anoxic phase, PAOs are able to use their stored PHAs for biomass growth and replenishment of cellular glycogen and uptake soluble phosphate to restore intracellular polyP. Similar to PAOs, GAOs can also uptake VFAs in anaerobic condition for PHA formation, but instead of using polyP for energy, they utilize glycogen as the primary source of energy. The intracellular polymers, including polyP, PHB, and glycogen, therefore play important roles in the EBPR process.

Although substantial progress has been made toward elucidating the mechanisms of EBPR and the factors that influence the EBPR system ecology and performance, great knowledge gap still exists in the fundamental understanding of the biochemistry and metabolic pathways employed by the functionally relevant populations in this biological system. Currently, identification of key populations involved in EBPR mostly relies on phylogenetic based molecular techniques such as fluorescence in situ hybridization (FISH) or PCR-based

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methods.[3,4] And, monitoring of EBPR-relevant populations has been limited to the species whose phylogenetic information has been obtained. Although *Accumulibacter* and *Actinobacteria*-like PAOs, have often been found to dominate in acetate-fed EBPR systems as well as in full-scale EBPR plants[5,6] it is recognized that the phylogenetic diversity of PAOs and GAOs in full-scale EBPR systems are likely larger than those known.[3,7] Complementary polymer staining techniques have been employed to assist in the quantification of total PAOs, such as DAPI staining and Neisser staining of polyP.[8,9] Up-to-date, there is no method available for quantifying total GAOs and only limited types such as *Competibacter*-type and *Defluvicoccus*-type can be detected due to the few candidate GAOs identified.[10−12]

Furthermore, linking the EBPR performance activities with the relative microbial population community structure has been rather difficult. Advanced techniques such as FISH combined with microautoradiography are useful to identify and understand the in vivo physiology, diversity, and activity of certain functional microbial groups in biological processes,[13] but this method suffers from the same limitation of the number of FISH probes available or the microbes identified.[14] Recently, flow cytometry was used as a novel fluorescent staining technique, which allows for reliable quantification of PAOs and revealing polyphosphate accumulation dynamics of not-yet cultivable bacteria in EBPR.[15] But the ability to monitor other key populations (e.g., GAOs) and related metabolic activities is yet to be developed. Recently, we have developed Raman microscopy analysis methods that can simultaneously reveal the dynamics at the resolution level of single cells of important intracellular polymers involved in the EBPR process.[16,17] In this study, we further explored the application of Raman microscopy approach for identification and quantification of functionally relevant populations, namely PAOs and GAOs, in EBPR system based on their unique Raman Spectrum of respective polymers (indicative of metabolic state). The technique was validated by comparison with conventional quantification methods (e.g., FISH and DAPI staining). In addition, diversity and heterogeneity of single cell level intracellular polymer spectrum at various stages during the EBPR cycle were revealed which provided insights into the EBPR metabolic biochemical pathways.

EXPERIMENTAL METHODOLOGY

Lab Scale EBPR System. A laboratory scale continuous flow EBPR system was established to enrich for organisms (PAOs/GAOs) that could be used for the analysis of polyphosphate, PHB and glycogen inclusions inside the cells. The Reactor included an anaerobic zone, an anoxic zone and followed by two-stage aerobic zones and the configuration allowed for both A2O (anaerobic-anoxic-oxic) and UCT (University of Cape Town) modes of operation. The reactor was maintained in a controlled temperature room with temperature ranging from 20 to 22 °C. The hydraulic retention time (HRT) and solids retention time (SRT) of the system were maintained at 18 h and 8 days, respectively. The composition of synthetic wastewater feed was according to Schuler and Jenkins.[18] Phosphorus was added as 35.6 mg/L sodium phosphate monobasic ($NaH_2PO_4{\cdot}H_2O$) (8 mg-P/L). The organic portion of the feeding consisted of 744 mg/L sodium acetate ($CH_3COONa{\cdot}3H_2O$) (350 mg COD/L) and 15 mg/L of casamino acids. Detailed performance data is reported elsewhere.[19]

PAOs/GAOs Population Analysis. Presence of PAOs in the reactor was confirmed with phosphate removal performance evaluation, Neisser and DAPI staining[8,20] as well as FISH. Oligonucleotide probes targeting *Eubacteria* (EUB or total bacteria), *Accumulibacter* PAOs, *Actinobacteria* PAOs, *Competibacter* GAOs, and *Defluvicoccus* cluster 2 GAOs were used for FISH. Detailed listing of probes can be obtained in Supporting Information (SI) Table S1. The DAPI staining, FISH protocol and hybridization conditions used were previously described.[5,21] DAPI staining was carried out at 50 μg/mL of DAPI for 1 min, which allows to identify the overall bacteria population (stained blue) as well as cells containing a large amount of polyP (stained bright yellow) and therefore allowing the estimation of the fraction of PAOs among the total cells.[9] Hybridized cells were observed with an epifluorescent microscope (Zeiss Axioplan 2, Zeiss, Oberkochen, Germany). For the quantification of the relative proportion of the target types of cells, around 20 micrographs were collected with random fields of view from the same slide/sample. Then, average abundance of the target cells was calculated as the relative proportion of fluorescing area having the target label (PAO/GAO) compared to the area of the total population (EUB/DAPI) using DAIME (Digital Image Analysis in Microbial Ecology) software version 1.3.1 (http://www.microbial-ecology.net/).[22]

Raman Data Acquisition. Details on the acquisition of spectra and more details related to Raman microscopic analysis can be found in our previous reports.[16,17] Briefly, Raman spectra were acquired using a WITec, Inc. (Ulm, Germany) Model CRM 2000 confocal Raman microscope. Excitation (ca. 30 mW at 633 nm) was provided by a Helium/Neon laser (Melles Griot, Carlsbad, CA). Spectra were collected at a 0.33 μm grid, with a dwell time of 10 s. Relative quantity of each polymeric content in each individual cell was evaluated based on the Raman intensity (peak height in the unit of charged coupled device (CCD) counts) of each signature peak that was identified and standardized for each of the polymers[17]. Samples subjected to Raman analysis were homogenized by pushing the cells through 26 gauge needle syringe for at least 20 times to obtain uniform distribution of cells (confirmed by microscopic observations) and then samples were prepared on optically polished CaF_2 windows (Laser Optex, Beijing, China) according to Majed et al.[16] For each sample, Raman spectra for at least 40−45 single cells were examined. The statistical assessment of the samples size, reproducibility and, the validation of the Raman analysis results via comparison with chemical analysis results are shown in our previous reports and key summaries are provided in the SI (Figures S1−S3).

Polymer Dynamics During EBPR Cycle. In order to study the dynamics of inclusion of polymers in the cells and assess the PAO and GAO populations, phosphate release, and uptake tests were performed. Details of phosphate release and uptake tests can be found in Gu et al.[7] For this particular study, the batch testing consisted of 90 min of anaerobic phase with acetate addition (50 mg-acetate/L) followed by 240 min of aerobic phase. For the intracellular detection of polyP, PHB and glycogen using Raman microscopy, at least 6−9 samples were taken throughout the phosphorus release and uptake test at 15−90 min interval. The samples were subject to both Raman analysis and chemical analysis. Each sample (10 mL) was filtered through 0.45 μm filter papers and were analyzed for orthophosphate (PO_4^{3-}), nitrate (NO_3^-) and acetate (CH_3COO^-) using DX-120 ion chromatograph (Dionex

Benelux, Belgium); for total organic carbon (TOC) and chemical oxygen demand (COD) using Tekmar/Dohrmann Phoenix 8000 UV-Persulfate TOC analyzer and HACH COD vials, respectively.

RESULTS AND DISCUSSION

Metabolic State-Based Identification of EBPR Populations via Raman Spectrum. Observation of over thousands of intracellular polymer spectrum in individual cells from samples taken during different EBPR cycle phases in lab-scale acetate-fed EBPR reactor showed that there are only eight unique Raman spectra that were repetitively identified, representing eight different combinations of intracellular polymeric inclusions (including no polymeric inclusions) as previously reported[17] (SI SFigure 1). Based on our current understanding of EBPR metabolic pathways and the dynamics of the synthesis and depletion of various intracellular polymers, these different combinations of intracellular polymers detected by Raman analysis are expected, corresponding to various cell metabolic states during the anaerobic–aerobic EBPR cycle for either PAO or GAO cells as shown in Figure 1. Although, it is still not clear whether PAO and GAO are two phylogentically and/or physiologically distinct groups of organisms, based on current generally accepted definition of PAOs or GAOs during EBPR process,[23−25] we propose the potential identification of PAOs or GAOs based on their expected possible polymer spectrum described as follows: cells containing polyP with various combinations of glycogen and PHB polymers, including those containing polyP, PHB and glycogen, polyP and PHB, polyP and glycogen, or polyP only, are all considered as candidate PAOs. This is similar to current approaches of using DAPI staining of polyP for quantifying total PAOs in EBPR samples.[9,15] The presence of different intracellular polymer combinations and the rationale for assigning candidate PAOs are described as follows (Figure 1): (1) cells containing polyP, PHB, and glycogen: they represent PAOs that perform EBPR metabolism and follow the generally accepted biochemical pathways; (2) cells containing polyP and PHB: represent the PAOs that may have depleted glycogen to be below detection limit by Raman method, such as at the end of anaerobic phase; (3) cells containing polyP and glycogen: likely represent PAOs that may have depleted PHAs near the end of the aerobic phase, or under the anaerobic phase, they may represent PAOs that have very low level (nondetectable) of PHB. It may also represent cell that do not store PHB but other forms of PHAs such as polyhydroxyvalerate (PHV), polyhydroxy-methyl-butyrate (PH2MB), or polyhydroxy-methyl-valerate (PH2MV)) that can not yet be accurately quantified with Raman;[17] (4) cells containing only polyP: It is possible that some of the polyP-containing cells are not active in EBPR since polyP formation has been found for cells experiencing stress conditions irrelevant to EBPR.[27] There maybe a small number of cells whose intracellular glycogen levels are below detection limit due to heterogeneity among individual cells.[43] However, the latter is unlikely the only reason to explain the relatively high relative abundance of poly-P-only containing cells through out the EBPR cycle. Further discussion of implications of the PAOs that containing various polymer combination and potential link to EBPR metabolic pathways is provided in later section.

For identification of GAOs, we considered cells containing both glycogen and PHB, as well as cells those containing only glycogen, as candidate GAOs (Figure 1). For cells containing

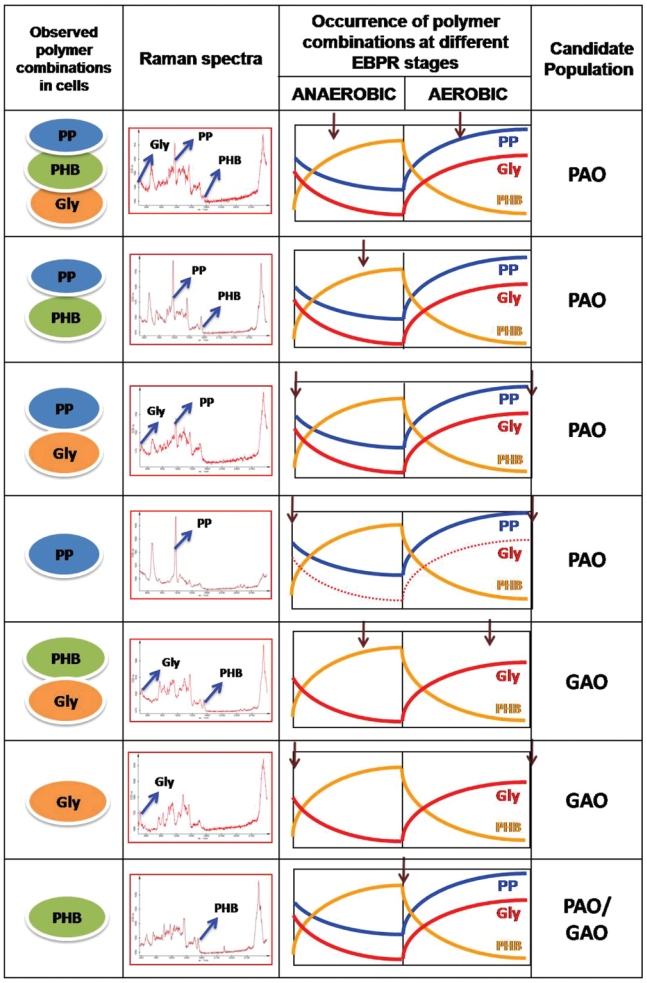

Figure 1. Proposed method for identification of PAOs and GAOs based on unique Raman spectrum of different combinations of polymers at different metabolic stages of EBPR. Only eight unique Raman spectra (including no polymer inclusion) have been repeatedly identified. Arrows indicate the specific points of time or stages that the respective combination of polymeric inclusion is expected to occur and to be mostly likely detected in PAOs or GAOs cells via Raman during the EBPR cycle.

both PHB and glycogen (no polyP): they likely represent GAOs that are expected to contain these two polymers. We do not rule out the possibility that they could include some PAOs that may have depleted polyP at the end of anaerobic phase. For cells containing only glycogen: they likely represent GAOs since comparatively higher amounts of glycogen inclusions are associated with GAOs.[28] The possibility that these cells represent PAOs is eliminated since no period during EBPR cycle would possibly witness simultaneous depletion of polyP and PHB, leaving only glycogen for PAOs. There are cells that have only detectable intracellular PHB, which can present either PAOs that probably have consumed glycogen and polyP, or GAOs that have consumed glycogen, for example, at the end of anaerobic phase of EBPR cycle.

To validate the proposed method for identification of functionally relevant populations in EBPR using Raman microscopy, we quantified the abundances of the PAOs and GAOs using conventional methods including two different staining methods and FISH analysis, and compared the results

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with those determined based on Raman polymers spectrum analysis (Table 1).

Table 1. Comparison of Relative PAO and GAO Population Abundance in Lab-Scale EBPR Reactor Determined by Raman Spectrum with Those Determined by Conventional Polymer Staining and FISH Methods

Type of Method Employed	Population Type	Population Fraction ± Standard Deviation
Conventional Methods		
DAPI poly-P staining	Total PAOs	58.5 ± 8.7
Neisser poly-P staining	Total PAOs	65.5 ± 9.2
FISH	*Accumulibacter* PAOs	29 ± 6.5
FISH	*Actinobacter* PAOs	21 ± 7.6
FISH	*Competibacter* GAOs	31 ± 5.2
Raman Method[a]		
Raman Spectrum Analysis	Total PAOs	63 ± 3.6
Raman Spectrum Analysis	Total GAOs	33 ± 1.4

[a]Results based on at least three replicates of samples taken from aerobic phase of the EBPR cycle.

From Raman measurements, calculated relative abundance of PAOs was 63 ± 3.6% in our lab-scale acetate-fed EBPR system, which is consistent with the total PAO fraction determined by DAPI Staining (polyP stain) of 58.5 ± 8.7% and by Neisser staining of 65.5 ± 9.2%. FISH allowed for phylogenetic identification of PAOs belonging to *Accumulibacter* and *Actinobacter* groups and the total of these two groups accounted for about 50% of the bacterial cells and 74% of total PAOs. Both lab-scale and full-scale acetate-fed EBPR systems have been reported to enrich for *Accumulibacter*-like PAOs previously.[6] And *Actinobacteria*-like PAOs have been detected in high numbers (as high as 35% of total population) in full scale EBPR systems by Kong et al.[26] Presence of such considerable fractions (21% of total population in Table 1) of *Actinobacteria* in the present study reactor could be attributed to the presence of amino acids (in form of casamino acids) in the feeding, although their contribution to EBPR is still not well understood as they are believed to be unable to assimilate acetate into PHAs.[29]

Currently, there is no method available for quantification of total GAOs in an EBPR system. Identified candidate GAOs such as *Competibacter*, *Defluvicoccus* can be monitored using FISH or PCR based methods. *Competibacter* (targeted by *GB* probe) was found to be about 31 ± 5.2% of the total bacteria cells in our reactor. Raman analysis indicated that there was about 33 ± 1.4% of the total bacterial cells that exhibited GAO phenotype, which is actually very close to the relative abundance of *Competibacter*, indicating that it may be the dominant GAO type in our system. Kong et al.[12] also reported the presence of *Competibacter* GAOs as targeted by *GB* probes ranging from 10 to 50% of total population in both lab scale and full scale acetate-fed EBPR systems. *Defluvicoccus* type GAOs were not detected in our reactor (data not shown).

These results demonstrated that the proposed method to identify and quantify functionally relevant populations in EBPR system using Raman microscopy is generally valid and the metabolic state-based measurements of active PAOs and GAOs can provide a new alternative approach for monitoring populations and the ecological dynamics of EBPR process. The advantage of Raman method is that cells are identified based on their PAO or GAO phenotype activities, indicated by their intracellular polymer presence, rather than based on their traditional FISH/q-PCR techniques that can only detect those populations whose genetic information are available.

Diversity and Heterogeneity of Metabolic States among PAOs and GAOs. Based on the above proposed identification approach, distribution of candidate PAOs and GAOs that contained different combinations of intracellular polymers during the EBPR cycle were evaluated. Figure 2a shows the relative abundance and distribution of polyP containing cells (candidate PAOs) with five different combinations of polymers detected at different time points during the P release and uptake batch test (see SI SFigure 2 for substrate profiles of substrates during the batch testing). Figure 2b shows the distribution of cells with glycogen alone or with both glycogen and PHB (candidate GAOs) observed during the EBPR process. These results revealed diversity and heterogeneity among PAOs and GAOs cells with various intracellular polymers combinations and their temporal changes. The total PAOs and GAOs population abundance were determined as the sum of all cells that have been characterized based on the intracellular polymers combinations as described previously. It is noted that PAOs and GAOs may be underestimated at certain stage of the EBPR cycle based on the Raman spectrum since some polymers could be present at levels below the detection limit. For example, the slightly lower

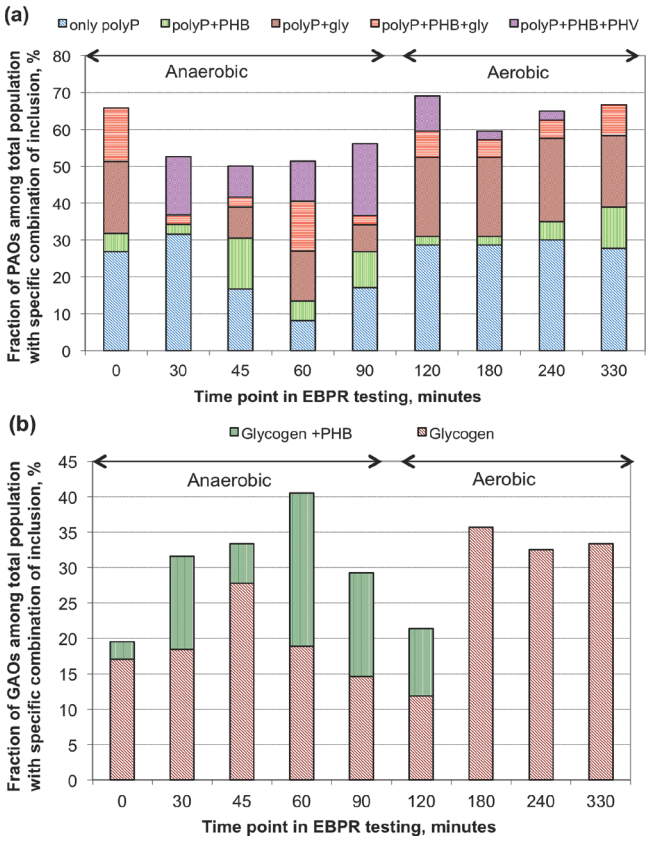

Figure 2. (a) Distribution of PAOs (polyphosphate-containing cells) with five different combinations of intracellular polymers at different time points during the EBPR cycle in a phosphate release and uptake batch test; (b) Distribution of glycogen-containing cells with either glycogen only or a combination of glycogen and PHB at different time points during the EBPR cycle in a phosphate release and uptake batch test.

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level of PAOs and GAOs detected near the end of extended anaerobic phased is likely due to the depletion of polyP and glycogen in some PAO and GAO cells, respectively. Note that the PHB-only containing cells accounted for about 7−9% of the total cells in samples taken from the anaerobic phase, which likely represent some PAOs and GAOs, and they were not accounted as either PAOs or GAOs. Nevertheless, the relative populations abundance of PAOs and GAOs determined based on the Raman method for samples taken in aerobic EBPR phase is rather consistent. Current approach of PAO quantification usually uses samples taken from the aerobic phase of the EBPR since signature intracellular polymer levels for PAOs and GAOs, polyP and glycogen, respectively, are expected to occur at higher cellular level at aerobic stage of the EBPR cycle. Similarly, we performed population quantification of PAOs and GAOs of the EBPR system based on Raman analysis of samples taken during the EBPR aerobic phase and compared with those determined using conventional methods as previously discussed.

Differentiated Polymeric Abundance Level Associated with PAO versus GAO Populations. Investigation of the abundance and fate of intracellular polymers in EBPR has traditionally relied on the chemical measurements of mixed enrichment cultures. As a result, the observed distribution and trend of glycogen and PHB in an EBPR process actually reflected the combined intracellular state of both PAOs and GAOs, which made data interpretation often complicated.[3] Based on the proposed identification of PAOs and GAOs population as described above, the PHB and glycogen content associated with PAOs or GAOs could be quantified separately. Figure 3 shows the differentiated and distributed measurements of PHB and glycogen inclusion levels between PAOs and GAOs at different time points for a typical anaerobic/aerobic batch testing EBPR cycle. The total PHB content in the sludge sample showed a temporal trend that is consistent with what is often observed for an EBPR cycle (Figure 3a). Overall, the PHB content associated with PAO cells (50−80%, average 62% of total anaerobic PHB content in all cells) was much higher than that with GAO cells (10−40%, average 31% of total anaerobic PHB content). However, considering the population abundance ratio of PAOs to GAOs (63%:33% = 1.91, see Table 1), in comparison to the ratio of the total PHB quantity associated with the two populations (averaged for all time points, 62%:31% = 1.96), it suggested that the rate and amount of PHB sequestration are likely similar for the two populations at individual cellular level.

Figure 3b shows the differentiated temporal trends and levels of glycogen associated with candidate PAOs and GAOs, respectively. Glycogen levels associated with both populations followed the trend of anaerobic depletion (concurrently with acetate depletion) followed by aerobic restoration as demonstrated previously with bulk measurements in PAO enriched and GAO enriched lab-scale EBPR systems.[28,30,31] In general, significantly higher proportion of glycogen accumulation was associated with GAO cells (average 78% of total aerobic glycogen content) compared to PAO cells (average 22% of total aerobic glycogen content). Relative percentage of glycogen inclusions distributed between PAO and GAOs is shown in Figure 4. These results provide cellular and population-level evidence that glycogen plays a key role as the sole source of energy for GAOs, whereas for PAOs, glycogen degradation is involved but it likely only provides minor energy in addition to that from polyP hydrolysis.[28,32]

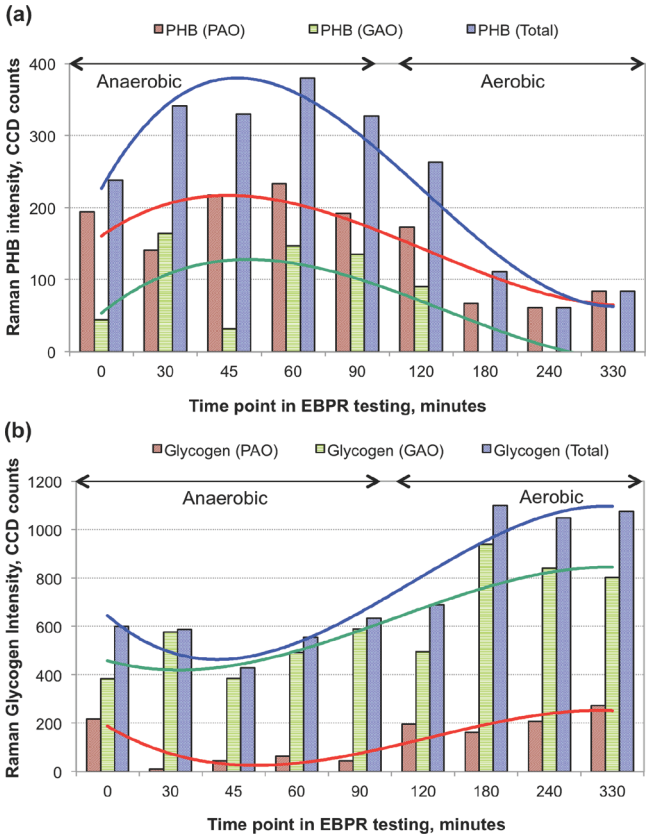

Figure 3. Distributed and total level of (a) PHB and (b) glycogen associated with PAOs and GAOs at different time points during a EBPR cycle in a P release and uptake batch testing. Solid lines represent trend lines for the respective sets of data.

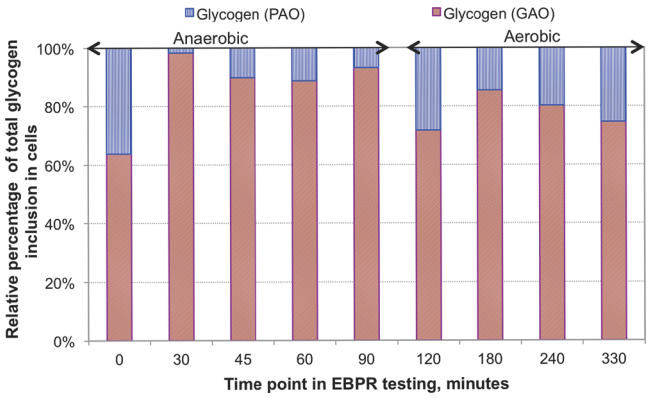

Figure 4. Distribution of glycogen inclusion between PAOs and GAOs based on Raman polymers spectrum analysis.

Distribution of Cells Containing Different Intracellular Polymeric Inclusions in EBPR Cycle and Insights into EBPR Metabolic Pathways. Uncertainties still exist regarding the mechanism and metabolic pathways in the EBPR process; particularly, the involvement of either TCA cycle or glycolysis pathway (Entner-Doudoroff versus Embden-Meyerhof-Parnas pathway) for energy and reducing power generation and, the possession and extent of utilization of these pathways by different phylogenetic PAOs or GAOs groups are still unclear.[33,34] As illustrated in SI SFigure 3, PAOs can use either glycolysis only, or use both glycolysis and partial TCA cycle (bypass through glyoxylate shunt)[36] or split TCA cycle

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(oxidative branch of TCA cycle or reductive branch of TCA cycle which is also called succinate-propionyl-coA pathway). For PAOs that employ glycolysis and TCA cycle, it is expected that they contain higher level of polyP + glycogen, with lower or depleted PHAs at the end of aerobic phase. Cells following these pathways, which contain both polyP and glycogen inclusions, accounts for approximately 40% of the total PAO cells at the end of aerobic phase, as shown in Figure 2a. However, the remaining of the polyP-containing cells, with or without PHB/PHV polymers inclusions, did not have detectable glycogen polymer, even though glycogen is expected to replenish and occur at higher levels during the aerobic phase. It is possible that some of the polyP-containing cells are not active in EBPR as discussed previously, or there maybe a small number of cells whose intracellular glycogen levels are below detection limit due to heterogeneity among individual cells. However, the rather high abundance of these nonglycogen−containing PAOs and their temporal trends along with the EBPR cycle suggest that it is also possible that these cells represent those that employ different metabolic pathways and therefore exhibit distinctive intracellular polymers profiles.

One possible explanation is that some of these PAOs employ TCA cycle only and do not involve glycolysis. If these PAOs rely on TCA alone for reducing power and energy without glycolysis pathway, they might not contain glycogen and, they only produce PHB, not any PHV. The detection of polyP+PHB containing cells in absence of any PHV (up to 13.5% at 45 min and 11% at 330 min, as shown in Figure 2a) during the anaerobic phase seems to support this hypothesis (Figure 2a). Dependence on TCA cycle alone for PAOs was proposed and evidenced by Zhou at al (2009).[37] They demonstrated that the *Accumulibacter*-enriched EBPR system, after depletion of the glycogen pool through starvation, was able to uptake acetate anaerobically and, reaction stoichiometry and kinetics comparison with the existing models led them to conclude the operation of TCA cycle as a sole source for NADH and ATP production.

Another possibility is that some of these PAO cells employ such pathway and metabolism that do not involve the known and identifiable storage polymers as previously mentioned. For example, *Actinobacteria* contributes to >30% of the total PAOs in our reactor, which have been found to assimilate only amino acids under anaerobic condition into storage compounds so far unidentified[26] and thus might comprise some of the only polyP-containing cells during aerobic phase in our study. Of course, further study is required to investigate these speculations. Nevertheless, our intracellular polymeric evaluation provides evidence of the metabolic diversity and variation in the employment of EBPR pathways and it is possible that some PAOs can operate with the combination of glycolysis and partial TCA, some may possibly rely on TCA cycle alone for reducing power and energy generation in anaerobic phase. For the TCA cycle to be operational, how the FADH2 generated in the TCA cycle could be oxidized anaerobically is yet to be resolved.[37] Furthermore, whether the metabolic diversity and/or heterogeneity observed are associated with phylogenetic diversity, metabolic switching among the same phylogenetic groups, or merely reflection of metabolic heterogeneity among individual cells remains unclear and requires further investigation.[38,39]

In summary, these results demonstrated the intracellular polymer and implicated metabolic variations among the PAO populations and provided evidence of diverse metabolic pathways possibly employed by subpopulations within PAO groups. Thus, the actual biochemical pathways and reaction stoichiometry employed by a specific PAO population may not be accurately reflected by the bulk measurements of the trends of polyP, glycogen and PHB during the EBPR cycle, as in most current EBPR studies. This may also explain the inconsistent and varying observations related to the processes mechanisms and stoichiometry employed by PAOs for reducing power generation under anaerobic conditions reported by different researchers.[35,37,40−42] These results imply the need for using isolates or relying on tools that can reveal cellular level information for further understanding of the fundamental mechanisms of EBPR.

Application of Raman Method in the Evaluation of EBPR Mechanism. In this study, we have successfully demonstrated the application of Raman microscopic method for metabolic state based identification and quantification of the relative abundances of functionally relevant populations (PAO and GAO) in EBPR system via simultaneous detection of multiple intracellular polymers. Dynamics of intracellular polymeric inclusions were evaluated and differentiated for each population group, which revealed for the first time, the level and trend of each polymeric inclusion associated with the respective population group. Cellular level information of intracellular polymers for different phenotypic groups at various metabolic stages of EBPR provided insights into the involvement of different EBPR metabolic pathways and therefore demonstrated the applicability of the method for better understanding of EBPR mechanisms. This method, combining with the traditional genetic-target based methods, has great potential in obtaining a more complete system level and individual cellular level evaluation of EBPR process. Further research will focus on applying this method to investigate the impact of different substrate and loading conditions on the dynamics of intracellular polymeric inclusion and metabolic state.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table S1: Details on oligonucleotide probes used for FISH in this study and their respective target groups; SFigure 1: Enlarged versions of Raman spectra corresponding to Figure 1; SFigure 2:Orthophosphate and acetate profiles during phosphorus uptake and release batch testing; SFigure 3: Schematic of anaerobic biochemical pathways employed by PAOs for energy (ATP) and reducing power (NADH) production and for intracellular PHAs formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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