Discovering Polyphosphate and Polyhydroxyalkanoate-Accumulating Organisms Across Ecosystems: Phenotype-targeted Genotyping via FACS-sequencing

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Abstract

Polyphosphate-accumulating organisms (PAOs) play a central role in enhanced biological phosphorus removal (EBPR) systems by coupling intracellular polyphosphate (polyP) cycling with carbon storage through polyhydroxyalkanoates (PHA). However, in natural environments such as soil and sediments, the term PAOs has been loosely applied to polyphosphate-containing organisms (PCOs) without consideration of their carbon storage capacity or phylogeny. This oversimplification overlooks a critical metabolic trait—surplus co-accumulation of polyP and PHA—that defines functionally active PAOs in engineered systems. This study presents a novel phenotype-targeted approach that integrates triple-stained fluorescence-activated cell sorting (FACS) with 16S rRNA gene sequencing, TriFlow-Seq, enabling the simultaneous detection, quantification, and phylogenetic characterization of functional PAOs capable of dual polyP and PHA accumulation. TriFlow-Seq was applied to microbial communities from full-scale and pilot-scale EBPR systems, as well as maize rhizosphere soils across multiple genotypes and fields. In EBPR systems, the method successfully enriched known PAOs, including *Candidatus Accumulibacter*, *Tetrasphaera*, *Dechloromonas*, and *Pseudomonas*, while also revealing novel candidate PAOs, particularly within *Rhodobacteraceae*. In agricultural soils, TriFlow-Seq uncovered a previously unrecognized and diverse PAO community, dominated by *Pseudomonas*, *Halomonas*, and *Nannocystis*. Notably, PAO community composition in the maize rhizosphere exhibited significant genotype-specific patterns, indicating plant genetic background influences the recruitment and enrichment of functional PAOs. These findings highlight the importance of exploring alternative PAOs and their specific metabolic preferences across environmental samples. By providing insights into the detection and characterization of bacteria of interest, flow cytometry

combined with phylogenetic analysis offers a promising avenue for advancing our understanding of PAOs and their roles in wastewater treatment and environmental processes.

1 Introduction

A diverse range of microbes contain intracellular biopolymers, including polyphosphate (polyP), polyhydroxyalkanoates (PHA), glycogen, wax esters, triacylglyceride (TAGs), trehalose, and cyanophycin.[1] These biopolymers serve versatile functions such as energy storage, nutrient cycling , ion balance, redox homeostasis, and stress resistance, allowing microbes to adapt to fluctuating environmental conditions.[1–3] Among them , polyP-accumulating organisms (PAOs) have been widely recognized as a key agent to achieve sustainable phosphorus (P) removal and recovery such as in enhanced biological phosphorus removal (EBPR) systems .[4,5] In EBPR systems, PAOs exhibit a unique metabolism: under anaerobic conditions, they utilize stored polyP as an energy source, releasing phosphate and concurrently accumulating PHA. Then under subsequent oxic or anoxic conditions, they take up phosphate and synthesize new polyP while using stored PHA to fuel growth.[6] The metabolic interdependence of dual intracellular polymers (polyP and PHA) is a defining feature of functionally verified PAOs in EBPR systems, such as *Candidatus Accumulibacter*,[6] *Pseudomonas*,[7] and *Dechloromonas*,[8] although some cannot accumulate PHA such as *Tetrasphaera*.[9] This unique metabolism provides competitive advantages for PAOs under fluctuating anaerobic/oxic conditions.[8,14,15]

Beyond EBPR engineering systems, there are increasing interests in exploring and identifying functional roles of PAOs in natural environments, as emerging evidence points to their ubiquitous presence and versatile functions.[10–13] However, PAOs in natural environments are often identified broadly as polyphosphate-containing organisms (PCOs)**,** with little knowledge of the

potential co-metabolisms with carbon (C) biopolymer storage. Here, we are specifically interested and thus propose a refined definition of functional PAOs in natural environments as organisms that co-accumulate both polyP and intracellular C, predominately PHA, to surplus levels, where the co-metabolism holds important ecological roles in these systems for following reasons. Firstly, as one of the dominant C storage biopolymers, PHA concentrations (~1-4μgC/g soil) are positively responsive to C availability, but are suppressed by nitrogen availability, suggesting its role as an alternate C allocation strategy for stoichiometric balancing and stress response.[16,17] The co-storage of C biopolymers and polyP likely enhances microbial adaptability to nutrient fluctuations and contributes to broader nutrient cycling and ecosystem functioning. This dual-storage strategy distinguishes specialized PAOs exhibiting resource-dependent surplus storage from organisms that accumulate polyP primarily for stress responses or cation sequestration.[17–19] Secondly, our preliminary results indicate that up to 73.7% of polyP-accumulating cells in crop soil contain C storage biopolymers with significant Pearson correlation between polyP-accumulating cells and polyP-C-accumulating cells ($R=0.8$, $P<0.001$) (Figure S1). Lastly, the inability of polyP-synthesizing fungal organisms, such as arbuscular mycorrhizal fungi, to produce PHA provides a clear demarcation between bacterial and fungal PAOs. Fungal PAOs were more extensively studied, whereases relatively little is known about bacterial PAOs in natural environments.[24] Therefore, we focus on identifying bacterial microorganisms exhibiting surplus storage, defined by their specific ability to accumulate "substantial" polyP and PHA beyond immediate biological maintenance, distinguishing them from microbes that maintain constitutively low levels of these biopolymers for basic cellular functions (e.g., *Escherichia coli* or *Bacillus subtilis*).[20,21]

Despite the ecological, biogeochemical, and engineering significance of PAOs, identifying functionally active and phylogenetically novel PAOs in complex environmental matrices remains

challenging.[10,22–24] Conventional approaches rely heavily on gene-centric profiling such as polyP-metabolism biomarkers (i.e. ppk) or metagenomics to identify and quantify previously known PAOs across aquatic and terrestrial systems,[25,26] yet these methods have limitations and can not reflect actual phenotypic traits such as PolyP accumulation.[25–27] Discovery of novel PAOs is often prohibited with *ppk* gene-based detection that rely on prior genetic information of the targeted PAOs and, it also has low specificity for detecting PAOs with unqiue polyP accumulation ability as the polyP-synthesizing gene *ppk* is ubiquitous across microorganisms.[27,28] Moreover, polyP metabolism is not solely regulated by *ppk* gene, as alternative proteins, such as actin-like Arp proteins, have been proposed to exhibit PPK-like functions in bacteria.[29] Methods for detecting PAOs that co-accumulate polyP and PHA in natural systems have never been reported. A few recent studies have employed fluorescence-activated cell sorting (FACS) to phenotypically track the polyP accumulation traits and resolve the phylogeny and taxonomy of PAOs from EBPR systms,[30,31] although these studies stains only for polyP, without detecting the co-metabolism polymers like PHA.[31] In addition, FACS-based PAO detection in soil samples have never been reported.

In this study, we developed an integrated approach combining triple-staining fluorescence-activated cell sorting (FACS) with genomic sequencing, termed here as TriFlow-Seq. Beyond polyP-based PAO detection, TriFlow-Seq enables the simultaneous detection of functionally active PAOs with both polyP and PHA, where sorted cells were subsequently subject to 16S rRNA amplicon sequencing for phylogenetic identification. To validate this method, we applied an independent phenotypic detection technique—Single-Cell Raman Spectroscopy (SCRS)—an established approach for the simultaneous detection of intracellular polyP and PHA in EBPR PAOs,[23,32–35] in parallel for comparison. We further demonstrated the successful application of

TriFlow-Seq across diverse environmental samples, including activated sludge from four full- and pilot-scale EBPR facilities and rhizosphere soil from maize croplands in the U.S. By addressing key limitations of conventional methods, this approach enhances our ability to resolve the identities of bacterial PAOs beyond known taxa across diverse environmental systems, from soils and sediments to water bodies.

2 Material and Methods

2.1 Sample collection.

2.1.1 Activated sludge samples from EBPR systems

Biomass samples from the aerobic zone were collected from three different full-scale EBPR plants from Hampton Roads Sanitation District (HRSD) that have effective P removal via EBPR activities thus containing active PAOs, namely in HRSD Nansemond Treatment Plant (Suffolk, VA, USA), HRSD Virginia initiative Treatment Plant (Nortfolk, VA, USA), HRSD Atlantic Treatment Plant (Virginia Beach, VA, USA), as well as from one pilot-scale side-stream EBPR (S2EBPR) (VA, USA). The collected activated sludge samples from EBPR systems were stored at 4 °C, transported overnight to the laboratory, and activated with phosphorus release/uptake test (Text S1, Figure S2) while sample from pilot-scale S2EBPR was directly fixed onsite as described in section 2.4.

2.1.2 Soil samples

Sixteen maize genotypes, six inbred and ten hybrid lines (Table S1), were each planted in a randomized complete block design (n=2) across two fields respectively with standard planting density (24 plants/plot), each characterized as Lima Silt Loam with a 0-3% slope at Musgrave Farm in Aurora NY (42.733317, -76.652394). Rhizosphere samples were collected at tasseling (VT) in accordance with Hanway et al.[36] Briefly, from each plot, 3 randomly selected plants were

uprooted with loosely attached bulk soil shaken off, root adhered soil was taken as rhizosphere soil, pooled by plot and stored and transported at 4ºC for sample pre-processing within 24 hours.

2.2 Microbiota isolation

2.2.1 Rhizosphere microbiota

The microbial fraction of the soil was isolated via detached from 1 g of soils using detachment detergent (3 mM sodium pyrophosphate, 0.5% Tween20, 0.35% PVPP in 1xPBS) in a 1:20 ratio shaken at 200 rpm on an orbital shaker for 30 mins at room temperature as previously described in Deng et al., (2019). Following detachment, to prevent re-aggregation, detached soil community was washed 1x with 0.4 mM EDTA dissolved in 1X PBS (pH ~7.2) for excess extracellular cation removal followed by 2x washing with 1x PBS.

2.2.2 Activated sludge sample

Sludge samples were collected in the middle of the aerobic phase from each condition and were centrifuged to remove supernatant. The remaining pellets were washed three times with 1X PBS for excess cation removal.

2.3 Cell fixation

The fixation buffer was prepared according to previously described protocol,[38,39] containing 5 mM Nickel Chloride ($NiCl_2$), 5 mM Barium Chloride ($BaCl_2$), 10 % Sodium azide (NaN_3) in HEPES Buffer (25 mM), pH = 7.2. The remaining pellets were resuspended in a 5 ml fixation buffer, vortexed for 10 s, and were stored at 4 °C for 1 day.

2.4 Triple-staining

Fixed sludge samples were washed three times with phosphate-buffered saline (PBS: 0.4 M Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.2) with centrifugation at 3200 g for 10 min while fixed soil samples were washed three times with 1X PBS with centrifugation at 6000g for 10 mins at

4°C. Fixed and washed sludge (1 ml) were treated with 0.584 ml solution A (0.11M citric acid/4.1mMTween 20 in distilled water) for 20 min, washed and resuspended in 1ml of PBS buffer. Fixed, washed, and resuspended (5 mL) soil pellets were similarly treated, with 1.6mL solution A for 20 mins, washed, and resuspended in 5mL of PBS.

DNA, polyP and PHA of the cells were stained with SYBR Green I (SG) (Ex 488 nm / Em 515 nm), tetracycline hydrochloride (TC) (Ex 390 nm / Em 560 nm) (Sigma Chemical Co., USA), and Nile red (NR) (Ex 532 nm / Em 610 nm) (Sigma Chemical Co., USA), respectively–each validated in previous studies.[30,40–43] First, TC was applied as a 2 mg/ml stock solution in milli-Q water (0.225 mM in cell suspensions) and incubated for 10 min.[39] NR stock solution (10 mg/ml in DMSO) was added to 1 ml of cells a as final concentration of 0.05 mg/ml, and then incubated for 10 min.[44] Lastly, 100X SG was applied into the cells, and it was incubated for 4 hours.[37] All incubation steps were done at room temperature in the dark.

2.5 Floc Disaggregation

Unlike activated sludge, rhizosphere samples are particularly prone to aggregation due to root exudates, mucilage, and extracellular polymeric substances (EPS), which can reduce sorting efficiency and risk nozzle clogging. A disaggregation process was therefore developed to further prevent re-aggregation during FACS. The protocol involves resuspending the stained pellet in 3 mL of 1X Trypsin-0.5mM EDTA for 1 minute, followed by trypsin deactivation using 1X FBS (1:1 ratio). Prior to FACS, trypsinized samples were diluted in 1X PBS to achieve a proper threshold rate of 1500 events s^{-1}.

2.6 FACS settings and Validation of FACS gating strategy

Cell sorting was carried out using the 100-micron nozzle on Fusion Aria III cell sorter (BD, U.S.) equipped with a blue laser (488nm excitation) with 530 nm band pass (BP) filter for SG, a violet

laser (405nm excitation) with 525 BP filter for TC, and a yellow-green laser (561nm excitation) with a 610 BP filter for NR. The blue laser was also used to analyze forward scatter (FSC) which is related to cell size and side scatter (SSC) which is related to cell granularity. Manual gating was performed and summarized in supplementary material (Text S2, Figure S3). Bacterial cells were sorted in purity mode at a rate less than 1500 cells per second. To obtain sufficient DNA for the following DNA preparation and analyses, up to 10^5 cells were sorted.[45] The cells were sorted into sterile 1.5 mL micro tubes and frozen for further analysis.

To ensure the reliability and accuracy of the proposed TriFlow-Seq in identifying and quantifying PAOs in complex microbial communities, we employed two independent validation methods.

We first verified the PAO enrichment via fluorescent confocal microscopy. Approximately 4000 sorted cells were collected to validate that all sorted cells were positive with SG, TC and NR. Unsorted cells as control and sorted cells were first filtered through black polycarbonate filter (0.22 um pore size) to concentrate cells. The filters were placed onto slide glass with a drop of antifade reagent. The validation of the gating methods was carried out by observation through confocal microscopy (Carl Zeiss) with 800X magnification and three excitation/filter sets: blue light (BP 450–490 nm, FT 500 nm, LP 515 nm), green light (BP 510–560 nm, FT 565 nm, LP 590 nm) and ultraviolet (BP 340–380 nm, FT 400 nm, BP 435–485). Quantification of TC and NR-stained cells relative to SG-stained cells was performed using Daime (version 1.2).[46]

Additional validation was conducted by comparing FACS results with SCRS analysis, an independent method previously developed by our group quantifying polyP and PHA-containing cells.[32,33] Samples were prepared on optically polished CaF_2 windows (Wilmad LabGlass, Buena, NJ). Raman spectra were acquired using a Horiba LabRam Evolution (Horiba, Kyoto, Japan) equipped with 50X objective (Olympus LMPLFLN 50x, Tokyo, Japan) in accordance with

established protocol.[23,47,48] Relative polymer content in each individual cell was evaluated based on the signature peak intensity (CCD counts) of each polymers.

2.7 DNA extraction and Amplicon sequencing

Genomic DNA of sorted soil and sludge cells was extracted using InstaGene matrix (Bio-Rad Laboratories, USA). Genomic DNA from unsorted raw soil and sludge was extracted via DNeasy Power Soil Pro kit (Qiagen, Valencia, CA, USA) carried out on the QIAcube® (QIAGEN) platform. The V4 region of the 16S ribosomal RNA (16S rRNA) genes from both sorted and unsorted samples were amplified and sequenced using primers 515F and 806R at University of Connecticut-MARS (Microbial Analysis, Resources, and Services) facility. Details of sequencing were listed in Text S3.

2.8 Statistical Analysis

The Spearman correlation coefficients and corresponding *p*-value were computed using Python3 *Scipy 1.10.1* package. Principal Coordinates Analysis (PCoA) was performed using *R vegan* (2.6-4) to assess beta diversity based on Bray-Curtis dissimilarities of normalized relative abundance data. The ordination plot was visualized using *ggplot2* (3.4.4).

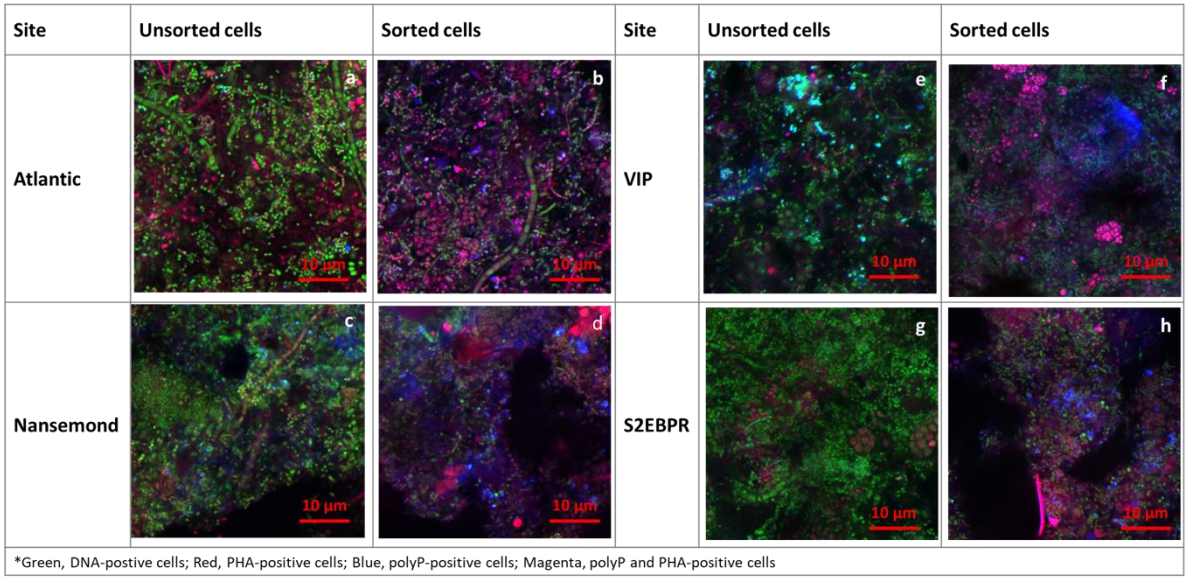
3 Results and discussion

3.1 Development of FACS TriFlow method for sorting functional PAO with dual accumulation of polyP and PHA

The effectiveness of the triple-stained FACS approach for identifying and sorting PHA-containing PAOs were tested with EBPR systems that are known to contain PAOs (positive control environmental samples). The density of the triple-stained cells before and after the FACS enrichment were visualized using confocal microscopy (Figure 1A). A significant and evident enrichment of candidate PAO cells that contain both polyP and PHA were shown in the sorted

samples (Magenta) compared to the original sludge, which was validated in all four tested EBPR systems. In addition, relative abundances of PHA-positive and polyP-positive cells as of total cells (SG-stained cells) were semi-quantified using digital image analysis (Figure 1B). NR-stained and TC-stained cells were clearly enriched in all FACS sorted samples in comparison to the original sludge samples. These results demonstrated that our gating strategy in the FCAS analysis effectively led to dual-polyp-PHA-accumulating PAO enrichment as expected, and the gating strategy employed was appropriate.

A)



B)

Sample	Unsorted cells (%)		Sorted cells (%)	
	NR+ (PHA-positive)	TC+ (polyP-positive)	NR+ (PHA-positive)	TC+ (polyP-positive)
ATL	50.3	35.6	99	98.3
NM	45.7	53.7	97.3	87.7
VIP	68.1	29.1	79.8	80.9
S2EBPR	19	6.8	96	83.6

NR+, Nile Red-stained cells; TC+, Tetracycline-stained cells.

Figure 1 Images from confocal microscopy for comparison between FACS sorted stain-positive cells and unsorted cells in activated sludge. Samples from Atlantic, Nansemond, VIP EBPR systems and a S2EBPR system, respectively; B) Relative abundance of NR (PHA)-positive and TC (polyP)-positive- cells in FACS sorted samples versus original unsorted samples by semi-quantitative confocal microscopic image analysis.

3.2 Validation of polyP-PHA-acumulating PAO quantification through TriFlow

Prior to implementing the TriFlow in soil samples, we validated polyP-PHA dual staining by comparing the relative abundance of polyP-containing cells through FACS with those determined by SCRS. SCRS, which targets the molecular composition of microbial cells, has demonstrated exceptional sensitivity and accuracy in detecting and quantifying intracellular polymers such as PolyP, PHA, and glycogen.[49] SCRS-based detecton of PAOs containing both polyP and PHA were demonstrated in our previous studies (ref.) Detailed sampling information and the linear correlation between the two methods are provided in Text S4.

We compared the quantification of PAOs with polyP-detection irrespective of intracellular PHA containment, as well as the quantification of dual polyP-PHA-accumjuting PAOs, across various microbial samples from EBPR systems, from bulk soil and from maize rhizosphere. A positive correlation between the percentage of polyP-containing cells identified through FACS and SCRS was demonstrated (Pearson coefficient r^2 = 0.6974158, *p*-value = 0.000309; Figure 2A). Importantly, the percentage of PAOs determined by FACS and SCRS falls in the same range. This significant and strong correlation between the two methods for detecting and sorting polyP containing cells validated the proposed FACS protocol for sorting PAOs.

We further compared the performance of triple-stained FACS and SCRS in identifying the relative abundances of putative functional PAOs (containing both polyP and PHA) across diverse samples including soil, rhizosphere, EBPR activated sludge, and isolate PAO strains (*T. elongata* DSM 14184) (Figure 2B). They demonstrate a weak linear (Pearson's r = 0.488) yet still a significant positive monotonic relationship (Spearman's r = 0.62, *p*-value = 0.009).

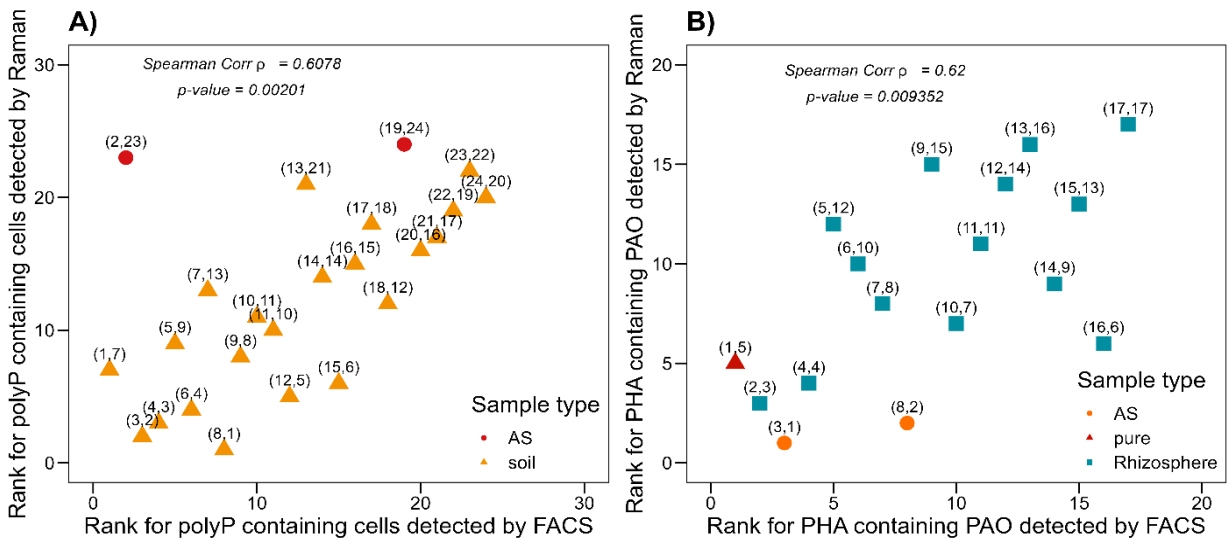


Figure 2 Spearman's rank correlation coefficients of PAOs and polyP-containing cells detected by FACS and SCRS. (A) polyP-containing cells, irrespective of PHA presence; (B) PAOs, defined as cells containing both polyP and PHA. The strong correlation between the two independent techniques validates the effectiveness of the triple-stained FACS in detecting and quantifying polyP-containing cells and PAOs in complex microbial communities.

The percentages determined by FACS were consistently lower than those identified by SCRS, likely stems from fixation-free characteristics of SCRS methods as discussed in the following section.It is important to note that FACS and SCRS employ fundamentally different phenotyping mechanisms, leading to distinct approaches in quantifying target cell populations. SCRS is a fixation-free method that can detect intracellular PHA and polyP based on the unique Raman spectral signature regardless of counterion type.[50] FACS, however, requires a fixation step that enhances dye penetration but permanently modifies cell wall and membrane permeability.[51] This modification typically results in a 10-20% loss of intracellular polymers, similar to observations with FISH fixatives.[52] In addition, the accuracy of FACS largely rely on the specificity and sensitivity of fluorescent dyes. Nile Red, although widely employed for PHA detection in flow cytometry, can also capture other lipophilic inclusions such as trehalose and triglycerides.[53] Similarly, tetracycline detects polyP through preferentially complexes with countercharge divalent (e.g., Ca^{2+}, Mg^{2+}) in polyP granules.[54–56] These methodological differences suggest that FACS may

slightly overestimate PHA-containing PAOs relative to SCRS method, consequently reducing the slope of the correlation curve. Furthermore, FACS employs size-based filtration (40μm cell strainer) to prevent flow cytometer nozzle clogging, while SCRS utilizes density-gradient centrifugation for cell extraction.[57] These procedural variations likely influence the organisms being analyzed, contributing to the observed differences in PAO abundance percentages. Despite these discrepancies, the consistent trend in PAO detection by both methods underscores the viability of the triple-stained FACS approach for identifying and quantifying PAOs in complex microbial communities.

3.3 Application of TriFlow-Seq in Identification and Quantification of PAOs in EBPRs

Combining triple-stained FACS with downstream 16S rRNA genes amplicon sequencing (termed as TriFlow-Seq) led to enrichment, quantification, and identification of both known and candidate PAOs in pilot and full scale EBPR plants. The relative abundances of known PAOs in the original sludge prior to the FACS sorting, versus those after the FACS enrichment were depicted in Figure 3.

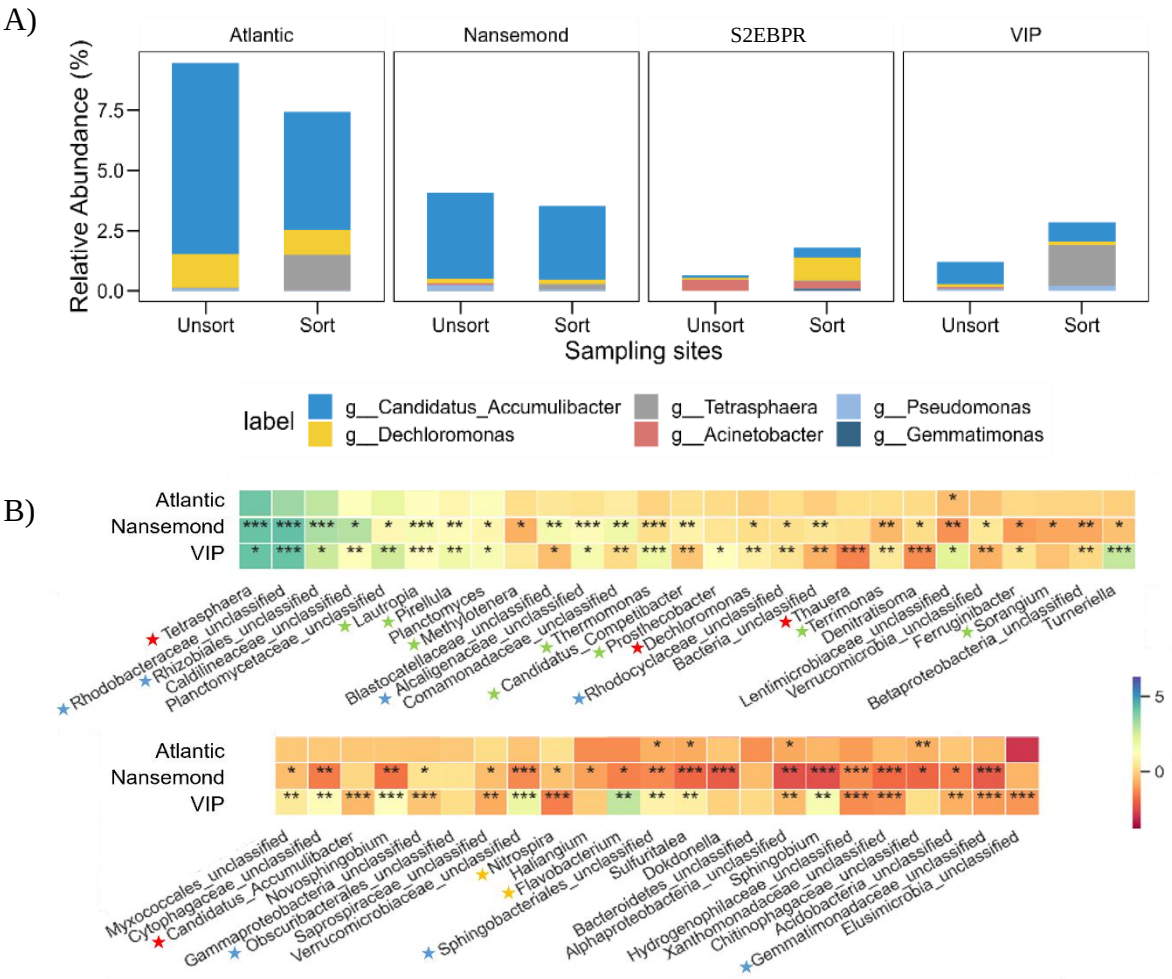


Figure 3 A) Relative abundances of known candidate PAOs before (left) and after (right) FACS sorting in three full-cale EBPR plants and one pilot-scale S2EBPR plant. These samples were activated with P release and uptake tests. The groups that had relative abudances below 0.1% were not shown; B) Differential abundance analysis for idnetification of unknown candidate PAOs at the genus level following FACS sorting. Heat map represents t-statistics derived from two-tailed t-tests on log10-transformed fold changes. Blue hues denote positive t-statistics (enrichment), while red hues indicate negative t-statistics (depletion). Statistical significance levels: *p < 0.05, **p < 0.01, ***p < 0.001. Red pentagram indicates the genus has been experimentally confirmed with PHA/polyP and PAO activities; Yellow pentagram indicates the presence of polyP and PHA; green pentagram indicates the the reference genome contains necessary genes for polyP and C storage synthesis; blue pentagram indicates that some genus within the Family have polyP accumulation ability. Detailed information can be found in Table S2. Analysis limited to the top 50 genera by abundance.

3.3.1 TriFlow-Seq enriched known PAOs in EBPR systems

TriFlow-Seq effectively identified previously known and expected PAO genera in EBPR systems, including *Pseudomonas*, *Dechloromonas*, and *Ca*. Accumulibacter[58–61] across all plant samples (Figure 3A), validating its capability to identify organisms known to contain both polyP and PHA in EBPR systems.

The sorted communities were characterized by the enrichment ratios, calculated as the relative abundance of each genus post-sorting divided by its pre-sorting abundance (log10 scale). Higher ratios indicate a greater enrichment of bacteria capable of accumulating both polyP and intracellular C polymers, thus reflecting the proportion of putative PAOs in that taxonomic group (Figure 3B).

Ca. Accumulibacter, despite being consistently identified as the dominant PAO in unsorted samples in three full-scale plants except S2EBPR pilot plant (1.2~4.0%) (Figure 3A), showed varied enrichment ratios among the EBPR plants (Figure 3B). Its relative abundance even decreased after sorting in the VIP samples. Interestingly, *Tetrasphaera* emerged as the most significantly enriched genus after sorting (Figure 3A and 3B). Although initially thought to lack PHA synthesis capability, recent studies have revealed that anaerobically PHA synthesis depends on clade composition of *Tetrasphaera*.[62–65] For instance, the *T. japonica* genome possesses PHA synthase (*phaC*) and was proved experimentally to synthesize PHA under anaerobic condition.[63] This evidence indicates that *T. japonica* or potentially uncultured *Tetrasphaera* that possess Accumulibacr-like PAO metabolism reside in full-scale EBPR.[66] Overall, the relative abundances of known PAOs were only 2 to 12% out of total PHA+polyP positive cells (Figure 3A), highlighting the larger phylogentic diversity of PAOs than our current understanding.

3.3.2 Discovery of candidate PAOs in EBPR

It should be noted that even within known PAOs, some sub-genus populations were not actively performing polyP and PHA accumulation, resulting in a negative enrichment factor, for example, Ca. Accumulibacter. Therefore, the selection of putative PAOs in sludge were based on (1) consistent detection across sorted communities, and (2) prior evidence of or genomic potential for polyP and PHA accumulation. A notable enrichment of putative PAOs was observed, including

unclassified *Rhodobacteraceae*, unclassified Rhizobiales, unclassified Caldilineaceae, *Lautropia*, etc (Figure 3B). The identified OTUs associated with these bacterial taxa were not classified to genus level, suggesting the potential presence of unknown genera, which could be candidate PAOs with or without classical EBPR metabolisms. Bacteria isolated from the order Rhizobiales were found to contain PHA and polyP.[67] An uncultured genus close to the *Rhodobacteraceae* family is speculated to be sulfur PAOs.[68] these new insights provide initial novel candidate PAOs and whether these putative PAOs can contribut to P rempval as those cronical PAOs require further investigation.

These results led to the following findings: (1) not all genetically identified *Ca*. Accumulibacter display metabolically active PAO phenotypes, as evidenced by their varied enrichment ratios; (2) *Tetrasphaera* showed unexpected and significant enrichment with variable intracellular PHA content, revealing phenotypic diversity beyond current understanding of this genus; and (3) The TriFlow-Seq method uncovered novel candidate PAOs from previously unrecognized taxonomic groups, expanding our understanding of PAO diversity in EBPR systems. Although a comprehensive comparison of the PAO population in the tested EBPR plants is beyond the focus of this study, this application has demonstrated that the TriFlow-Seq is a valuable tool for rapidly enriching targeted bacterial populations that exhibit the phenotypic traits characteristic of functionally relevant PAOs. This approach was further adapted and optimized to be applicable to more heterogeneous sample types, such as bulk soil, rhizosphere soil, and sediments, enabling the identification and characterization of PAOs in these complex environmental matrices.

3.4 Identification and Quantification of the Previously Unknown Rhizosphere PAO Community

3.4.1 Key Steps Modified for soil sample processing.

Significant challenges were faced in applying FACS to soil samples, which differ markedly from activated sludge in their physico-chemical properties and proneness to reaggregation. Traditional soil pretreatments using detergents or sonication quantifications prove inadequate for sorting low-abundance populations,[69,70] especially when high cell numbers are required for quality genomic reads. Key challenges include reaggregation during extended suspension, which reduces sorting efficiency and increases false positives,[71] and soil heterogeneity, which affects reaggregation rates due to variations in cation exchange capacity, plant and microbial protein activity, soil organic matter content and biofilm formation.[72] Our newly developed pretreatment method (Section 2.5) successfully overcomes these obstacles, demonstrating effectiveness across three diverse soil types: a silt loam from central NY, a soil fertilized with ammonium sulfate and high soil organic matter in Urbana Champaign Illinois, and a potting soil mixture. This method not only allows for effective sorting of low-abundance community members but also represents a significant step towards standardization in FACS preparation for varied soil types.

Notably, our soil pretreatment method achieved sorting efficiencies ranging from 77-92% across two soil series tested (n=8 samples), with an average of 85.5% (Table S3). This represents a substantial improvement, approximately 50% higher than the 35.75% average sorting efficiency obtained using standard WWT sample pretreatment (n=8 samples, Table S3). DNA extraction revealed the success of sorting with DNA quantification ranging from 1ng/ul to 16.3 ng/ul with downstream 16S rRNA amplicon sequencing quality control retrieving high quality reads (Phred >30) from all but one sequenced sorted sample (n=192).

3.4.2 Effectiveness of TriFlow-Seq in rhizosphere samples

The identity of active prokaryotic PAOs in the rhizosphere of maize are mostly unknown. For the first time, the bacterial PAO community in the maize rhizosphere of sampled Nested Association Mapping (NAM) and a subset of phenotypically diverse hybrids from the High Intensity Phenotyping Site (HIPS) panel were revealed through our triple stained FACS coupled to downstream 16S rRNA sequencing.[73] Enrichment profiles of key sorted taxa across maize genotypes were further investigated to identify candidate functional PAOs in maize rhizosphere.

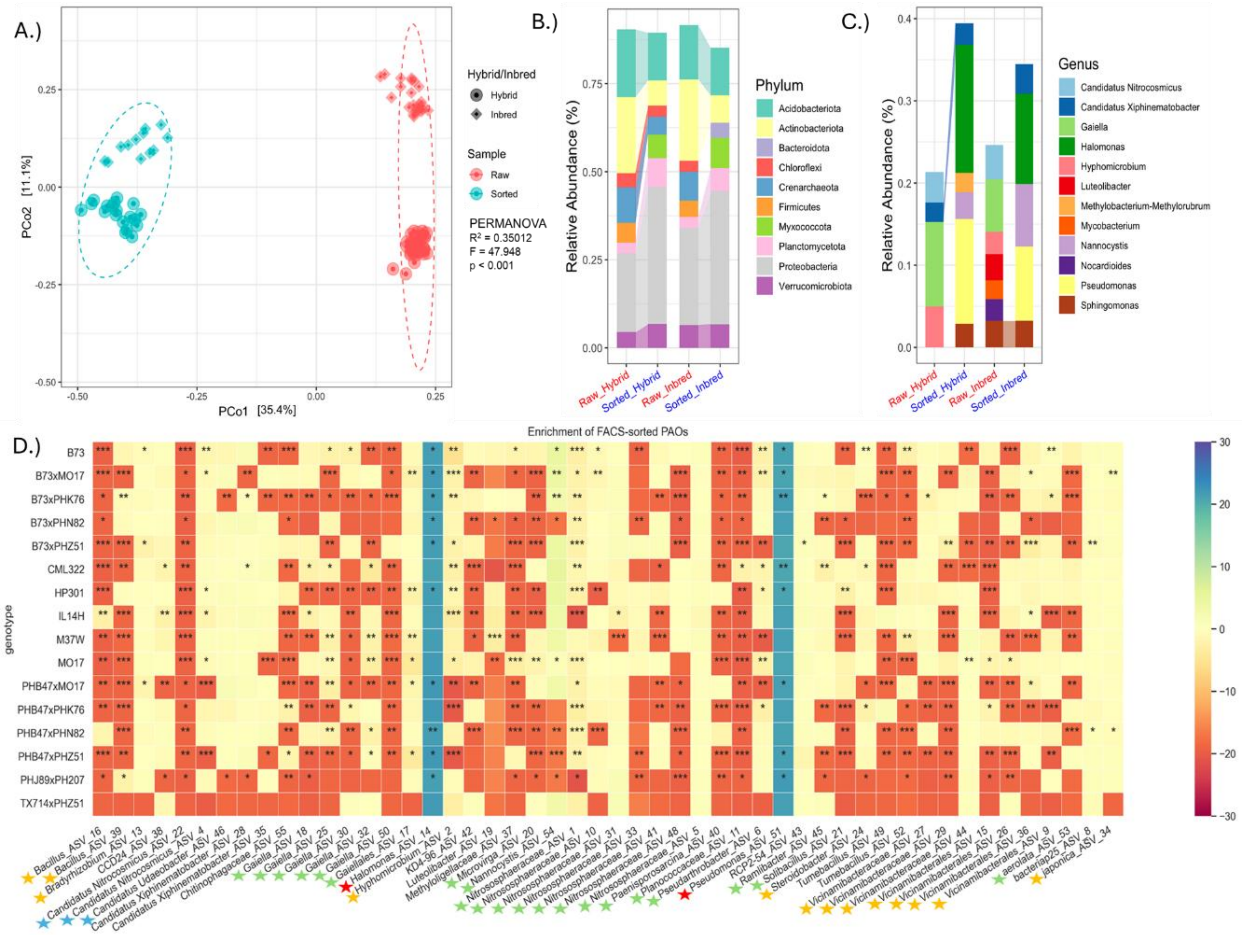


Figure 4. Identification of candidate rhizosphere PAOs and characterization of rhizosphere PAO community via coupled triple stained FACS and downstream 16S sequencing. A) Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity of unsorted raw and triple stained FACS sorted-16S sequenced communities across NAM (inbred) and HIPS (hybrid) lines; B) Phylum and C) Genus level community composition and relative abundance of unsorted raw rhizosphere community and FACS sorted-16S sequenced PAO community, separated by field, abundance filtered > 0.03; D) Differential abundance analysis of candidate rhizosphere PAO taxonomies in NAM/HIPS rhizosphere following FACS sorting-16S sequencing. Blue scale denotes positive enrichment in PAO community and red denotes negative enrichment, significance based on two-tailed t-test

with unscaled log10-fold change, *p < 0.05, **p < 0.01, ***p < 0.001. Red pentagram indicates the genus has been experimentally confirmed with PHA/polyP and PAO activities; Yellow pentagram indicates the presence of polyP and PHA; green pentagram indicates the reference genome contains necessary genes for polyP and C storage synthesis; blue pentagram indicates that some genus within the Family have polyP accumulation ability. Detailed information can be found in Table S2. Analysis limited to the top 50 ASVs by abundance.

To assess the effectiveness of TriFlow-Seq in identifying rhizosphere PAOs, beta and alpha diversity analysis were conducted to assess shifts in community structure post-sorting. Principal Coordinate Analysis (PCoA) revealed distinct clustering and significant separation ($p<0.001$, PERMANOVA) between the unsorted and sorted community along PCo1, which explained 35.4% of the total variance. This separation indicates effective isolation of a distinct sub-community (Figure 4A). Within both raw and sorted clusters, two significant sub-clusters emerged ($p<0.001$, PERMANOVA) based on maize genotypes (inbred versus hybrid) along PCo2, accounting for an additional 11.1% of variation (Figure 4A). This pattern indicates the sorted population retains its community fingerprinted from its original rhizosphere population. Alpha diversity was then conducted to understand impacts on community complexity. The sorted community exhibited significantly lower diversity ($p<0.001$) across all metrics, including Species Richness, Shannon Index, and Simpson Index (Figure S5), indicating that TriFlow-Seq successfully concentrated a highly specialized sub-community characterized by a few dominant taxa from rhizosphere soil.

Proteobacteria, Actinobacteria, and Acidobacteria were the most abundant phyla in both unsorted and sorted communities (63.2-66.2% and 59.1-59.5% respectively), aligning with typically observed community compositions in both maize rhizosphere and activated sludge communities (Figure 4B). Notably, the "highly social" Myxcococcota phyla, known for complex sterol synthesis and recently revealed in activated sludge to widely contain PAO-like *ppk1*,[74,75] was consistently detected only within the sorted communities (6.58-8.54%) (Figure 4B). While putative PAOs such as *Tetrasphaera*, *Ca.* Accumulibacter, and *Dechloromas* were detected within the sorted community, their combined prevalence <1% suggests minimal contribution to the

rhizosphere processes. Rather, *Pseudomonas* (9.03-12.7%) and *Halomonas* (11-15.6%), both putative PAOs identified in EBPR, were the predominate genera detected in the sorted communities. Additionally, *Nannocysitis* (3.25-7.56%), a *Myxcoccota* genus capable of polyP accumulation,[76,77] was consistently detected across sorted communities regardless of soil types or maize genotypes. The taxonomic composition of sorted communities further supports the efficacy of our method in identifying putative PAOs as well as taxa with known genomic potential for polyP and internal C accumulation.

3.4.3 Discovery of new PAOs in maize rhizosphere

Following confirmation of the efficacy of our TriFlow-Seq in identifying PAO or PAO-like populations, candidate functional rhizosphere PAOs were identified using selection criteria similar to those established for activated sludge systems. These criteria were adapted to address the inherent heterogeneity of soil systems, which results in variable phenotypic profiles and diverse metabolic activities of putative PAO. Enrichment profiles at the sub-genus level revealed selective dominance of specific ASVs within the predominate sorted genera, leading to the identification of three candidate rhizosphere PAOs. Among these, two predominant putative PAO ASVs exhibited positive enrichment across all maize lines: *Halomonas* ASV_14 (21.1-22.1-fold) and *Pseudomonas* ASV_51 (20.7-22-fold) (Figure 4D). Additionally, *Nannocystis* ASV_54, most closely related to *Nannocystsis exedens* (93.68% 16S rRNA sequence simialrity, BLASTN), exhibited positive enrichment (1.14-4.46-fold) across 68.75% of samples localized to sampled NAM lines (including B73), and most B73 parent hybrid lines ($p<0.05$) (Figure 4D). Analysis of the available annotated genome (GenBank GCA_002343915.1) of *N. exedens* revealed the presence of *ppk1*, *ppx*, sterol and steroid biosynthetic pathways.[78] While this study does not aim to exhaustively compare PAO populations across maize genotypes, our proof-of-concept analysis

revealed distinct patterns of PAO association with different maize genetics, which intrigues further investigation, .

These results, for the first time, revealed the characteristics, diversity, composition, and distribution pattern of rhizosphere PAO community. The emerging evidence points to the important roles of PAOs in rhizosphere communities and processes, including halotolerance, phytohormone production, siderophore production, beneficial antagonism/antibiotic production, and nutrient cycling.[79–81] Our proposed TriFlow-Seq method enables the discovery of new PAOs in agricultural soil or other natural environments, which will expand our understanding of the identity and diversity of PAOs in the natural environments.

4 TriFlow-Seq: Achievements, Applications, and Future Prospects

Linking phenotype-based detection and genotype-based characterization provides valuable insights into the discovery of organisms of interest. This study developed and validated the proposed TriFlow-Seq method and demonstrated two distinct applications: identification of functional PAOs in wastewater treatment and resource recovery facilities; and elucidation of unknown PAOs with previously unrecognized roles in P and C cycling within the maize rhizosphere. By successfully modifying FACS preprocessing protocols for soil samples, we enabled the identification of candidate PAOs in complex natural environments including soil and sediment systems. Our findings revealed unexpectedly high PAO prevalence and distinct phylogenetic patterns across different maize genotype lines, establishing a pioneering approach to explore PAO identities and functions in rhizosphere systems. Future applications of TriFlow-Seq could be enhanced through several approaches: (1) incorporation of additional phenotyping targets as new staining materials become available, potentially revealing greater PAO diversity; (2) integration with downstream mini-metagenomics or other omics techniques to obtain deeper

functional and metabolic insights into candidate PAOs; and (3) combination with FISH to increase method specificity for targeting particular microbial groups. Despite current limitations, this technique demonstrates significant potential for identifying novel PAOs within complex microbial consortia, advancing our understanding of microbial polyP metabolism and phosphorus cycling across diverse ecosystems.

5 Acknowledgement

This study was partially supported by the Cornell Institute for Digital Agriculture (CIDA), and Center for Research on Programmable Plant System (CROPPS) funded by the National Science Foundation (Grant No. DBI-2019674). We thank Dr. Lydia Tesfa, Jaclyn Mahoney, and Michael Sledziona from the Flow Cytometry Facility of the Biotechnology Resource Center at the Cornell University (RRID:SCR_021740) for their technical assistance with FACS experiments. Special thanks are given to the generous support of operators and staff of HRSD for their assistance with activated sludge sample collection for the Water Research Foundation project（Grant No. 4901). We thank Dr. Michael Gore and Liam Wickes-Do for their generous time and effort in curating, planting, tending, and growth of maize NAM and HIPS Panels.

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