MONITORING MICROORGANISMS IN BIOLOGICAL PHOSPHORUS REMOVAL PROCESSES

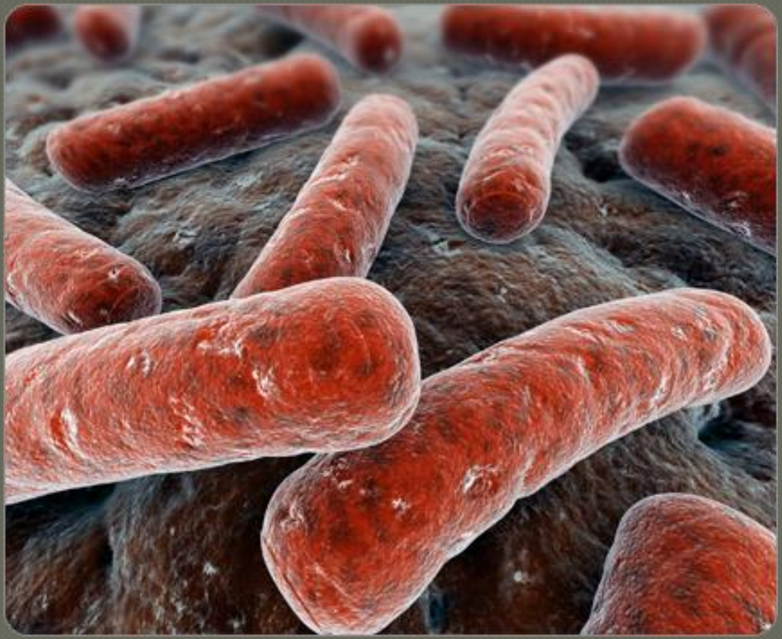
Toni Glymph-Martin
Senior Environmental Microbiologist
toniglymph@msn.com
www.toniglymphmartin.org

Total Phosphorus

- Total influent phosphorus includes:
  - Particulate
  - Soluble
- Particulate phosphorus can be removed from wastewater through solids removal
- Soluble can be removed by chemical precipitation or microbial uptake.

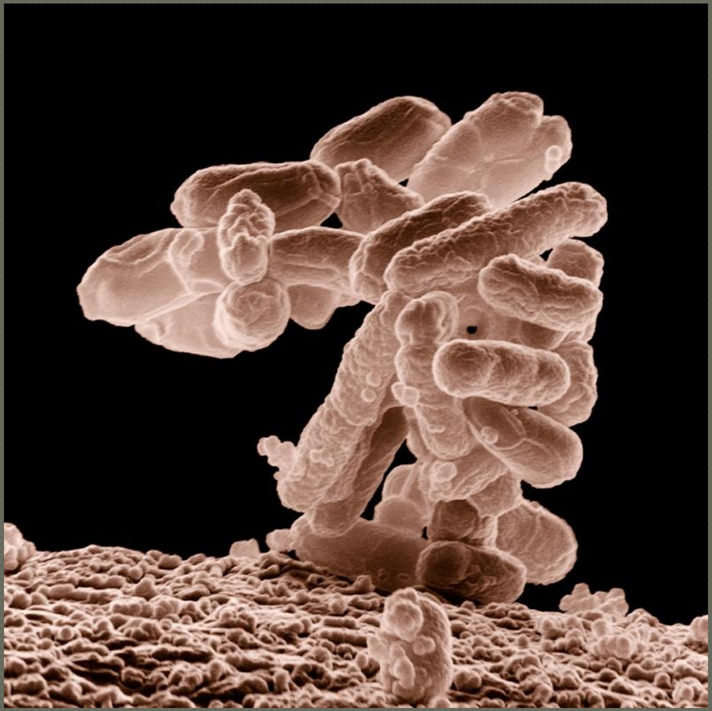
Biological Phosphorus Removal

- Enhanced Biological Phosphorus Removal (EBPR) is accomplished by phosphorus (polyphosphate) accumulating micro-organisms (PAOs) that occur naturally in the activated sludge treatment process.



Polyphosphate Accumulating Microorganisms (PAOs)

- PAOs are a group of bacteria, that under certain conditions, facilitate the removal of large amounts of phosphorus from wastewater.
- PAOs accomplish the removal of phosphate by accumulating it within their cells in the form of polyphosphate granules.



Polyphosphate Accumulating Microorganisms (PAOs)

- PAOs are not the only bacteria that can accumulate polyphosphate within their cells
- This is a widespread ability among bacteria.
- However, PAOs have characteristics that other bacteria do not have that makes them more suitable for use in the wastewater treatment process.

Polyphosphate Accumulating Microorganisms (PAOs)

- PAOs have the ability to consume simple carbon compounds in the absence of nitrate or oxygen (anaerobic conditions).
- Most other bacteria cannot consume under these conditions therefore anaerobic conditions give PAOs preferential access to the simple carbon compounds in the wastewater influent.

Polyphosphate Accumulating Microorganisms (PAOs)

- In the EBPR process, the growth of PAOs is encouraged by cycling them between anaerobic and aerobic conditions.
- Under anaerobic conditions, PAOs convert volatile fatty acids (VFAs) such as acetate or propionate to polyhydroxyl alkanoate (PHAs) compounds such as poly-β-hydroxybutyrate (PHB).

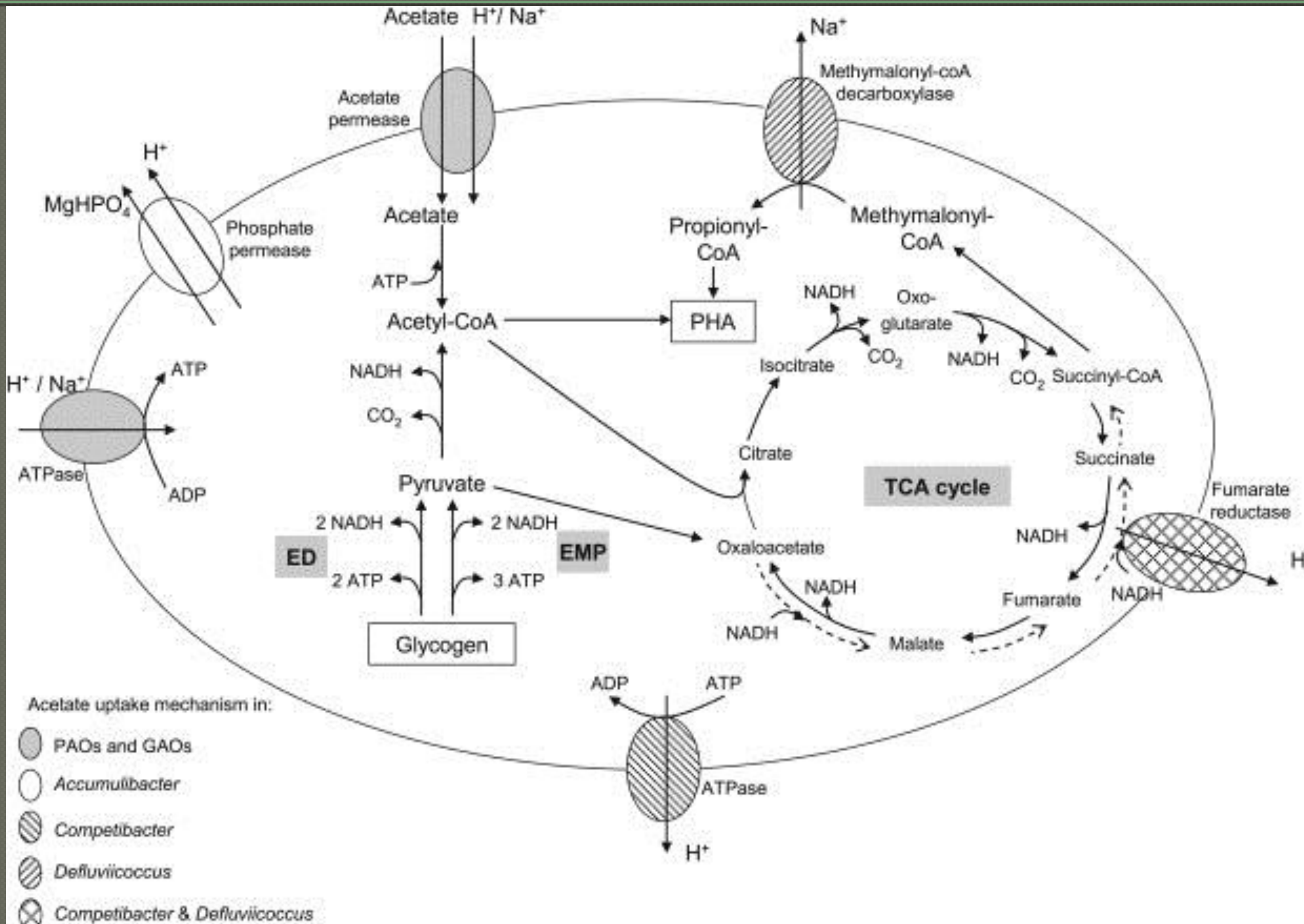
Polyphosphate Accumulating Microorganisms (PAOs)

- PAOs use energy generated by the breakdown of polyphosphate and glycogen molecules, to convert VFAs to PHBs
- This breakdown results in the release of phosphorus.

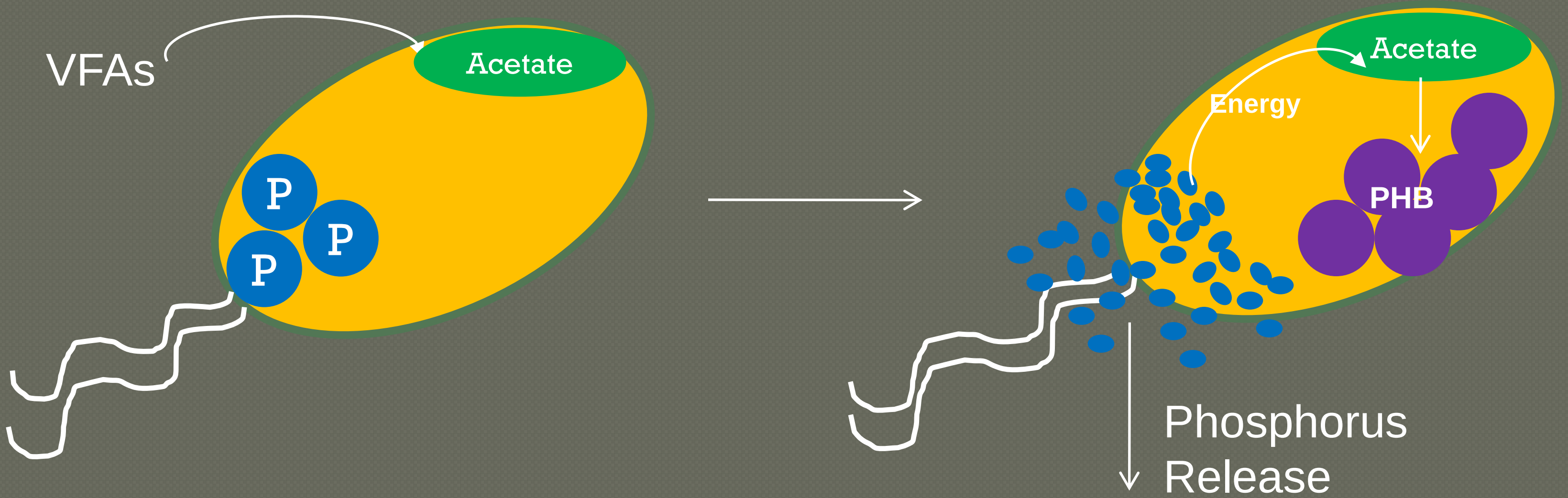
Polyphosphate Accumulating Microorganisms (PAOs)

- When followed by aerobic conditions PAOs use the stored PHBs as energy to take up the phosphorus that was released plus additional phosphorus in the wastewater.
- Phosphorus is stored in the cell as polyphosphate.
- This process renews the polyphosphate pool in the return sludge so the process can be repeated again.

Polyphosphate Accumulating Microorganisms (PAOs)

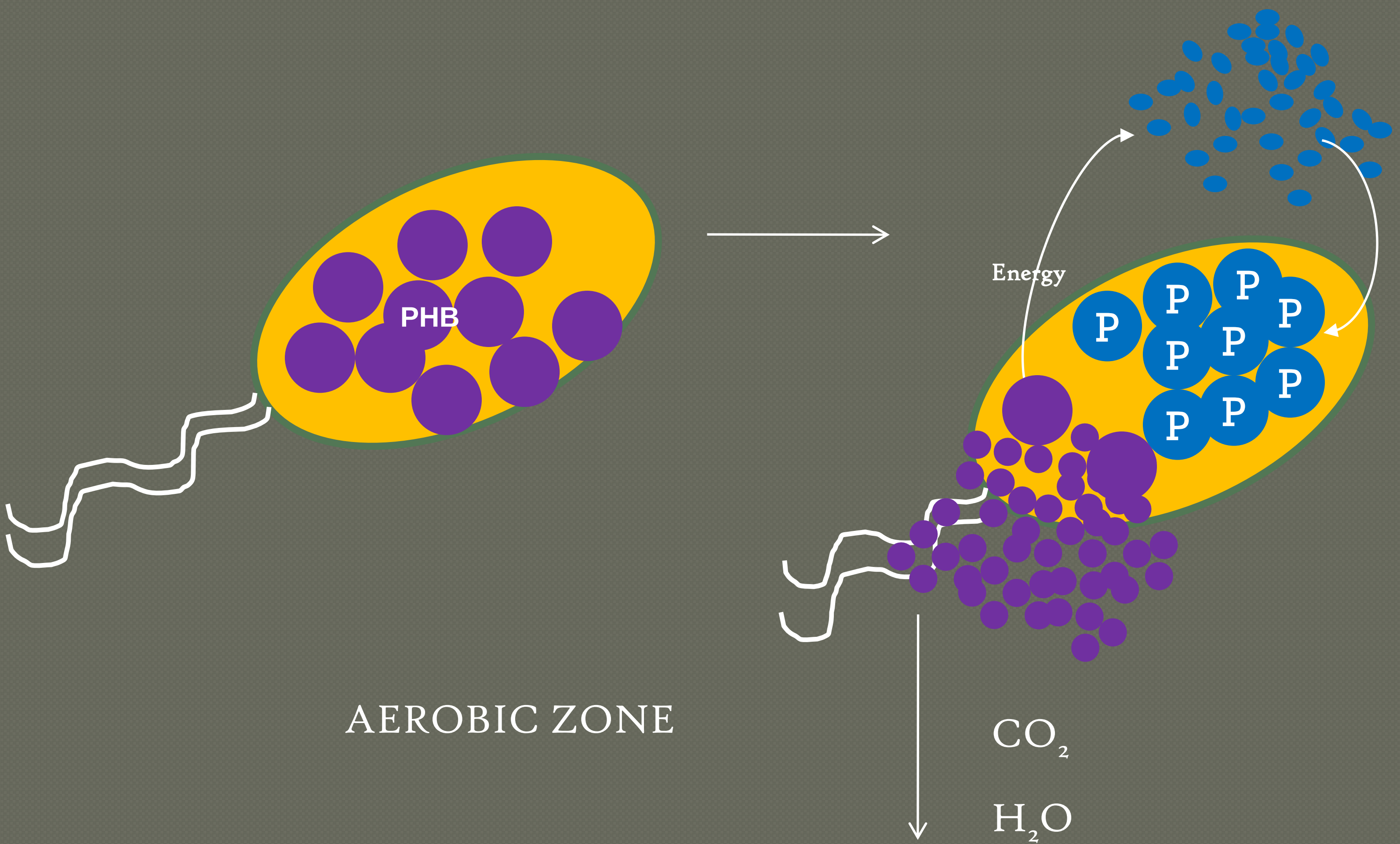


Polyphosphate Accumulating Microorganisms (PAOs)

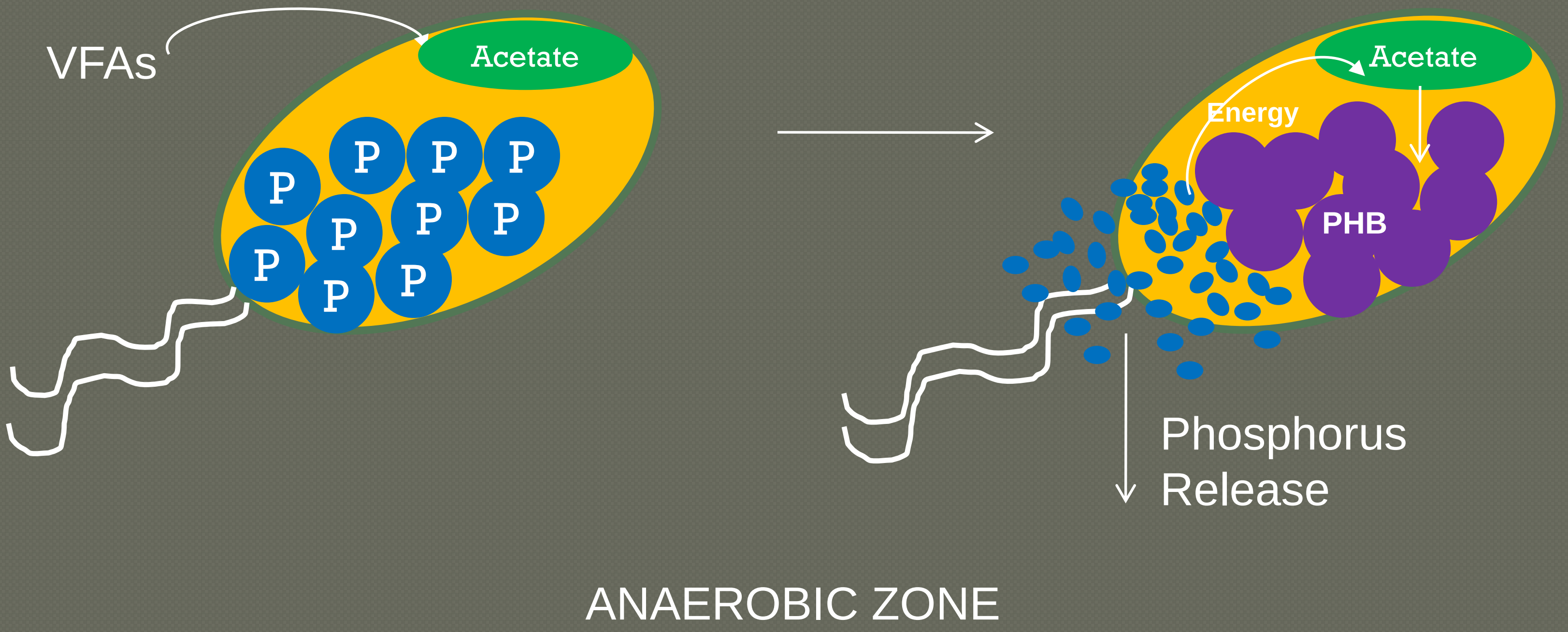


ANAEROBIC ZONE

Polyphosphate Accumulating Microorganisms (PAOs)



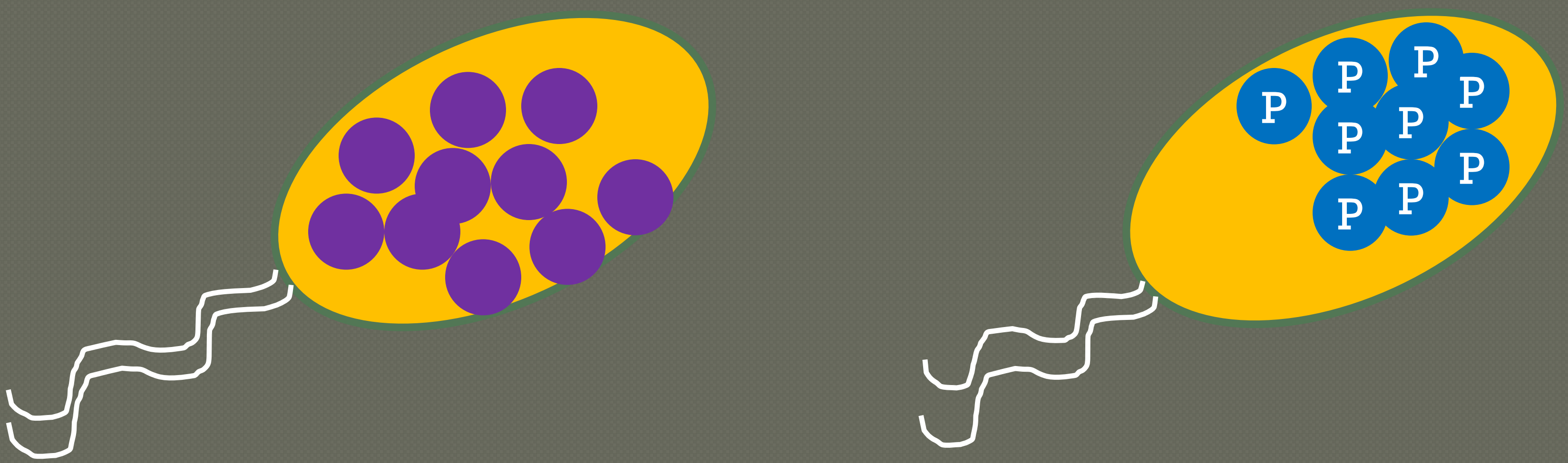
Polyphosphate Accumulating Microorganisms (PAOs)



Polyphosphate Accumulating Microorganisms (PAOs)

ANAEROBIC ZONE

AEROBIC ZONE



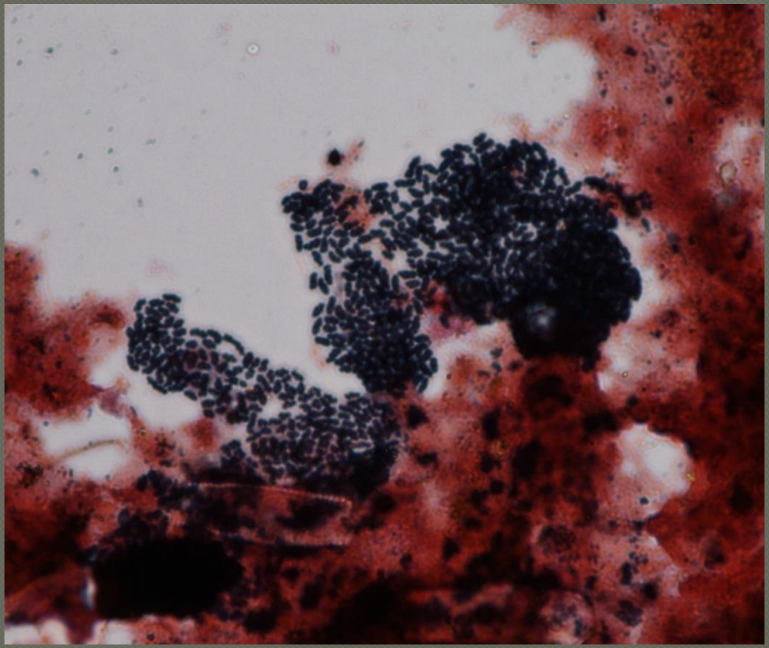
Phosphorus uptake in the aerobic zone is directly related to the amount of PHB stored in the anaerobic zone.

Polyphosphate Accumulating Microorganisms (PAOs)

- PAOs can be visualized by conventional microscopy after staining.
- In the anaerobic zone, PHB, a lipid-like polymeric ester is stored in the PAO cell as discrete inclusions or granules.
- The Sudan Black (PHB) staining method should be used to evaluate the abundance of PAOs in sample collected from the anaerobic zone.

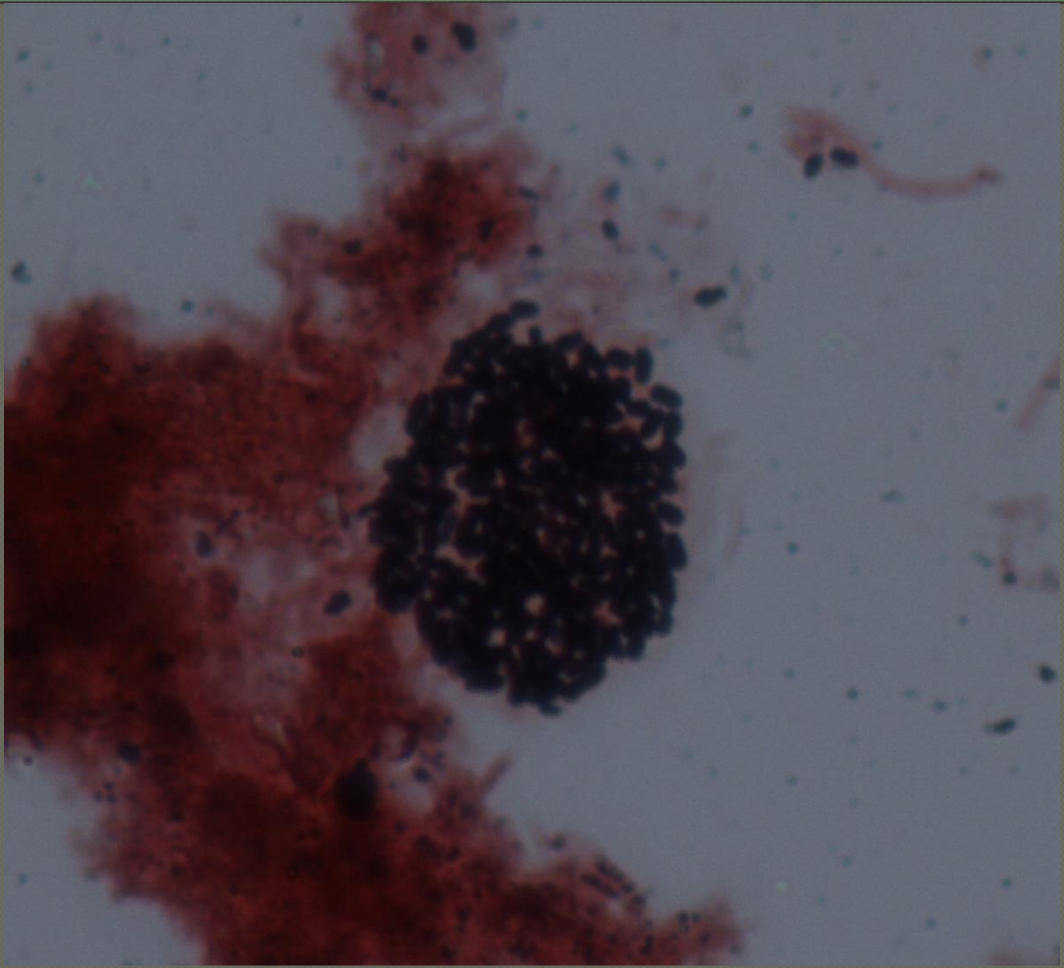
Polyphosphate Accumulating Microorganisms (PAOs)

- The ability of the lipophilic Sudan Black stain to dissolve in fatty material allows it to detect PHB granules, which would otherwise not be visible under light microscopy.
- However Sudan Black is not specific for PHB, and stains most lipophilic material.



Sudan Black Positive (PHB) Granules

Polyphosphate Accumulating Microorganisms (PAOs)



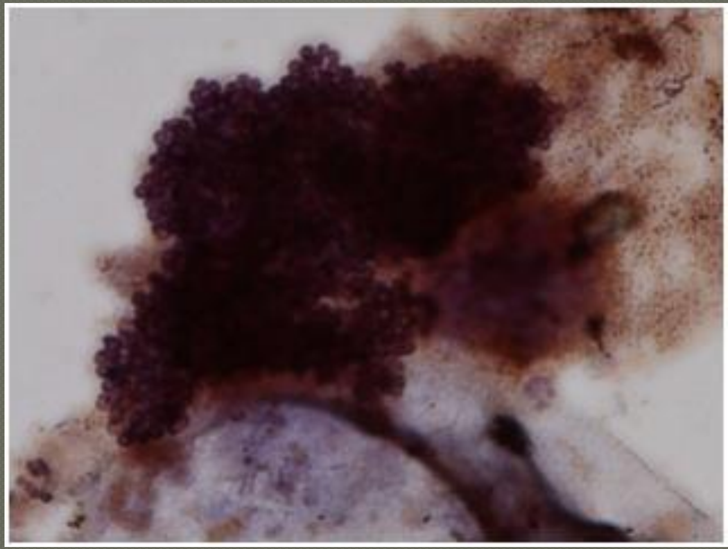
Sudan Black Positive (PHB) Granules

Polyphosphate Accumulating Microorganisms (PAOs)

- In the aerobic zone where phosphorus uptake occurs, phosphorus is stored in the PAO cell as polyphosphate granules.
- These discrete granules are not visible without staining.
- The Neisser staining methods should be used to evaluate the abundance of PAOs in the aerobic zone.

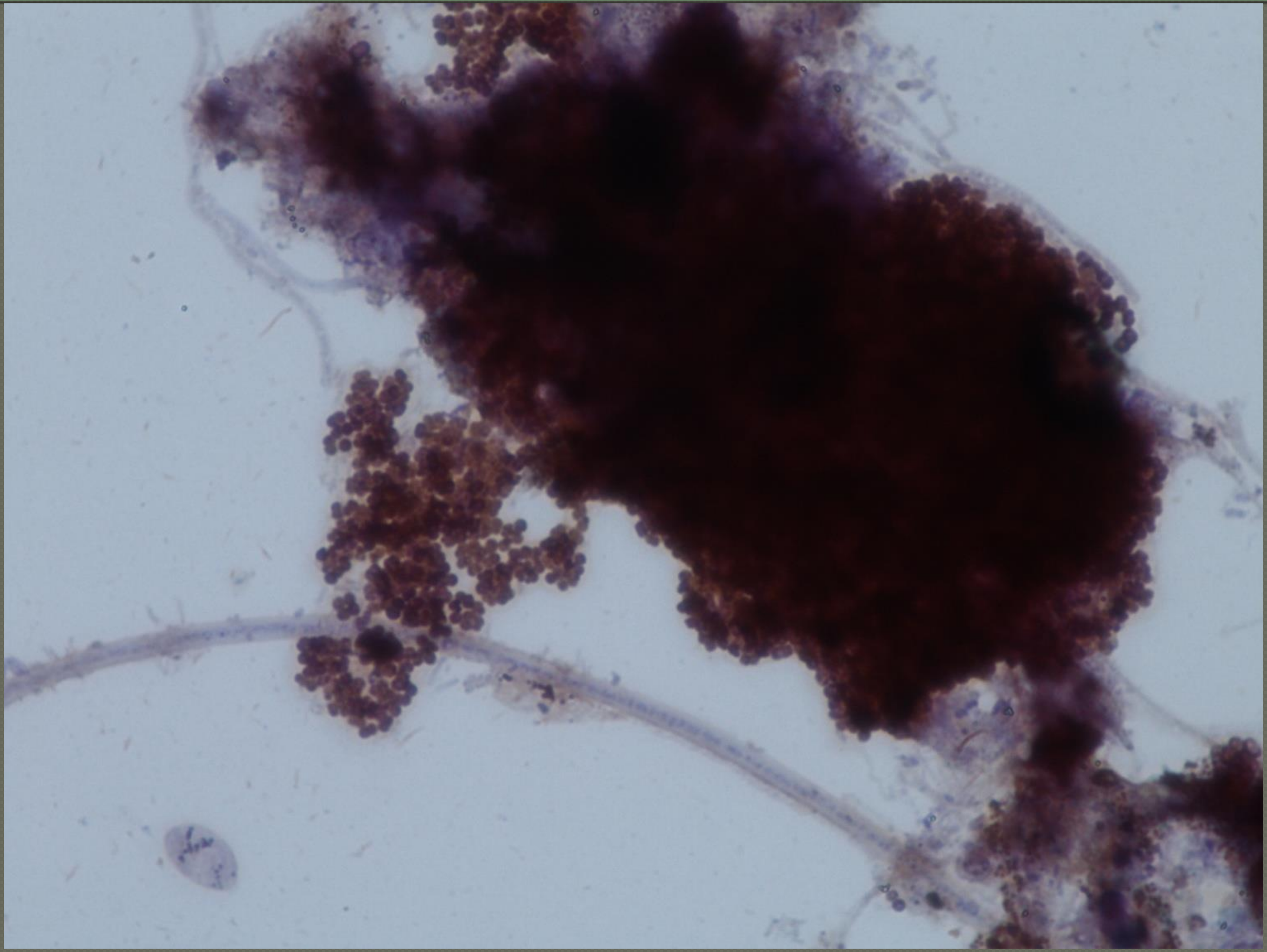
Polyphosphate Accumulating Microorganisms (PAOs)

- The active ingredient in the Neisser stain is Methylene blue which links to polymeric poly P chains, coloring them a purple-black color;
- The characteristic positive reaction of the Neisser staining method is a purple-black granule in a yellowish-brown background of the counterstained cells.



Neisser Positive (Poly-P) Granules

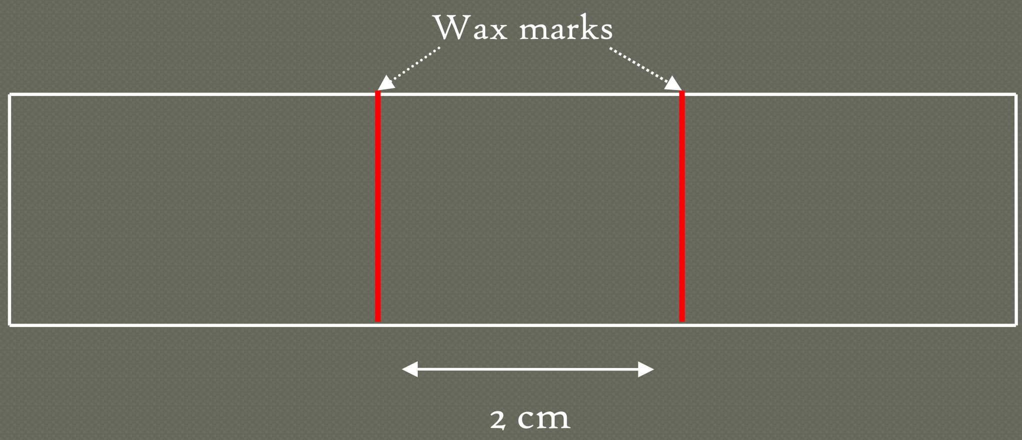
Polyphosphate Accumulating Microorganisms (PAOs)



Neisser Positive (Poly-P) Granules

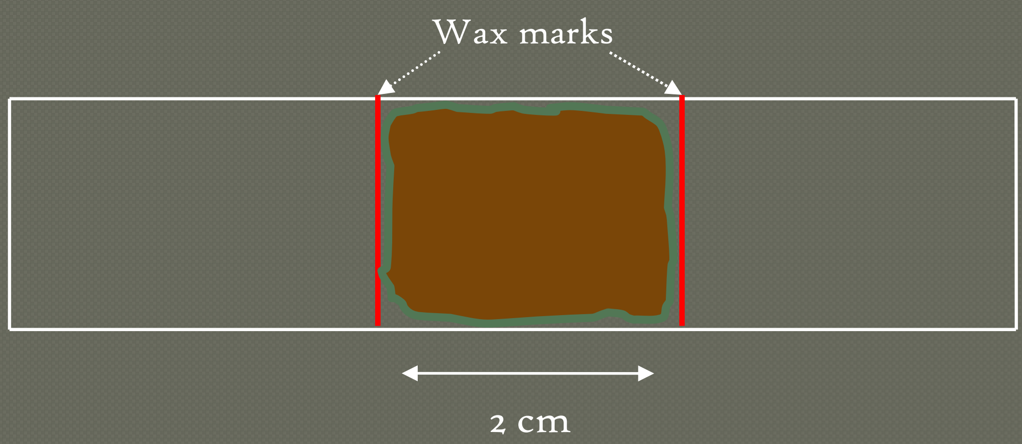
Microscopic Assessment of PAOs

- Collect a representative sample from both the end of the anaerobic zone and the end of the aerobic zone.
- Split a portion of each sample for MLVSS analysis.
- Each slide used for staining is marked with 2 lines spaced 2 cm apart using a wax pencil. The wax markings served as a border to contain and concentrate the sample within a fixed area.



Microscopic Assessment of PAOs

- Using a pipette, place 200μL of well mixed sample onto each marked slides (2 for the Sudan Black stain and 2 for the Neisser stain) and spread evenly within the marked area.
- Set aside to dry for 2 hours.



Microscopic Assessment of PAOs

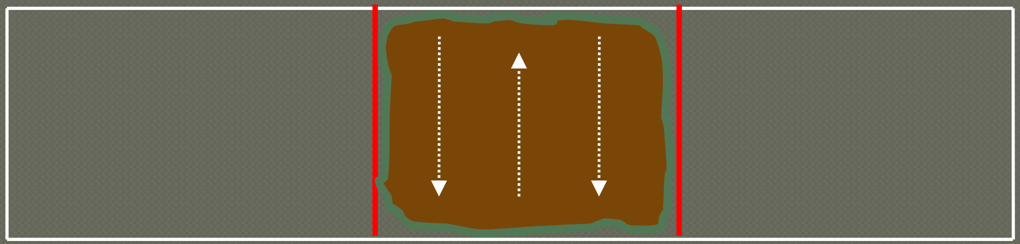
- Sudan Black Stain:
  - Solution 1 - Sudan Black B (IV), 0.3% in 60% ethanol
  - Solution 2 – Safranin O, 0.5% w/v aqueous
- Completely cover the smeared area with solution 1 and let stain for **10 minutes**. Rinse briefly with water.
- Completely cover the smeared area with solution 2 and stain for **10 seconds**. Rinse well with water and blot dry.
- Examine under oil immersion w/100x objective (do not use phase). PHB granules will appear as intracellular, blue-black granules within the cell.

Microscopic Assessment of PAOs

- Neisser Stain:
  - Solution 1 – 2 Parts Methylene Blue (95% ethanol; glacial acetic acid; distilled water) and 1 Part Crystal violet (10% w/v in 95% ethanol)
  - Solution 2 – Bismark Brown (1% w/v aqueous)
- Completely cover the smear with solution 1 and let stain for **30 seconds**. Rinse briefly with water.
- Completely cover the smear with solution 2 and let stain for **1 minute**. Rinse well and blot dry.
- 100x oil immersion; no phase; purple-blue granules

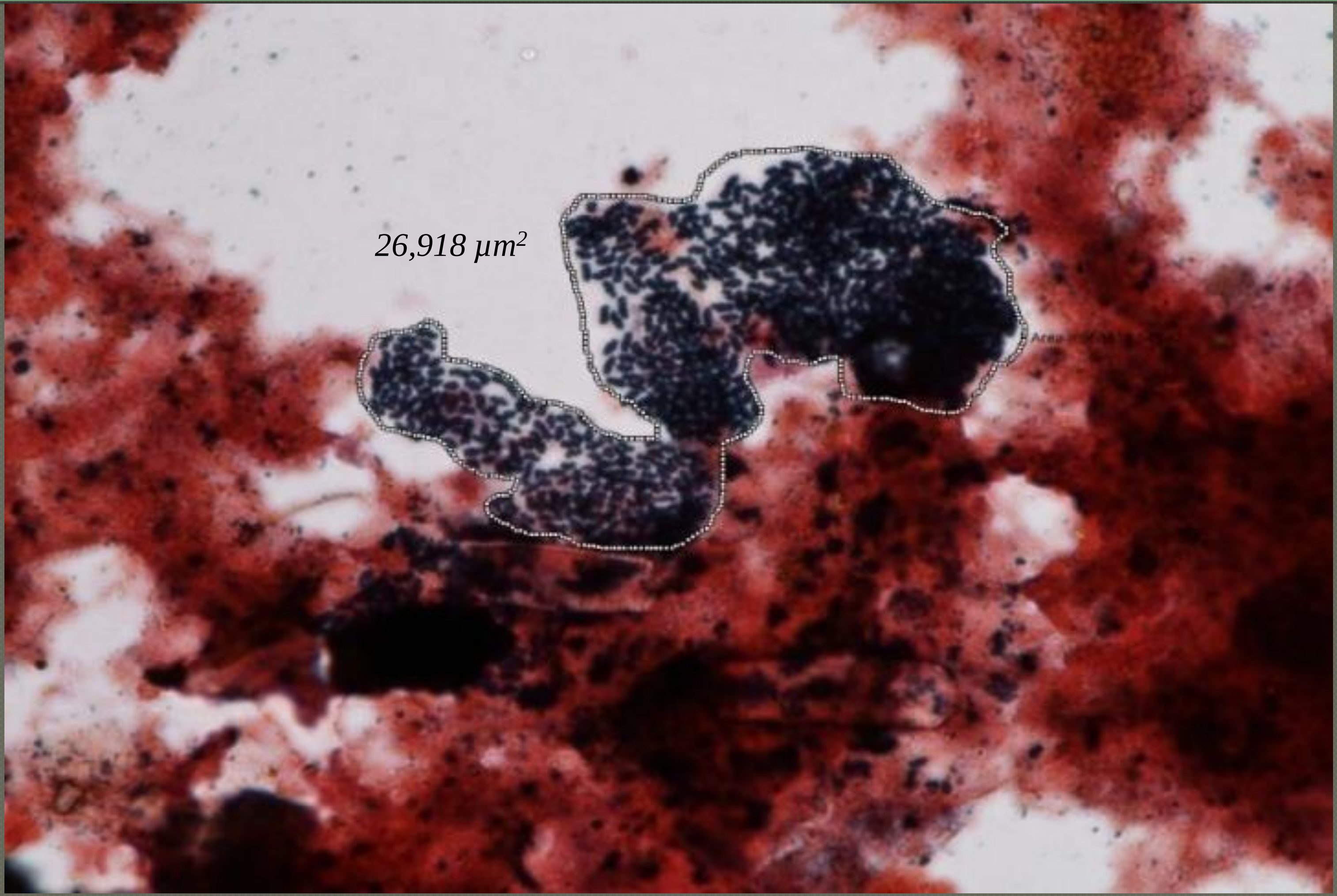
Microscopic Assessment of PAOs

- Make duplicate slides for each sample of mixed liquor and for each stain (2 slides/sample for each stain) and scan at 100X magnification using three (3) passes per slide.



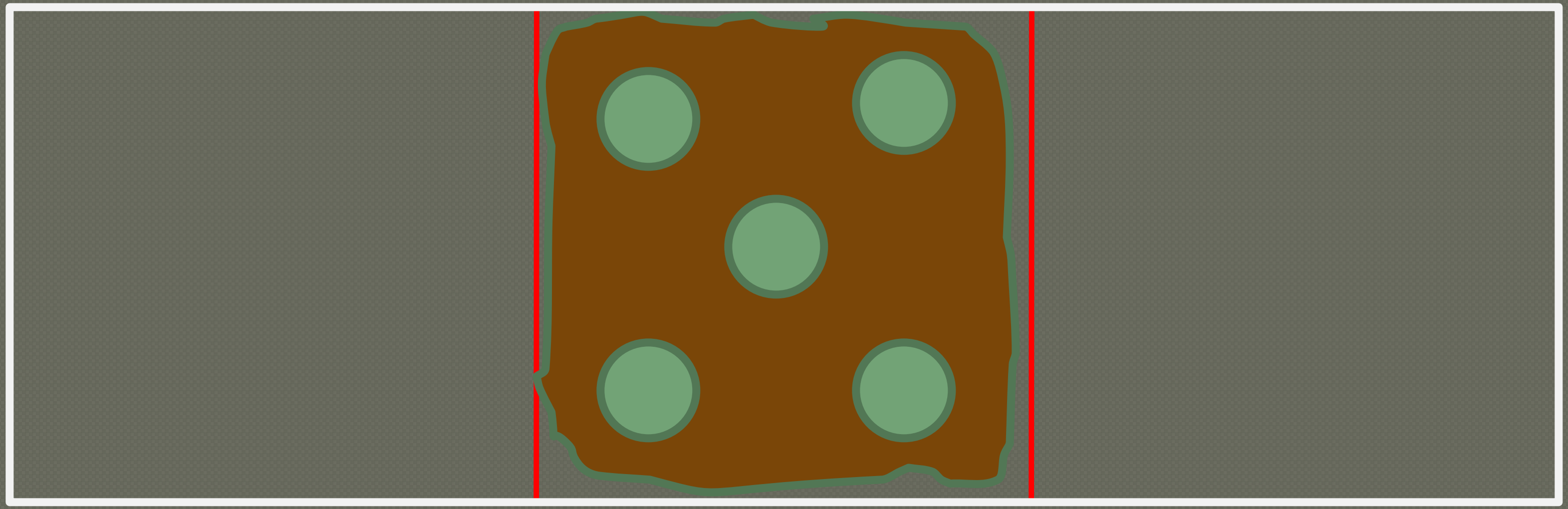
- Measure the area of each PAO cluster observed using a microscope equipped with imaging software. Determine the total area for each slide recorded in square micro-meters.

Microscopic Assessment of PAOs

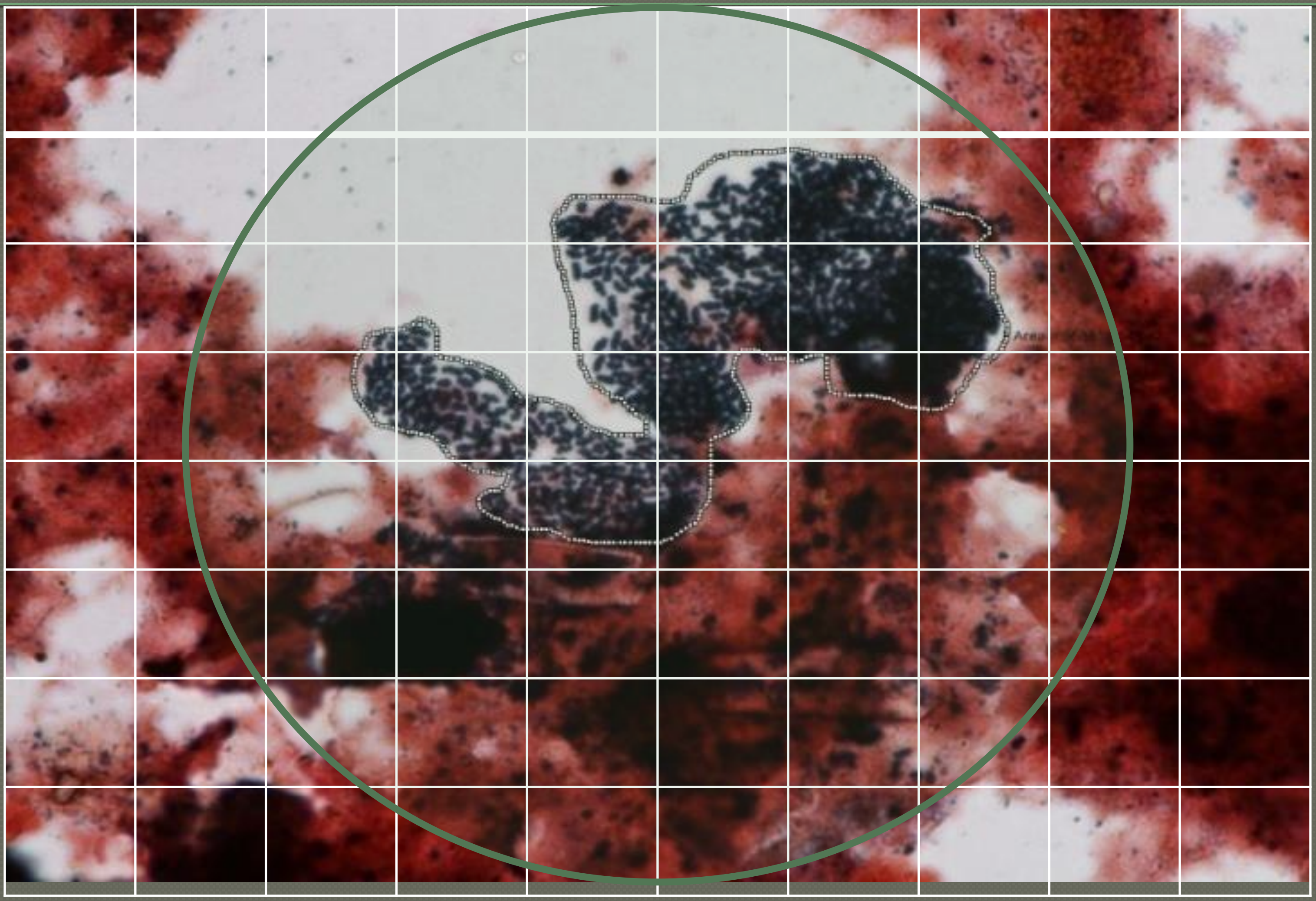


Microscopic Assessment of PAOs

- Select 5 fields and count the number of grids containing PAOs.



Microscopic Assessment of PAOs



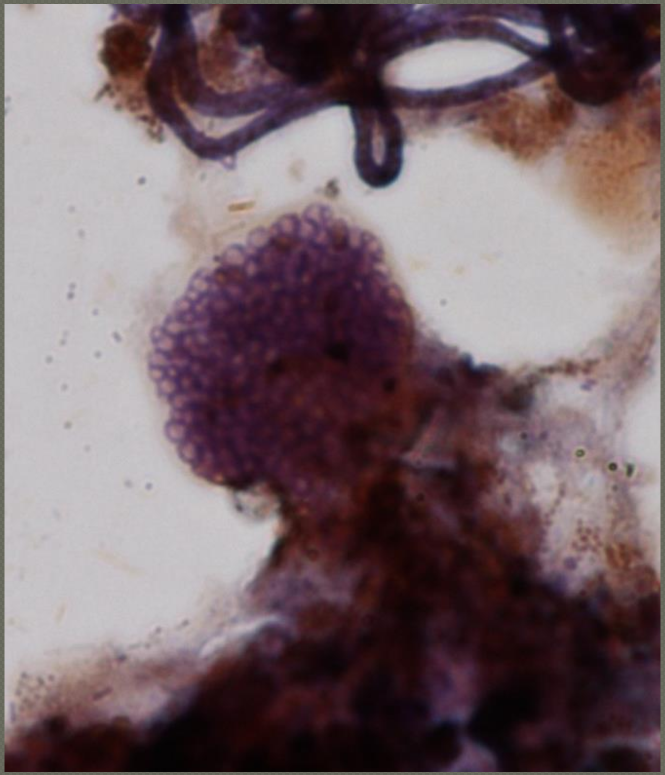
Microscopic Assessment of PAOs

- To determine the area of PAOs in one (1) mL of sample ($\mu m^2/mL$), multiply the total area by five (5).
- Total PAOs ($\mu m^2/mL$) = (Total area of PAOs /200μL) x 5
- To account for the mixed liquor concentration, the total PAOs ($\mu m^2/mL$) for each slide was divided by the MLVSS concentration and multiplied by 1000 to obtain the total area of PAOs per milligram of VSS.

- Total PAOs (μm^2)/mgVSS = $\dfrac{\text{Area of PAOs } (\mu m^2) \times 1000}{\text{MLVSS}}$

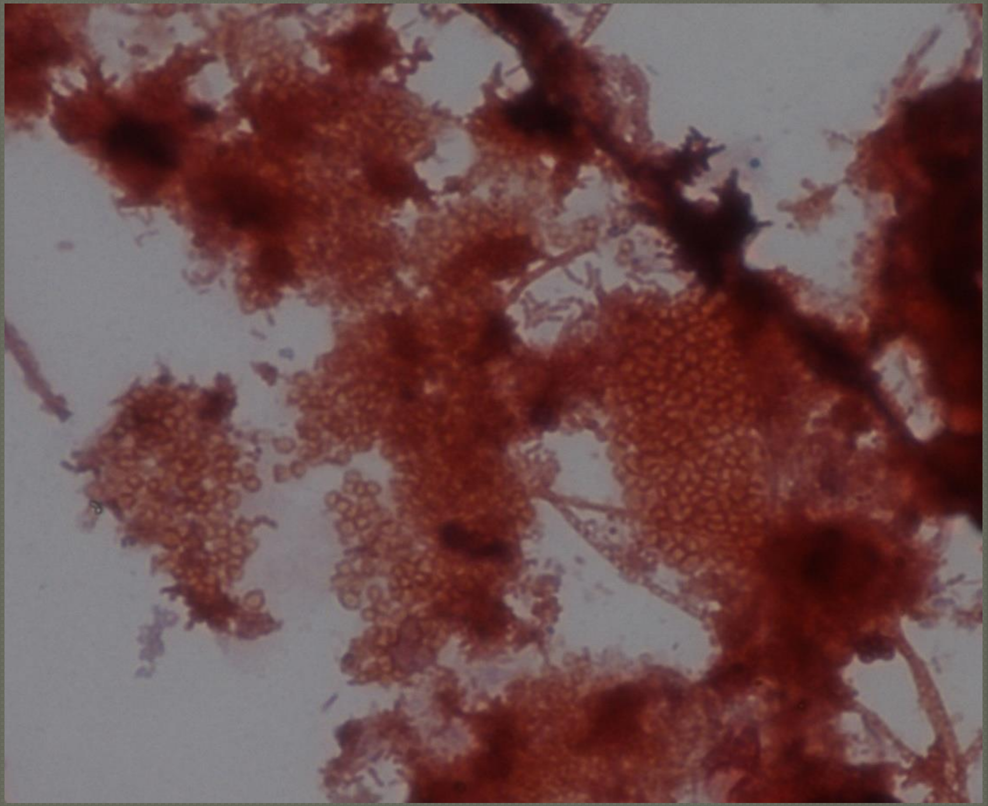
Glycogen Accumulating Microorganisms (GAOs)

- In the anaerobic zone of the EBPR system, an undesirable group of micro-organisms known as glycogen-accumulating organisms (GAOs) compete with the PAOs for VFAs.



PAOs vs GAOs

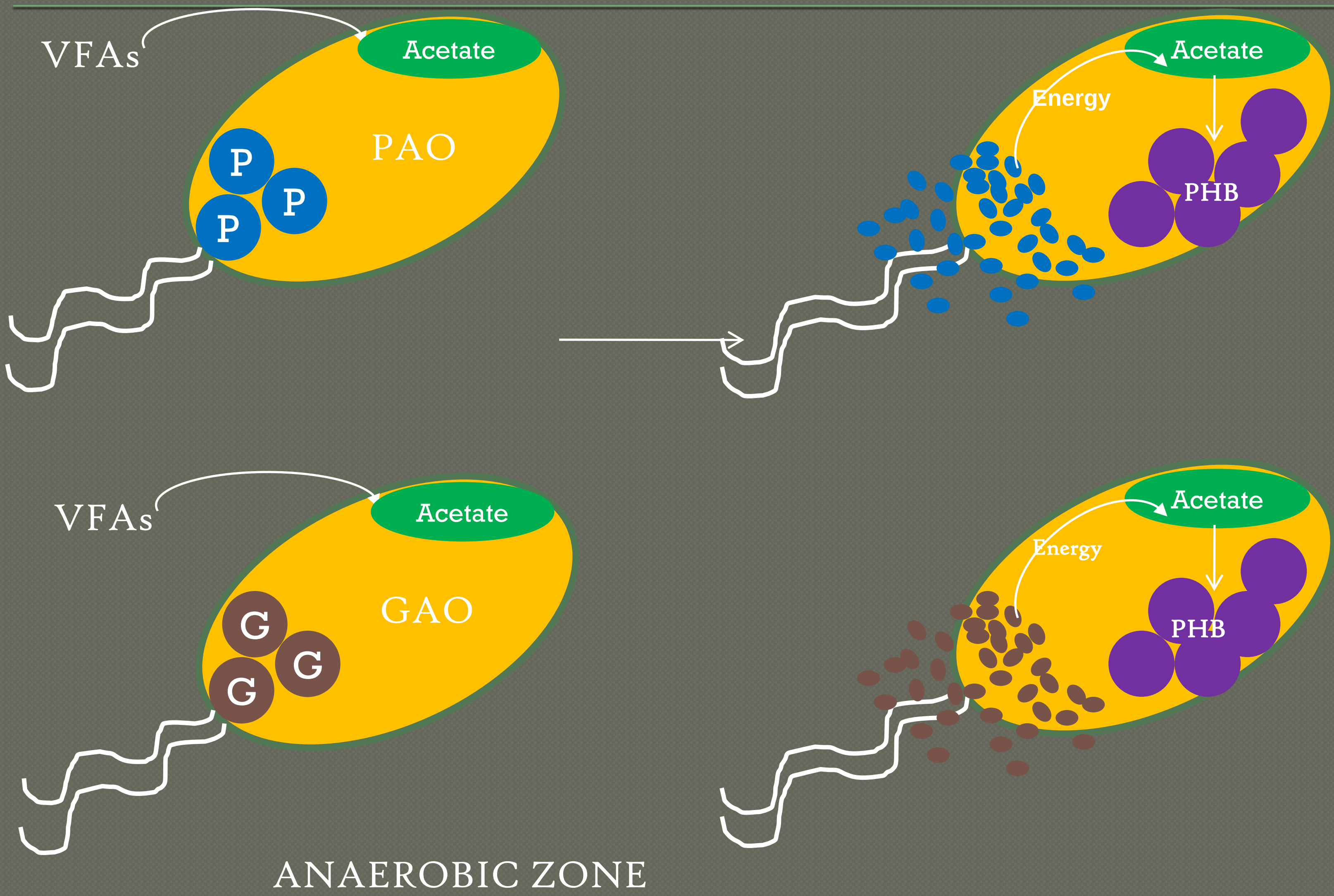
- Most GAOs are large cells with the ability to take up VFAs under anaerobic conditions, but unable to accumulate polyphosphate under the subsequent aerobic conditions.

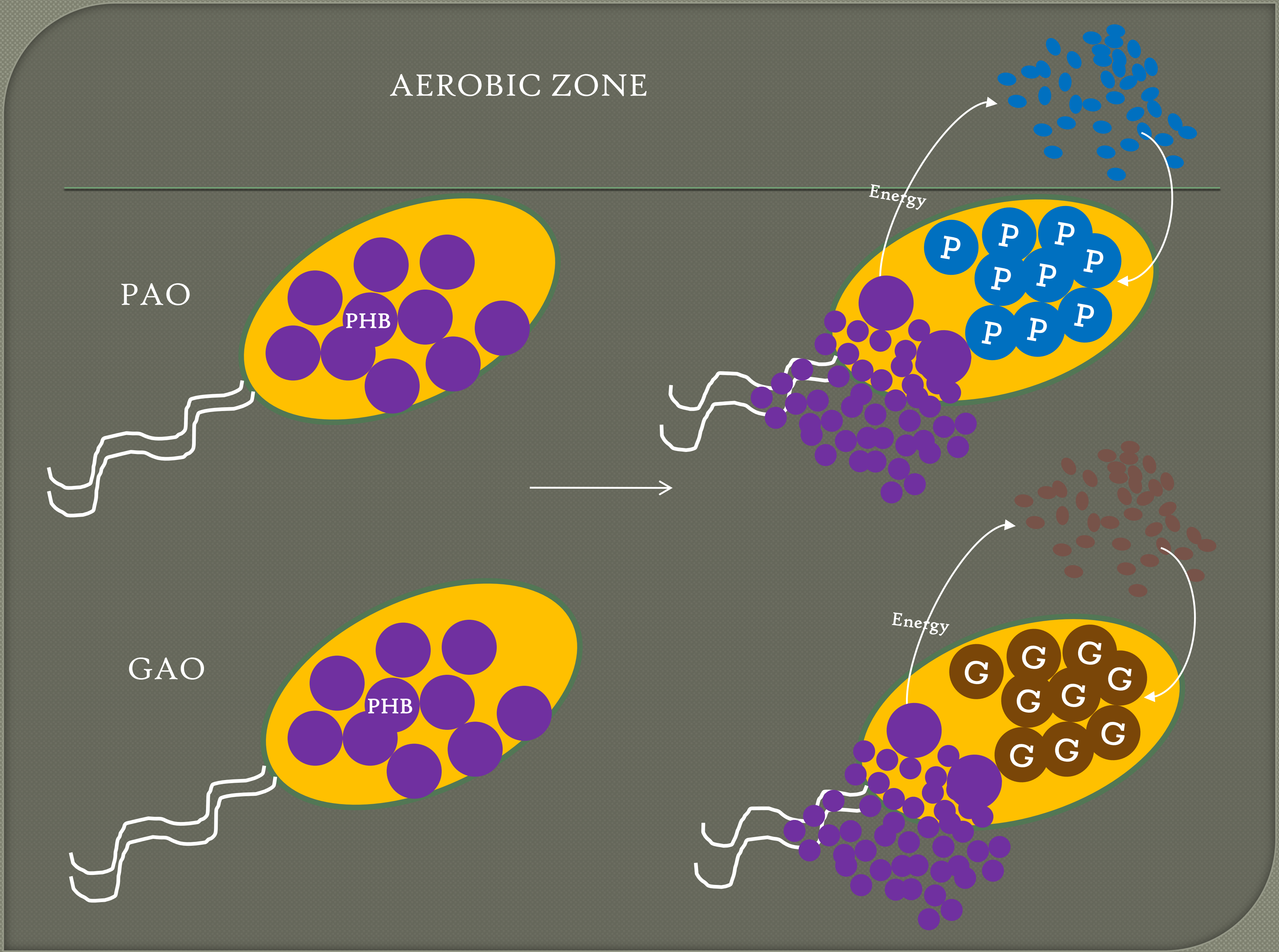


PAOs vs GAOs

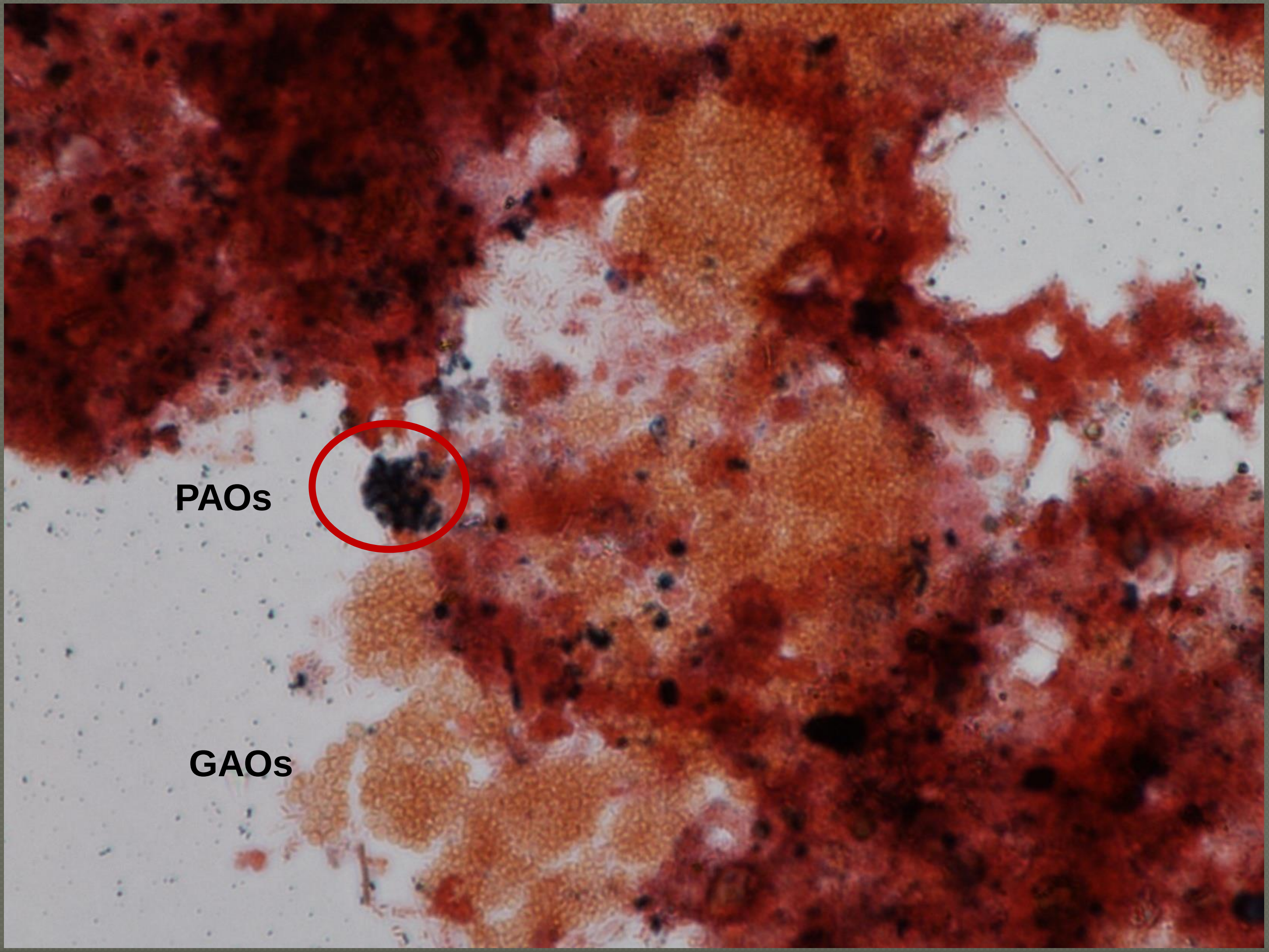
- Like PAOs, GAOs are capable of taking up the often-limited VFA substrates from EBPR systems anaerobically however, GAOs do not contribute to P removal.
- The presence of GAOs only serves to remove VFAs that would have ideally been metabolized by PAOs.
- Increasing numbers of GAOs can indicate problems in the BPR process.

PAOs VS GAOs


VFAs
Acetate
PAO
P
P
P
Acetate
Energy
PHB
VFAs
Acetate
GAO
G
G
G
Acetate
Energy
PHB
ANAEROBIC ZONE


AEROBIC ZONE
PAO
PHB
Energy
P
GAO
PHB
Energy
G

PAOs vs GAOs



GAOs VS PAOs

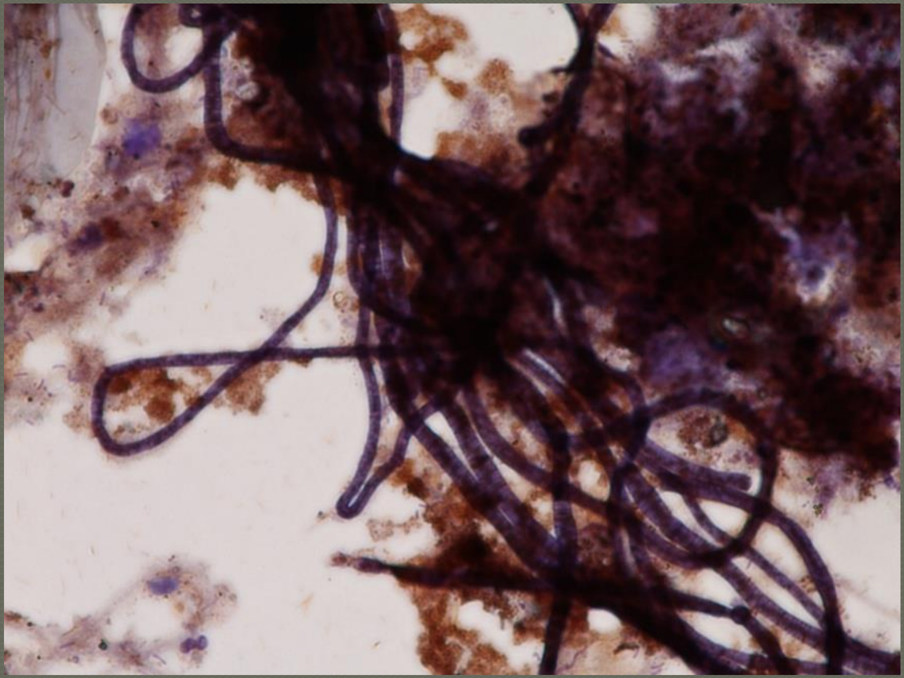
- The availability of VFAs in the anaerobic zone is critical to the success of the BPR process.
- Acetic acid and propionic acid are the dominant VFAs in domestic wastewater.
- GAOs do not grow as well on propionic acid compared to PAOs.
- Propionate may be a more suitable substrate than acetate for enhancing phosphorus removal in EBPR systems as propionate provide PAOs with a selective advantage over GAOs in the PAO–GAO competition.

GAOs VS PAOs

- Warmer temperatures stimulate GAO uptake rates more than PAO uptake rates.
- GAOs also do not grow well if the pH in the anaerobic zone is greater than 7.25
- A high operational pH has been shown to provide PAOs an advantage over GAOs.
- A pH increase from ~7 to ~8 can result in a dramatic improvement in the phosphorus removal performance due to population shifts favoring PAOs over GAOs.

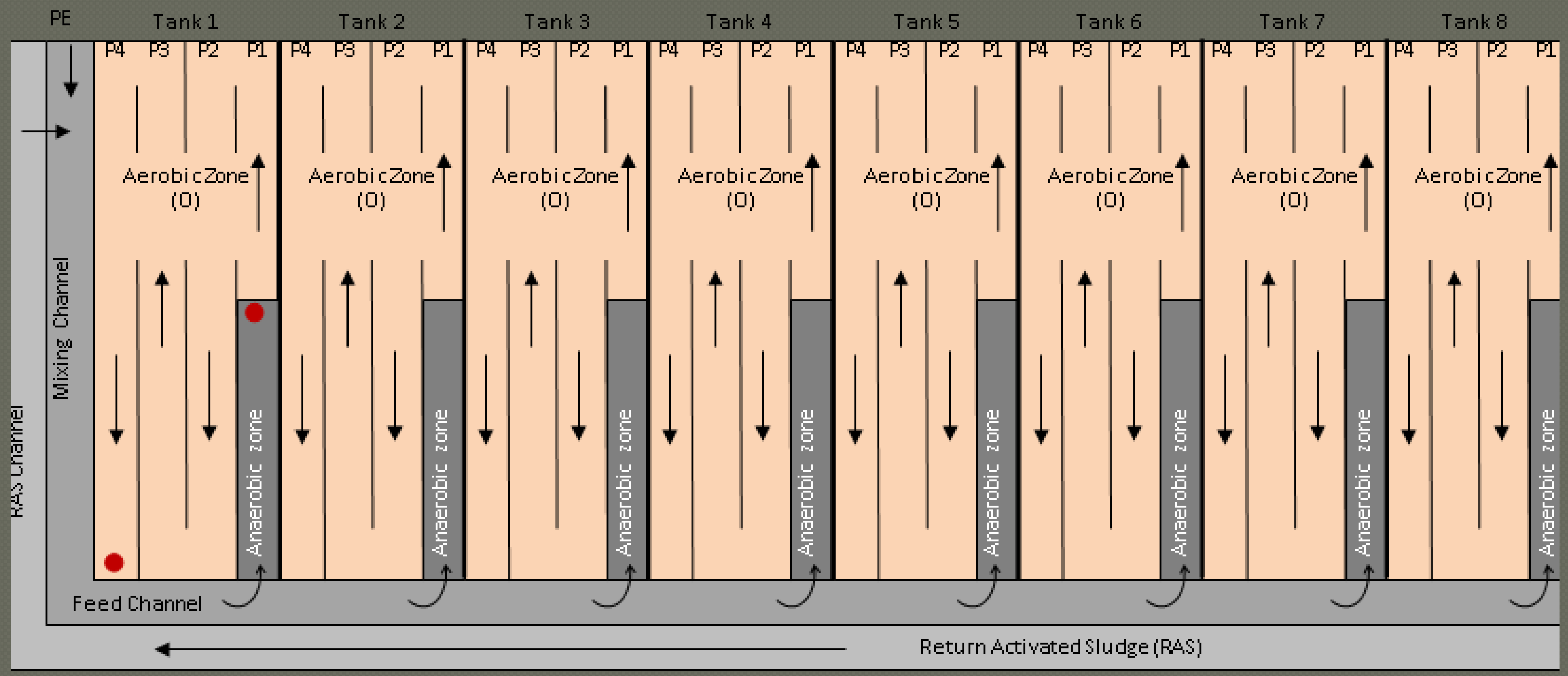
Filamentous Bacteria Concerns

- Filament type 021N increased significantly in the test battery.
- Type 021N is favored in the presence of organic acids and in low DO conditions



Case Study

Case Study



PE
Tank 1
Tank 2
Tank 3
Tank 4
Tank 5
Tank 6
Tank 7
Tank 8
P4
P3
P2
P1
Aerobic Zone
(O)
Anaerobic zone
Mixing Channel
RAS Channel
Feed Channel
Return Activated Sludge (RAS)

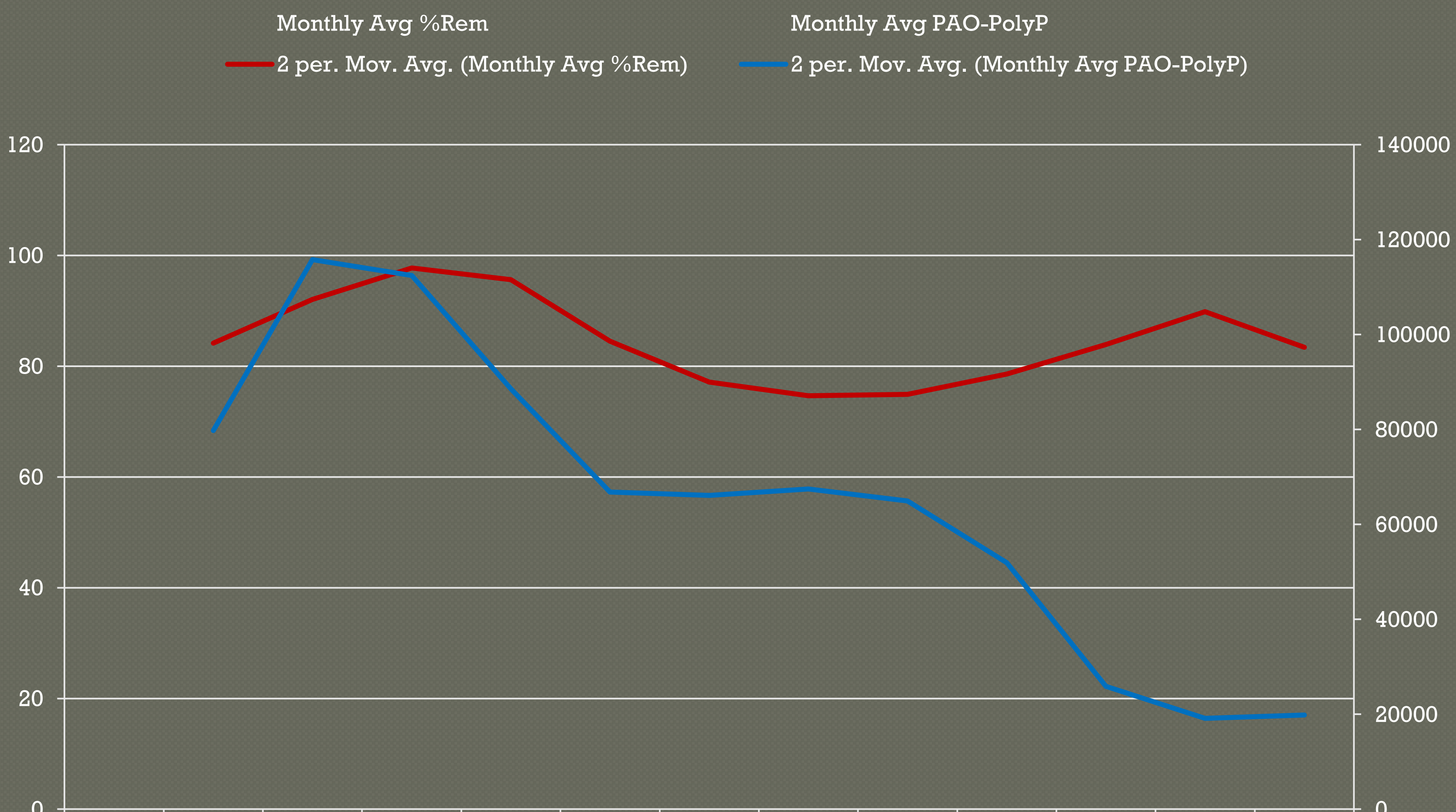
Case Study

- February 2015 - February 2016 Collected:
  - 55 samples each from anaerobic zone of all 4 batteries (Battery A, B, C & D)
  - 55 samples each from aerobic zone of all 4 batteries.
- Stained:
  - Anaerobic slides using Sudan Black (PHB)
  - Aerobic samples using Neisser
- Observed 880 slides
  - 440 anaerobic
  - 440 aerobic

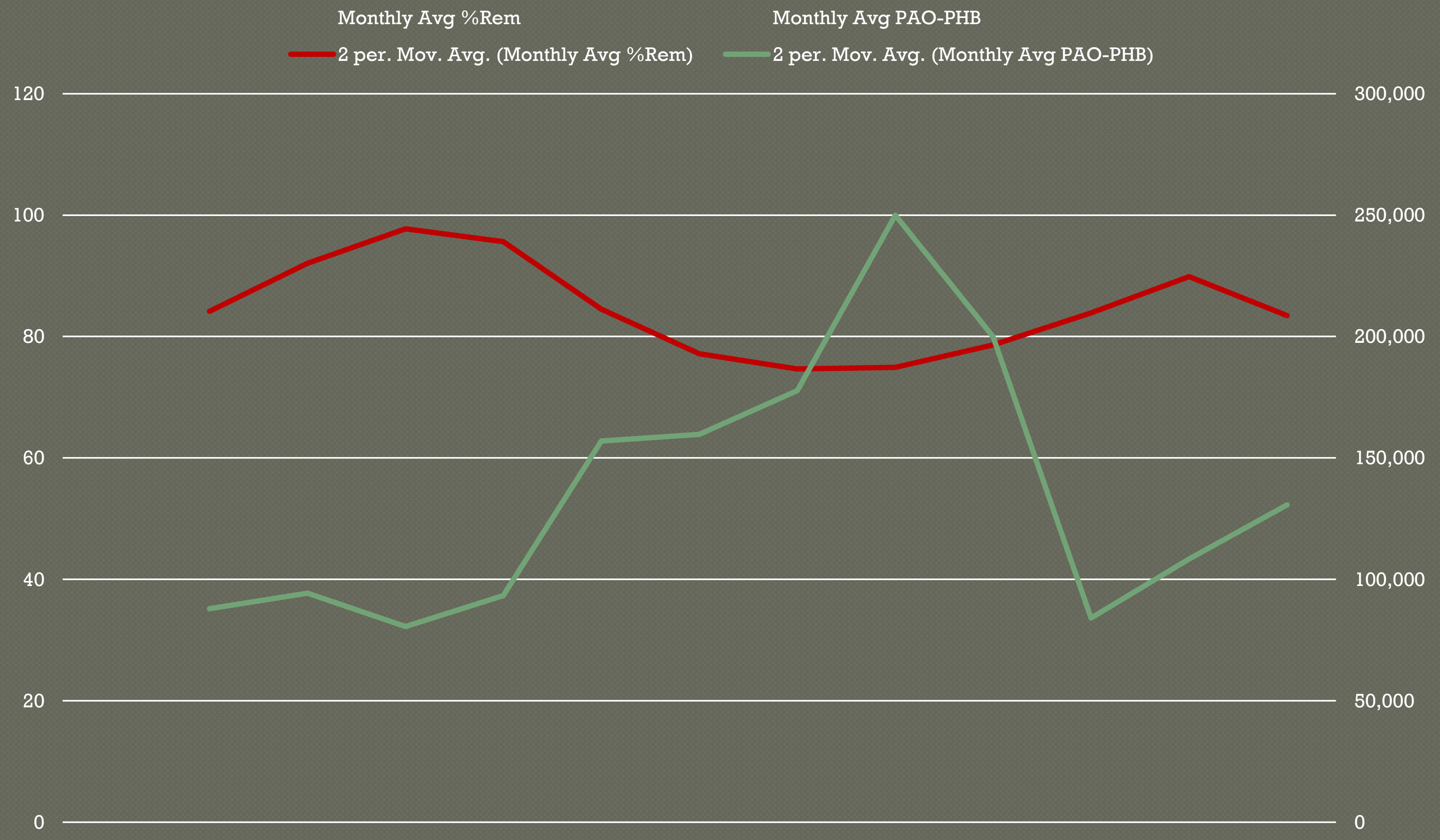
Case Study

- Results:
  - Total area of PAOs-PHB/mgVSS (anaerobic zone)
  - Total area of PAOs-PolyP/mgVSS (aerobic zone)
  - PHB:PolyP ratio
- Compared
  - % Phosphorus removal
  - Final effluent ortho-p
  - Influent BOD:Ortho-P ratio

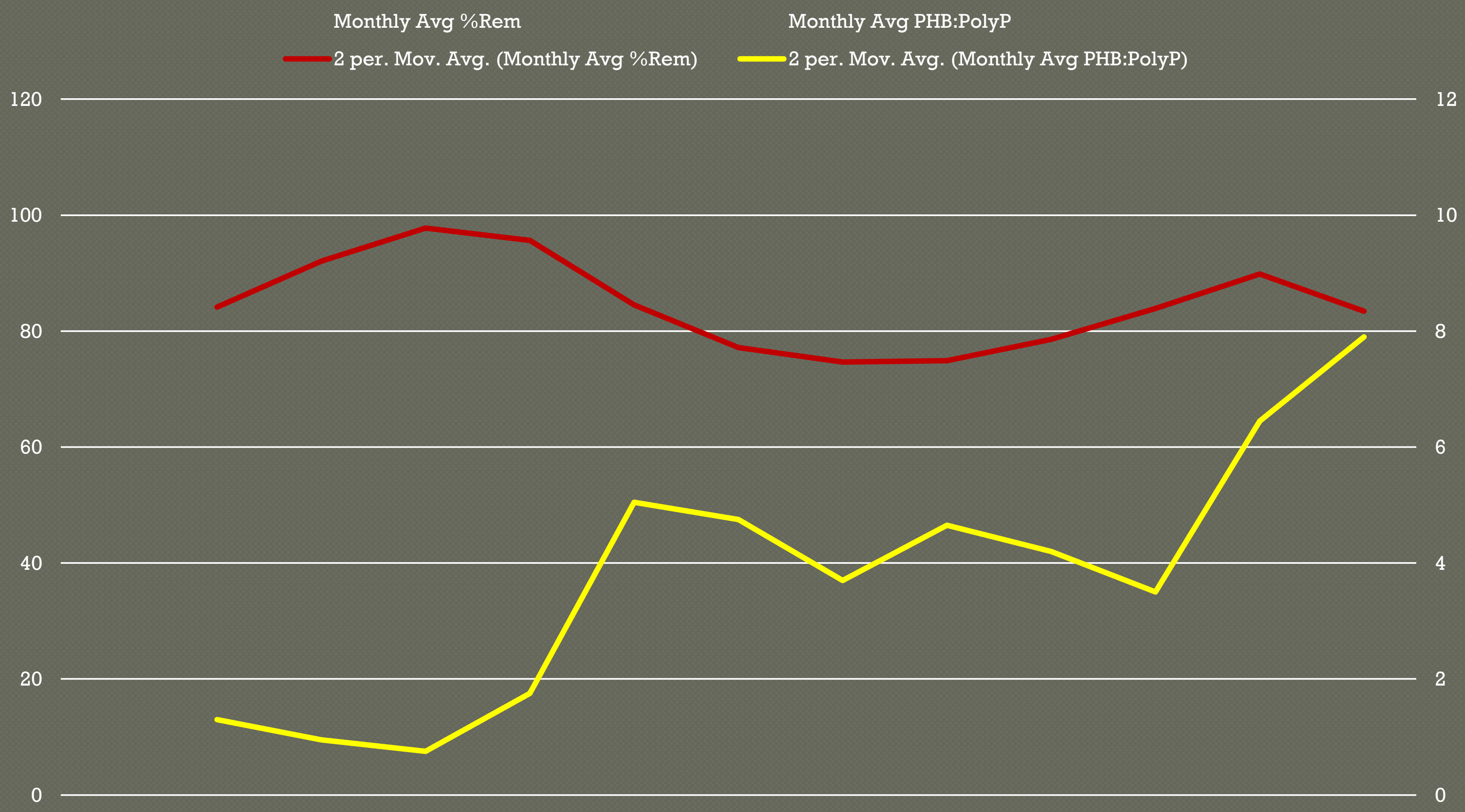
Case Study


Monthly Avg %Rem
Monthly Avg PAO-PolyP
2 per. Mov. Avg. (Monthly Avg %Rem)
2 per. Mov. Avg. (Monthly Avg PAO-PolyP)
120
100
80
60
40
20
0
140000
120000
100000
80000
60000
40000
20000
0

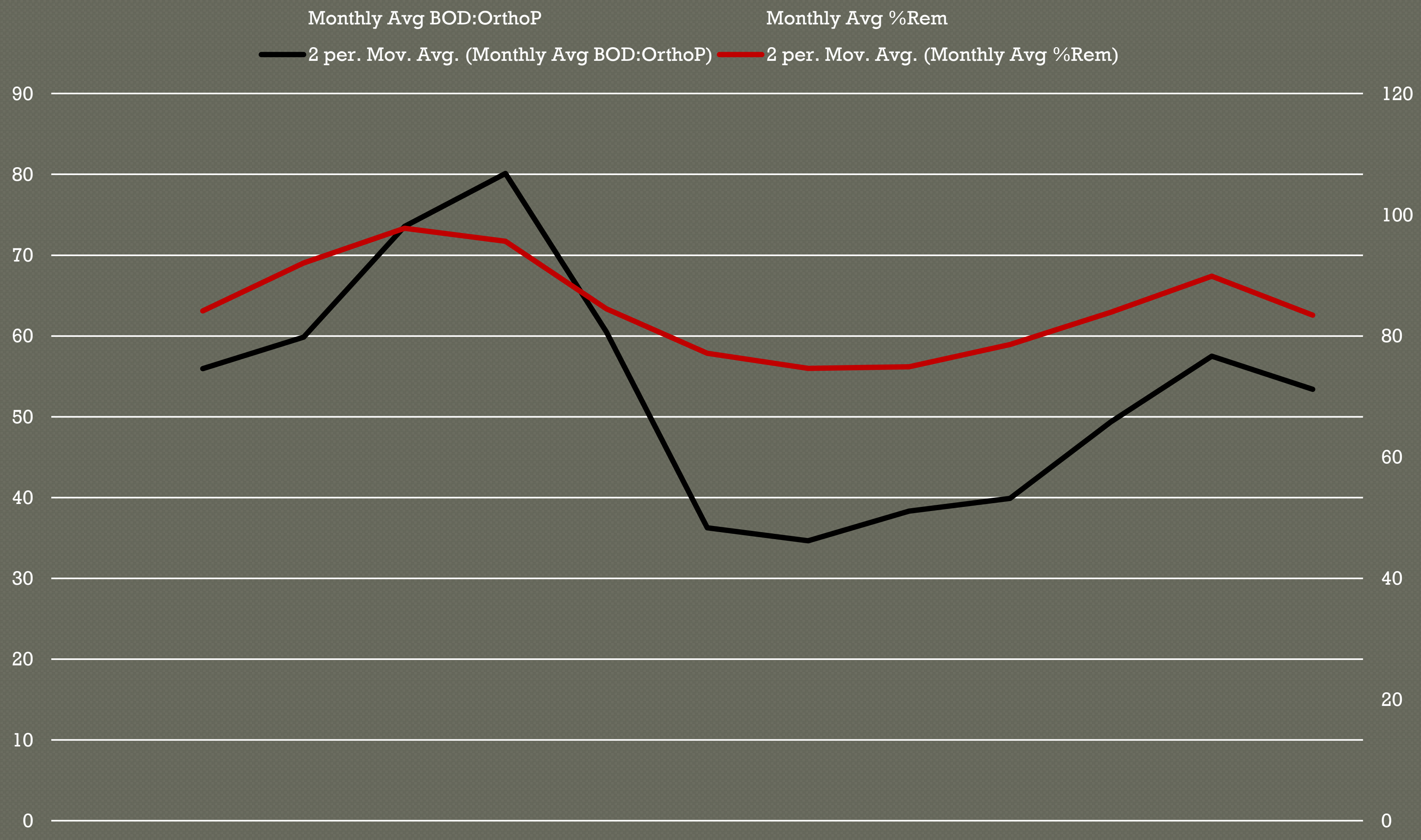
Case Study


Monthly Avg %Rem
Monthly Avg PAO-PHB
2 per. Mov. Avg. (Monthly Avg %Rem)
2 per. Mov. Avg. (Monthly Avg PAO-PHB)
120
100
80
60
40
20
0
300,000
250,000
200,000
150,000
100,000
50,000
0

Case Study

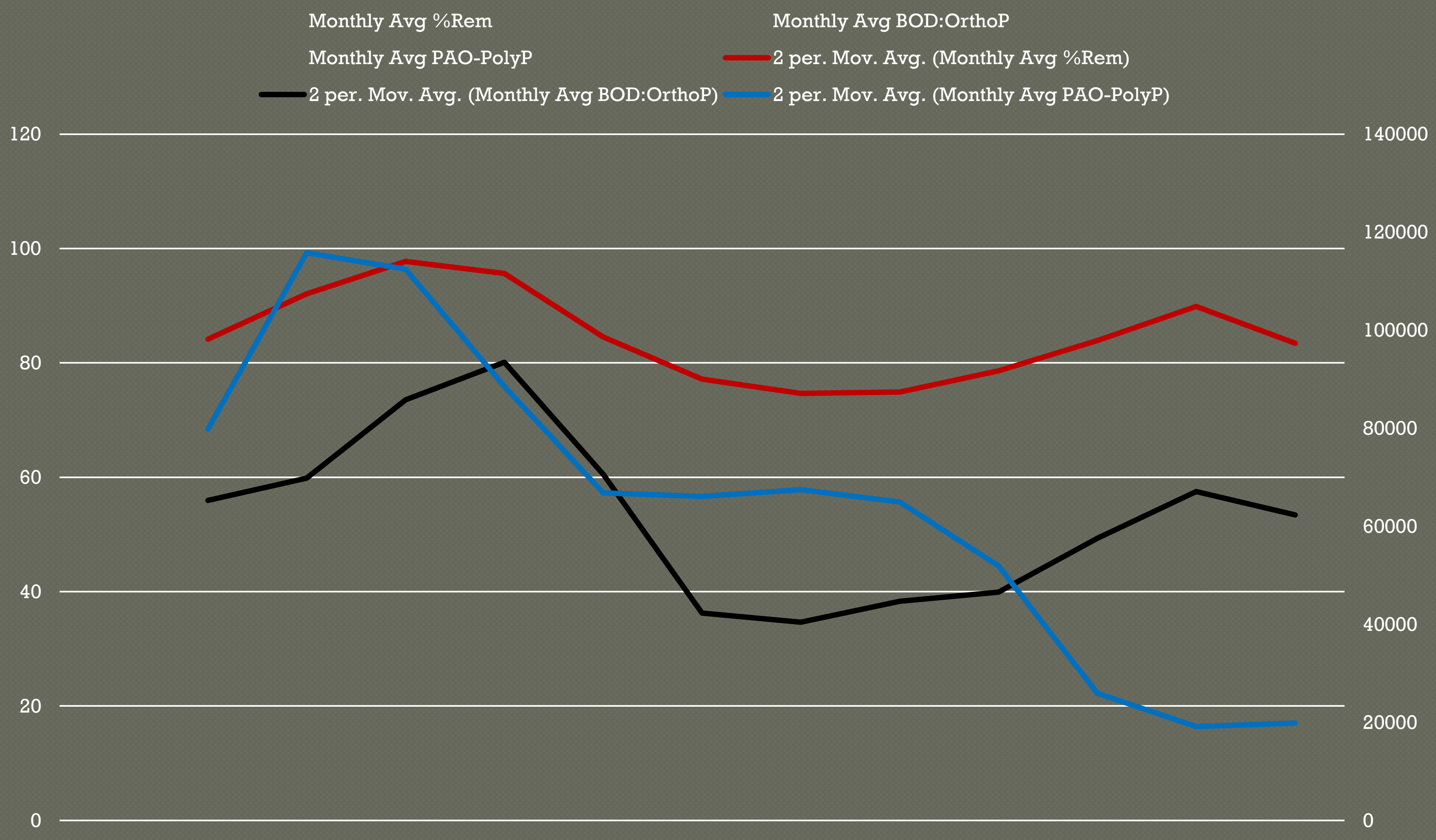

Monthly Avg %Rem
Monthly Avg PHB:PolyP
2 per. Mov. Avg. (Monthly Avg %Rem)
2 per. Mov. Avg. (Monthly Avg PHB:PolyP)
120
100
80
60
40
20
0
12
10
8
6
4
2
0

Case Study

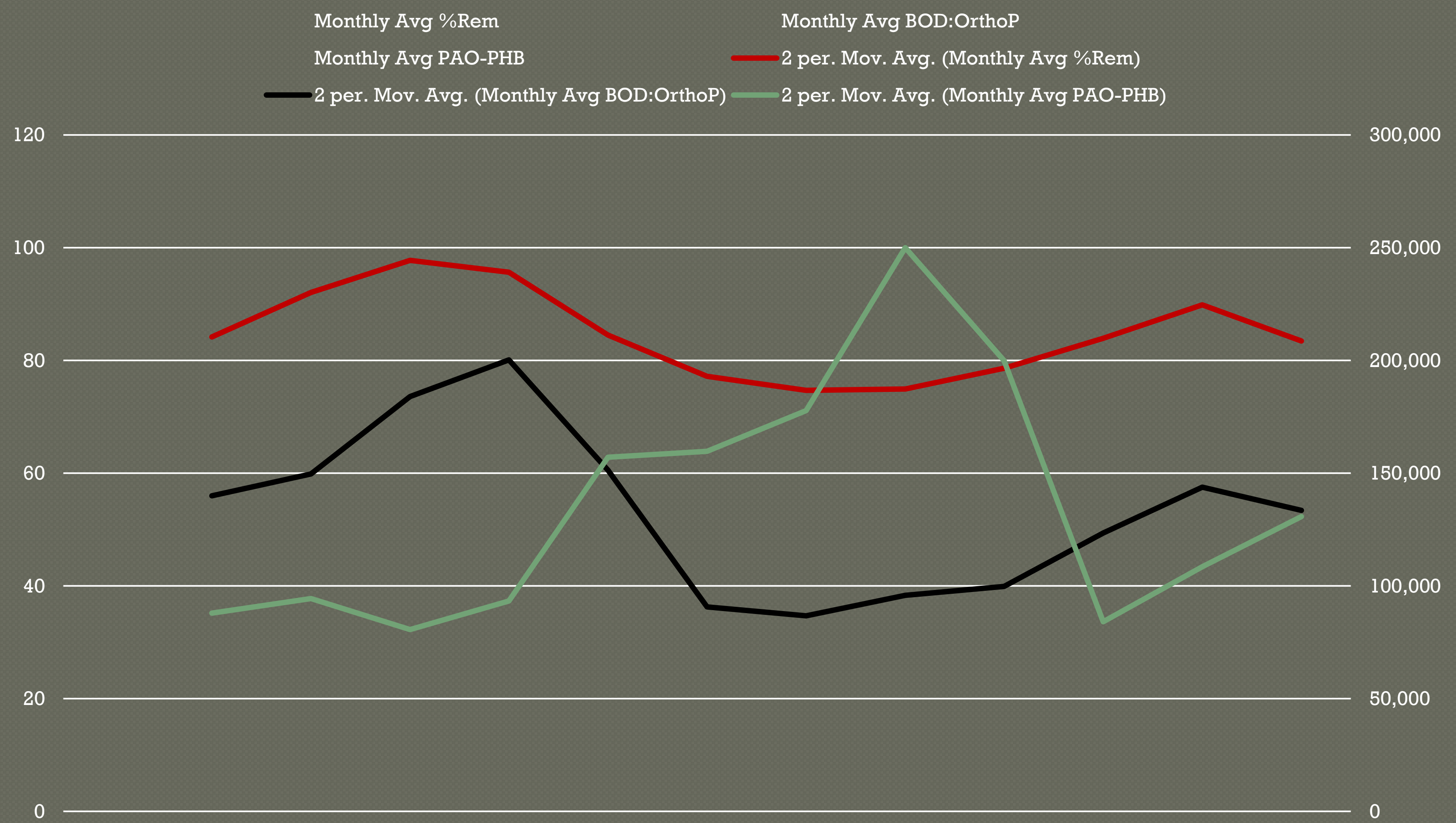


Monthly Avg BOD:OrthoP
Monthly Avg %Rem
2 per. Mov. Avg. (Monthly Avg BOD:OrthoP)
2 per. Mov. Avg. (Monthly Avg %Rem)
90
80
70
60
50
40
30
20
10
0
120
100
80
60
40
20
0

Case Study


Monthly Avg %Rem
Monthly Avg BOD:OrthoP
Monthly Avg PAO-PolyP
2 per. Mov. Avg. (Monthly Avg %Rem)
2 per. Mov. Avg. (Monthly Avg BOD:OrthoP)
2 per. Mov. Avg. (Monthly Avg PAO-PolyP)
120
100
80
60
40
20
0
140000
120000
100000
80000
60000
40000
20000
0

Case Study

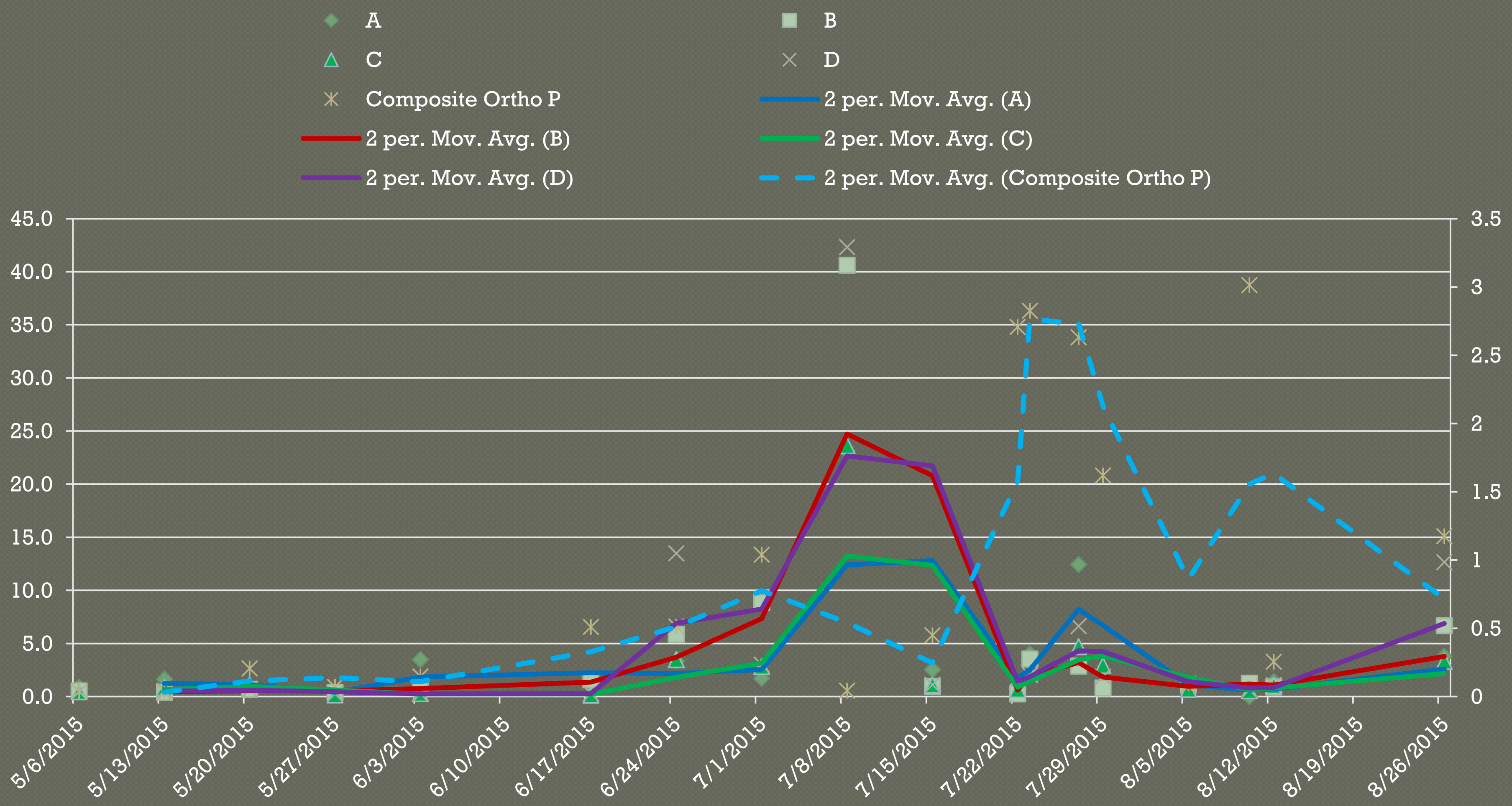


Monthly Avg %Rem
Monthly Avg BOD:OrthoP
Monthly Avg PAO-PHB
2 per. Mov. Avg. (Monthly Avg %Rem)
2 per. Mov. Avg. (Monthly Avg BOD:OrthoP)
2 per. Mov. Avg. (Monthly Avg PAO-PHB)
120
100
80
60
40
20
0
300,000
250,000
200,000
150,000
100,000
50,000
0

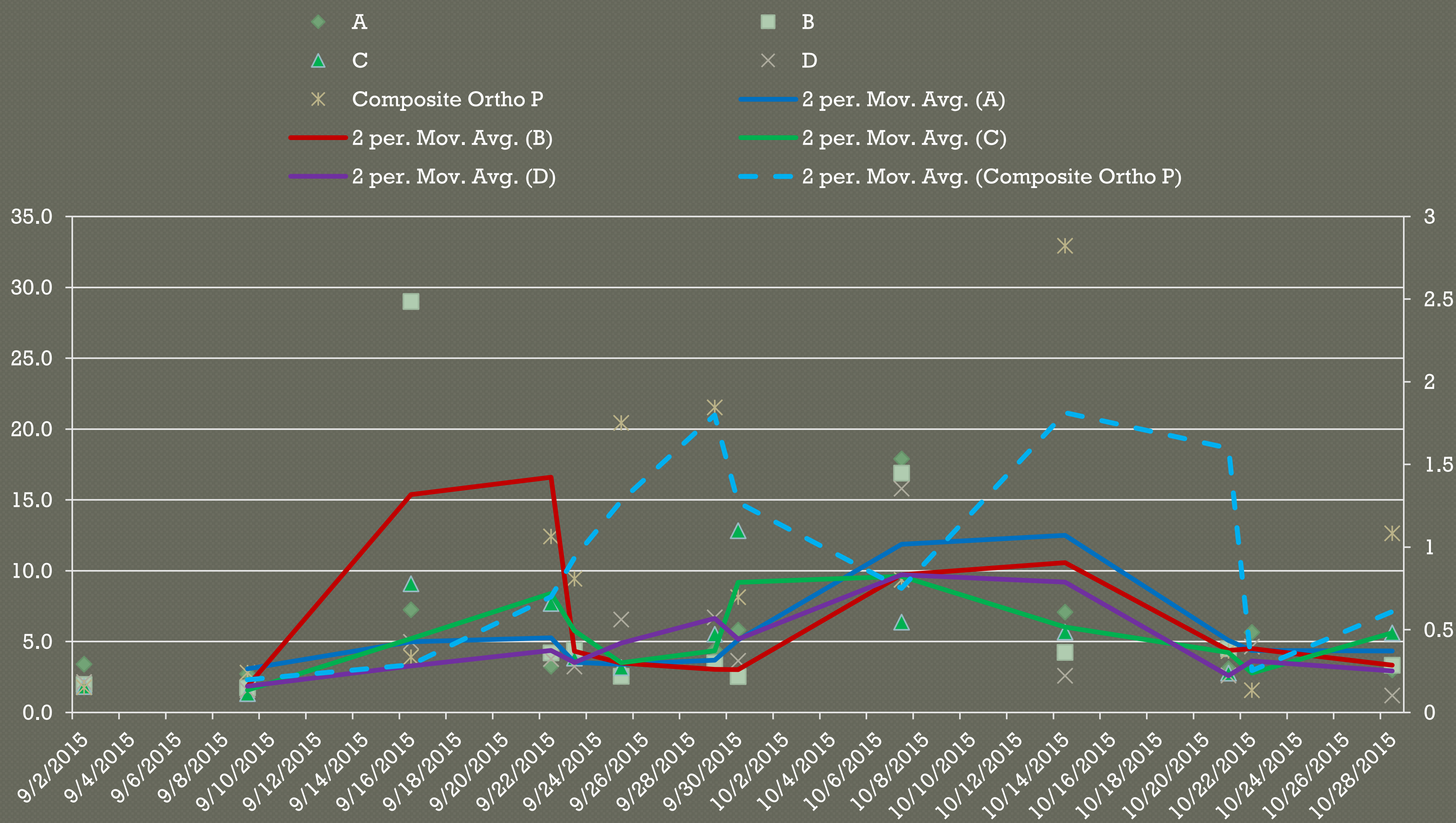
Case Study

- Some observations
  - Percent removal is directly related to influent BOD:Ortho-P ratio
  - PAOs-PolyP abundance is directly related to percent removal and influent BOD:Ortho-P ratio
  - PAOs-PHB is inversely related to percent removal and influent BOD:Ortho-P ratio
  - The closer the PHB:PolyP ratio is to 1, the better the performance

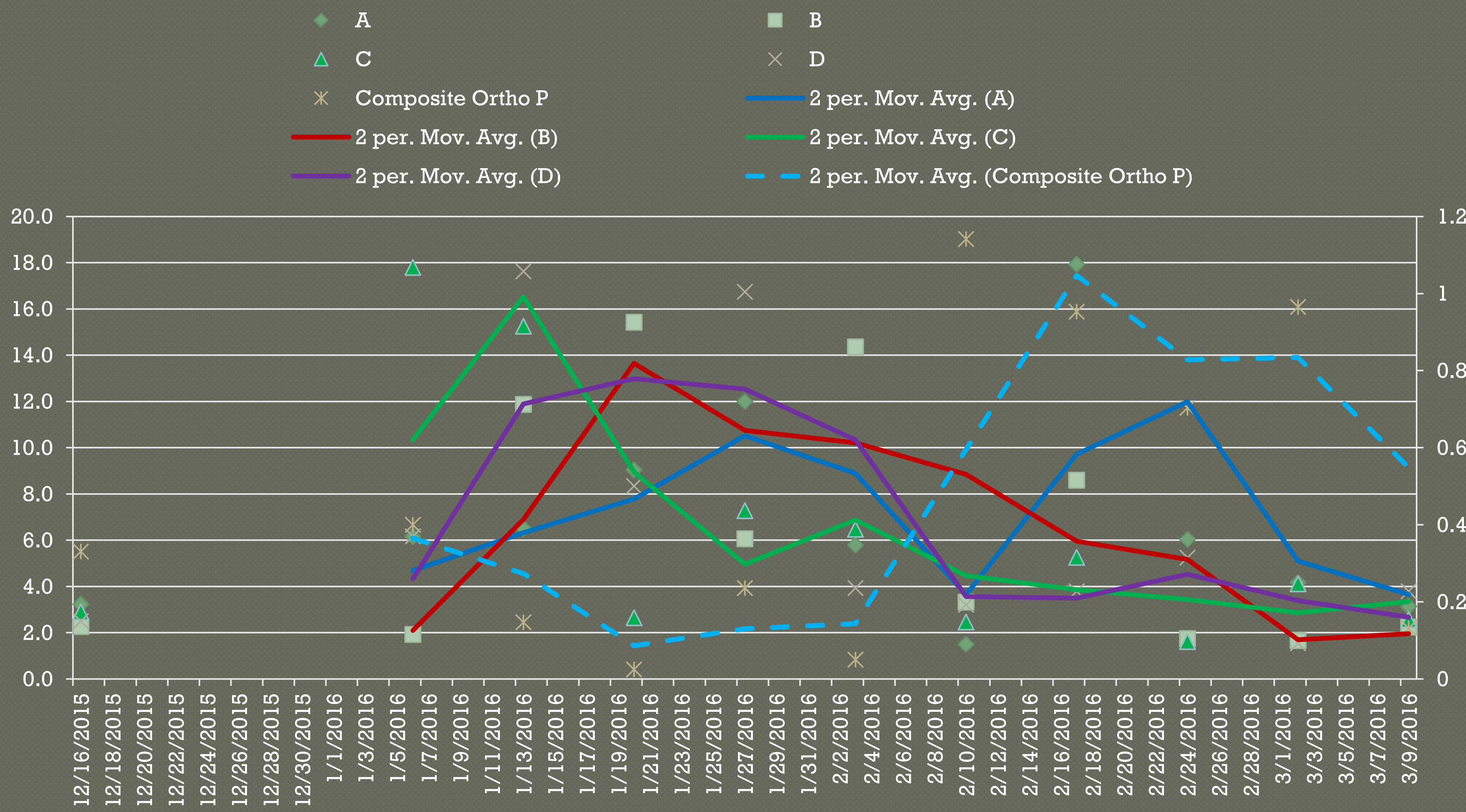
PHB:Poly-P Ratio & Outfall Ortho P



PHB:Poly-P Ratio & Outfall Ortho P



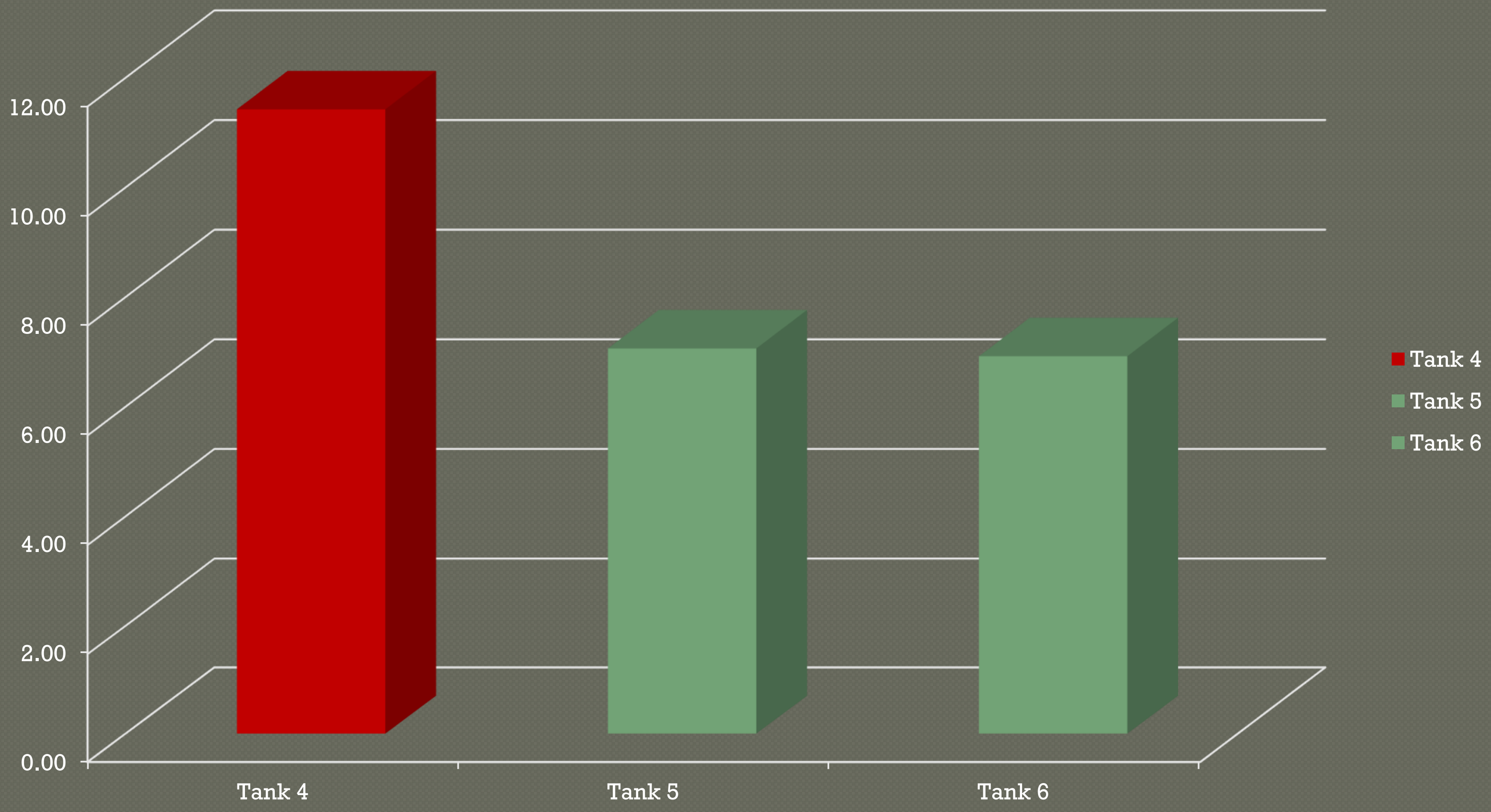
PHB:Poly-P Ratio & Outfall Ortho P



PHB:POLY-P RATIO

- The PHB:Poly-P ratio is a better indicator of EBPR system conditions
- 75 genera of bacteria that store PHB but not all can uptake phosphorus
- High PHB with low Poly-P (high PHB:PolyP ratio) indicates that other PHB storing microbes are competing for VFAs besides PAOs.

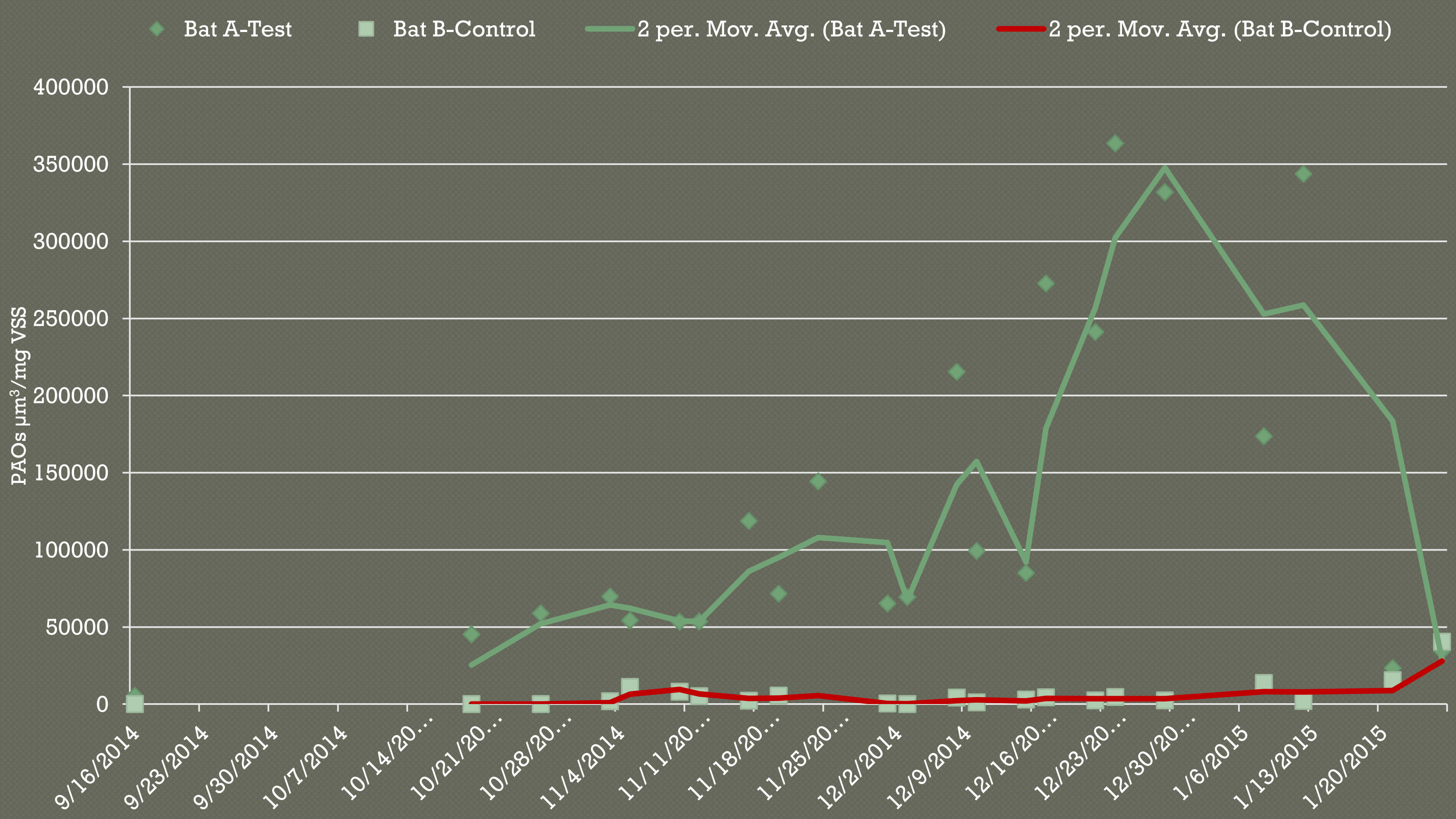
Case Study


12.00
10.00
8.00
6.00
4.00
2.00
0.00
Tank 4
Tank 5
Tank 6
Tank 4
Tank 5
Tank 6

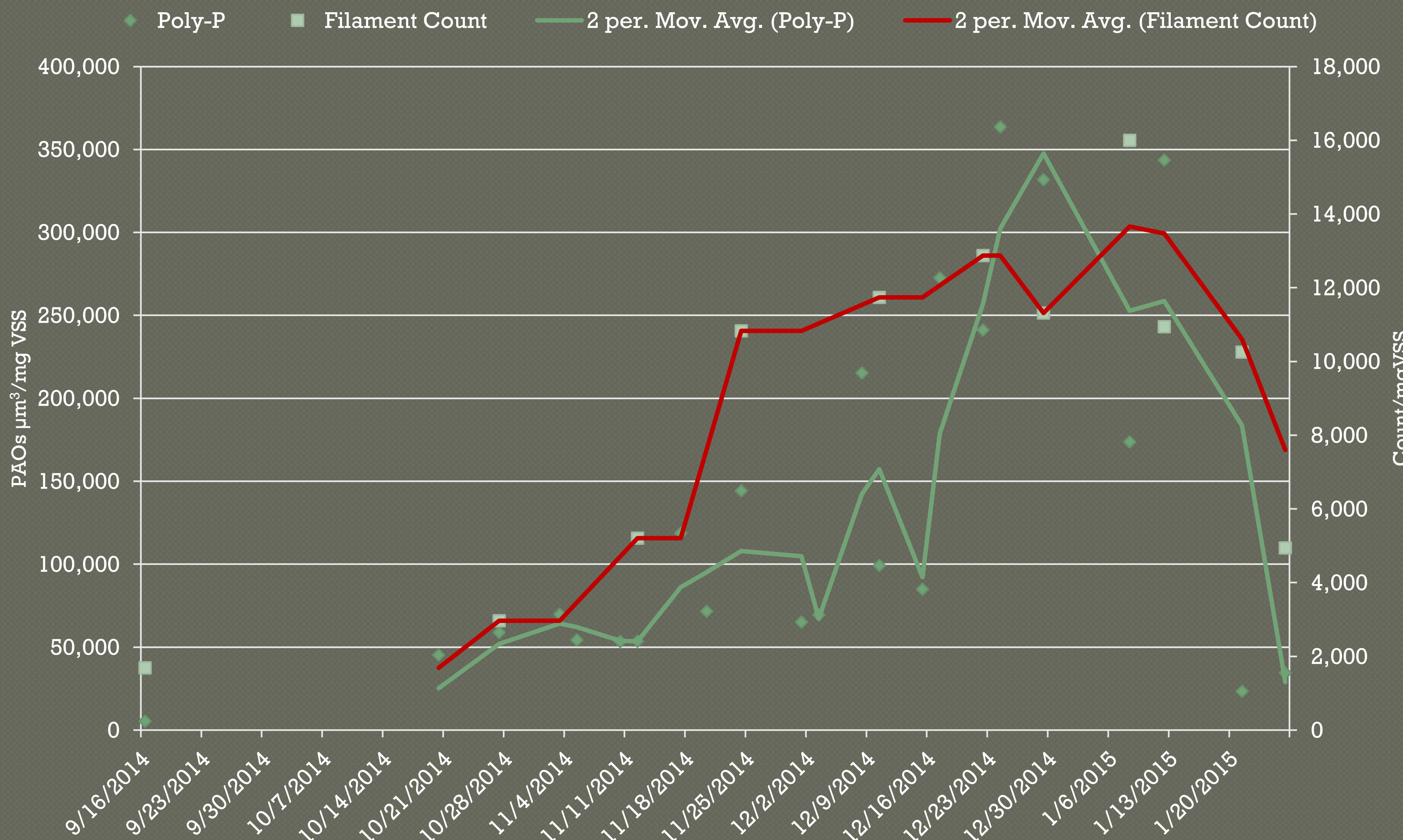
Carbon Supplements

- Influent Carbon concentrations did not meet design criteria
- External carbon source, MicroC 2000™ to address carbon deficit
- Glycerin-based, derived from renewable resources

PAOs with Carbon Addition



PAOs with Carbon Addition



Biological Nutrient Removal

- Biological nutrient removal is not going away!
- Biological nutrient removal systems require a little more skill than conventional activated sludge treatment processes.
- Microscopic assessment is just another tool in the toolbox.

ANY QUESTIONS ?