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DETERMINATION OF PHOSPHATE ACCUMULATING ORGANISM ACCUMULATION IN SEWAGE SLUDGE OF WASTEWATER

Master's Thesis (30 ECTS)

Curriculum Bioengineering

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Tartu 2023

DETERMINATION OF PHOSPHATE ACCUMULATING ORGANISM ACCUMULATION IN SEWAGE SLUDGE OF WASTEWATER

Abstract:

Phosphate-accumulating organisms (PAOs) are diverse bacteria that retain phosphate as polyphosphate. These bacteria play an essential role in various water treatment plants because they are used effectively to remove a proportion of the phosphorus in wastewater. Wastewater treatment plants (WWTPs) are vital in today's world, and the significance of phosphate in our lives and its relationship with these bioorganisms formed the main idea of this project. These plants use physical, chemical or biological methods to treat wastes in the water, and the abundance of these organisms will differ as each treatment plant has different management and environmental conditions. Therefore, in this study, biological detection of these organisms and a comparison of their abundance in several different wastewater treatment plants were carried out. Comparisons using a confocal microscope showed the same results trend when samples that had already been chemically measured were observed under the microscope stained with DAPI (4',6-Diamidino-2-phenylindole). The confocal microscope was successfully utilized to detect these organisms in the sludge in the wastewater. The comparison of these treatment plants regarding the abundance of these organisms and flow cytometry data was successfully performed.

Keywords:

Phosphate accumulating organisms, Wastewater treatment plants, DAPI, detection

CERCS:

B230 Microbiology

Kokkuvõte:

Fosfaati akumuleerivad organismid (PAO) on bakterid, mis säilitavad fosfaadi polüfosfaadina.Nendel organismidel on oluline roll erinevates veepuhastusjaamades, kuna neid saab efektiivselt kasutatda reovees oleva fosfori eemaldamiseks. Reovee puhastusjaamad (WWPT) koos selles uuringus/projektis kasutatud bioorganismidega on tänapäeva maailmas üliolulisel kohal ning seetõttu moodustas ka nendevaheline suhe selle projekti põhiidee. Reovee puhastusjaamad kasutavad nii füüsilisi, keemilisi kui ka bioloogilisi meetodeid, et reovett puhastada. Nende organismide arvukus/hulk on puhastusjaamades erinev, kuna iga puhastusseadme kasutusviis ja keskkonnatingimused varieeruvad. Just sel põhjusel on ka käesolevas uuringus nende organismide bioloogiline tuvastamine ja arvukuse võrdlemine teostatud mitmest erinevast reoveepuhastusjaamast kogutud andmete põhjal. Konfokaalmikroskoobi abil läbi viidud proovid, mis olid juba varasemalt keemiliselt mõõdetud ning DAPI (4',6-Diamidino-2-phenylindole)-ga värvitud, näitasid uuringutulemuste võrdlused sama tulemuseni suuduvat trendi. Nende organismide avastamiseks oli võimalik reovees sisalduvat muda konfokaalmikroskoobi abil edukalt ära kasutada. Uuringus kasutatud reovee puhastusjaamadest saadud info põhjal tehti edukas võrdlus nende organismide arvukuse kohta.

Võtmesõnad:
Fosfaate akumuleerivad organismid, reoveepuhastusjaamad, DAPI, tuvastamine.

CERCS:
B230 Mikrobioloogia

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS 6

INTRODUCTION 8

1 LITERATURE REVIEW 10

1.1 Polyphosphate Accumulating Organisms (PAOs) 10

1.1.1 Isolation of Polyphosphate Accumulating Organisms (PAOs) 10

1.1.2 Metabolism of PAOs 11

1.2 Wastewater Treatment Plants (WWTPs) 12

1.3 Enhanced Biological Phosphorus Removal (EBPR) 12

1.3.1 Factors Affecting EBPR Performance 13

1.4 Flow Cytometry 13

2 THE AIMS OF THE THESIS 14

3 EXPERIMENTAL PART 15

3.1 MATERIAL AND METHODS 15

3.1.1 MATERIAL 15

3.1.2 Preparation for the Methods 15

3.1.3 Fluorescence Microscopy 15

3.1.4 ImageJ 18

3.1.5 Flow Cytometry 18

3.1.6 Chemical Dosage Requirements 19

3.2 RESULTS 21

3.2.1 Microscopy Results 21

3.2.2 ImageJ Cell Count 22

3.2.3 Flow Cytometry Results 24

3.2.4 Comparing Microscopy and Flow Cytometry Data 27

3.3 DISCUSSION 30

SUMMARY 32

REFERENCES 33

Appendix 1: Microscopy Images 37
Appendix 2: Flow cytometry Data 39
NON-EXCLUSIVE LICENSE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC 47

TERMS, ABBREVIATIONS AND NOTATIONS

AS	Activated Sludge
C/N	Carbon/Nitrate Ratio
DAPI	4'1,6-Diamidino-2-Phenylindole
EA	Enzyme Assay
EBPR	Enhanced Biological Phosphorous Removal
EM	Electron Microscopy
EMP	Embden-Meyerhof-Parnas Pathway
ETC	Electron Transport Chain
FACS	Fluorescence-Activated Cell Sorting
FC	Flow Cytometry
FISH	Fluorescence in Situ Hybridization
FM	Fluorescence Microscopy
GAO	Glycogen Accumulating Organism
MS	Mass Spectrometry
NH_4^+	Ammonium
NMR	Nuclear Magnetic Resonance
OMICS	Omics methods
PAO	Phosphate Accumulating Organism
PBS	Phosphate-Buffered Saline
PHA	Polyhydroxyalkanoate
PO_4	Phosphate
Poly-P	Polyphosphate
SRT	Sludge Retention Time
TCA	Tricarboxylic Acid Cycle

VFA	Volatile Fatty Acid
WWTP	Wastewater Treatment Plant

INTRODUCTION

Phosphorus and phosphate are essential compounds already actively used in areas that profoundly affect our lives, such as agriculture, industry and biotechnology. Unfortunately, phosphorus is only obtained from a limited number of reserves worldwide. This means that phosphate has a sizeable economic share. Naturally, countries such as Morocco, which holds almost three-quarters of phosphate reserves, are in an advantageous position. At the same time, total phosphate reserves are approximately 67,000 Tg, and almost 220 Tg of phosphate is extracted annually (Schoumans et al., 2015). Therefore, recycling comes to mind, and at this point, wastewater has a vital share because studies have shown that there are not negligible amounts of phosphorus in wastewater (Tarayre et al., 2016).

It has been reported that microorganisms retain phosphate by various methods. Some of these are in the form of minerals or organic form. In some studies, *Halobacterium salinarum* and *Halorubrum distributum* were reported to retain $MgPO_4OH\text{-}4H_2O$. At the same time, Cyanobacteria have been observed to retain phosphate together with calcium in the sheat part of their body. However, it was concluded that polyphosphate was preferred as the most common form of phosphate retention in microorganisms in the general classification. PAOs have also been reported to be involved in the removal of phosphate in wastewater treatment plants (Tarayre et al., 2017)

The importance of the detection of PAOs in wastewater treatment plants has been emphasized in various studies. In the same way, the metabolic pathways of organisms have been studied to understand the pathway of phosphate-retaining organisms. For example, it has been reported that Tetrasphaera and Dechloromonas are involved in removing phosphorus by using fermentation or denitrification pathways. Likewise, the behaviour of these organisms in anoxic or anaerobic environments has been observed. Their performances under various conditions have been compared. It has been discussed how these organisms can play a role in the removal of phosphorus compounds by following a pathway together (Zhao et al., 2022).

Various methods have been used to detect PAOs organisms. It was also shown that these organisms do not grow as a pure colony in the environment, that the work on this subject is not easy and that various methods have been tried in order to develop a successful method, and that each method has advantages and disadvantages because it depends on the type of study to be carried out. For example, fluorescence in situ hybridization (FISH) and mass spectrometry (MS) methods can be used if only detection is desired. However, if

quantification and other properties are also desired, flow cytometry (FC), fluorescence microscopy (FM), X-ray, electron microscopy (EM), nuclear magnetic resonance (NMR), RAMAN Microscopy, enzyme assay (EA), or OMICS techniques can be used (Tarayre et al., 2016).

Detection of PAOs using sludge samples from wastewater treatment plants has been attempted by various methods. DAPI was used to stain the polyphosphate bodies, and this method indicated that the living cells were intact. There are differences in the concentration of DAPI in various sources, but the concentration we used was reported to be the optimized concentration in this study. Recovery of cells was performed by the FACS (Fluorescence-Activated Cell Sorting) method in many different studies. The purified samples at the colony isolation stage were identified by 16s rRNA gene sequencing. As a result of the analyses, it was concluded that living organisms containing polyphosphate could be stained with DAPI (Terashima et al., 2020).

1 LITERATURE REVIEW

1.1 Polyphosphate Accumulating Organisms (PAOs)

PAOs is the general name given to organisms that store phosphate as polyphosphate inside cells. Studies show that poly-P is the most common form of phosphorus retention. PAOs organisms are frequently used in wastewater recycling processes. PAOs organisms are used in Enhanced Biological Phosphorous Removal (EBPR) processes, but in the studies carried out, comparisons were made regarding efficiency and as a result. However, PAOs organisms are widely used in EBPR processes, and the desired values have yet to be reached (Tarayre et al., 2016). However, the fact that it is an environmentally friendly and economical system makes these systems more and more appealing day by day (Günther et al., 2009).

Each microorganism has different styles of phosphate retention. Some organisms, such as Halobacterium *salinarum* and *Halobacterium distributum*, retain phosphate in the form of $MgPO_4OH\text{-}4H_2O$ (Tarayre et al., 2017), but *Brevibacteria* is reported to retain it in the form of $NH_4MgPO_4\text{-}6H_2O$ (Tarayre et al., 2016). *Kuraishia capsulata* is reported that it could retain P as well (Kulakovskaya et al., 2012). Recent studies have found that Dechloromonas and Tetrasphaera also retain phosphate in their bodies, and it is predicted that they can be used in EBPR systems (Zhao et al., 2022). DAPI, used to visualize PAOs under the microscope, has been used in various concentrations in different sources. Likewise, the staining time also varies. It was observed from the studies that staining was performed with DAPI with a concentration of 5µg/mL for 1 hour in the dark (Hung et al., 2002), with DAPI with a concentration of 10µg/mL for 3 hours in the dark (Saia et al., 2017), and with 10µg/mL DAPI for 30 minutes in the dark (Terashima et al., 2020). Studies also show that the DAPI concentration can have a concentration between 1 and 50µg/mL (Mesquita et al., 2014).

1.1.1 Isolation of Polyphosphate Accumulating Organisms (PAOs)

Previous studies show that it is difficult to isolate these organisms, and various methods have already been tried (Liu et al., 2017). The detection of PAOs is based on the presence of poly-P and PHAs. However, it has been observed that the enrichment step is performed in some studies before detection (Morohoshi et al., 2003). While P is removed from wastewater in EBPR, the efficiency is found to be low. Likewise, the enrichment stage of polymers states that these primers are specific to organisms and that this process must be applied beforehand. Isolation in the presence of enriching components such as acetate and citrate was attempted but unsuccessful (Bao et al., 2007).

1.1.2 Metabolism of PAOs

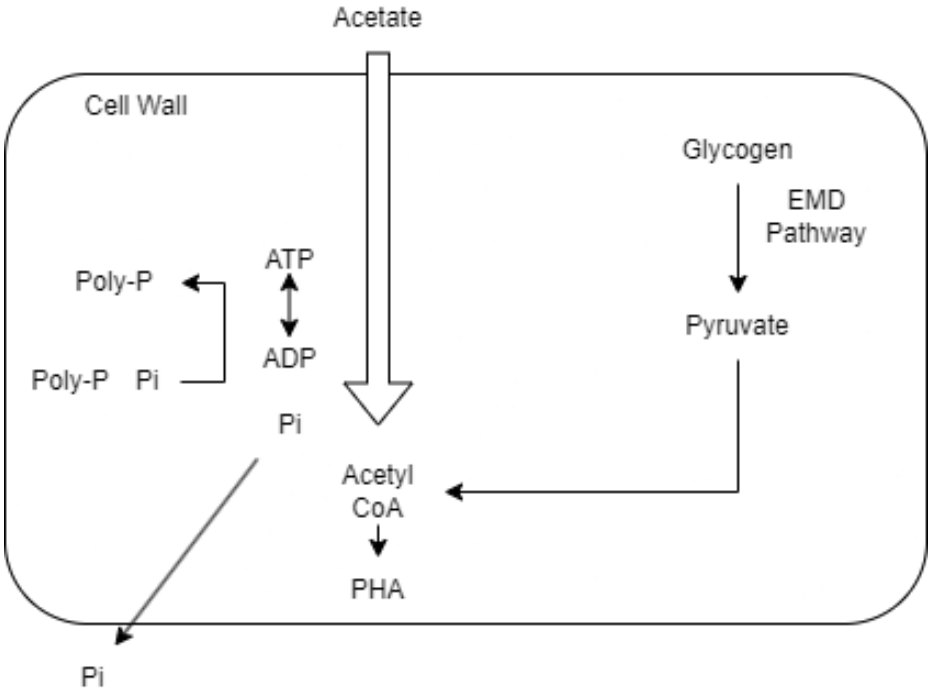


Figure 1. Metabolic pathway of P in PAOs under anaerobic conditions (Yuan et al., 2012)

Anaerobic and aerobic phases are crucial for PAOs because they are key factors determining whether phosphate will be released to the media. In anoxic conditions, these organisms try to survive by accumulating carbon from the media, which in wastewater is usually acetate. As the reaction occurs, P is released into the environment, converted to acetyl-CoA, and then converted into PHA.

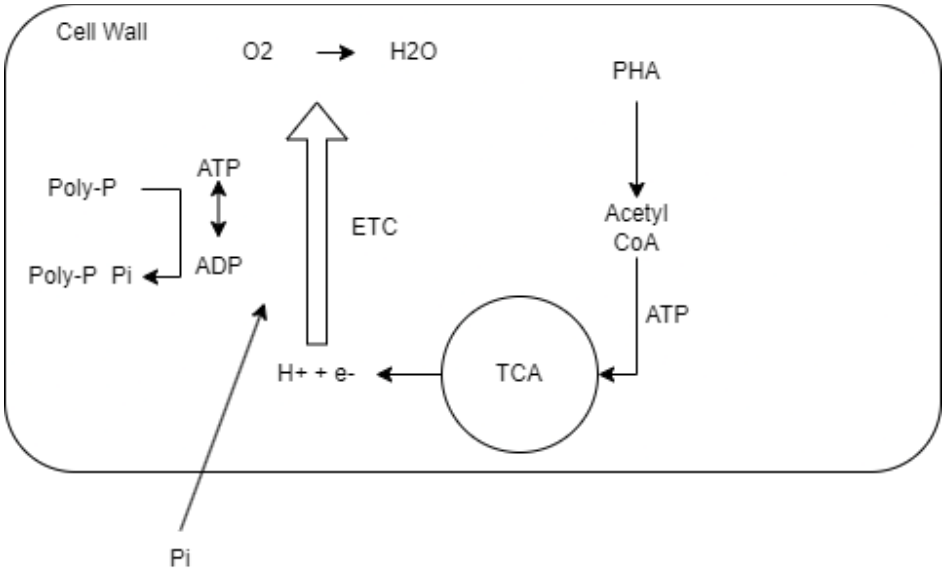


Figure 2. Metabolic pathway of P in PAOs under aerobic conditions (Yuan et al., 2012)

In the presence of oxygen, the organism uses the PHAs to grow (Tarayre et al., 2016).

1.2 Wastewater Treatment Plants (WWTPs)

Wastewater treatment plants are units used to eliminate the pollution caused by domestic and industrial wastes and are essential for our daily life. It has been determined that phosphate and phosphate compounds in wastewater cause eutrophication. Therefore, measures should be taken to protect the environment and water, and methods have been developed to filtrate phosphorus which causes eutrophication (Papp et al., 2022). In this context, it was determined that using the EBPR method to filtrate and separate phosphorus compounds is more accurate and efficient than conventional chemical methods (Meng et al., 2023).

1.3 Enhanced Biological Phosphorus Removal (EBPR)

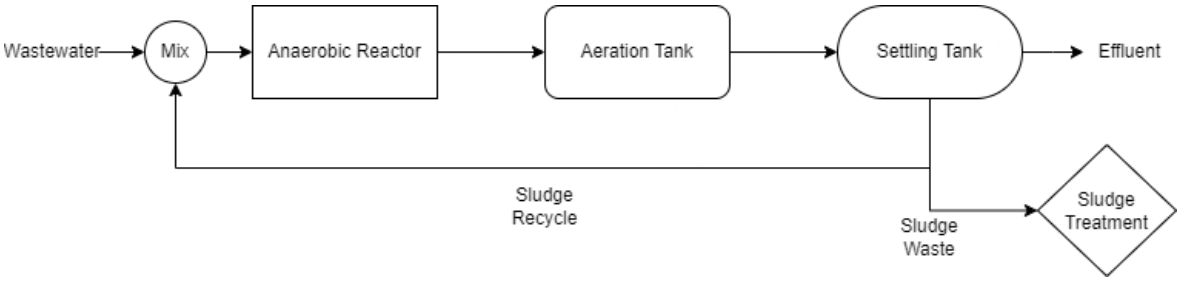


Figure 3. General enhanced biological phosphorus removal (EBPR) scheme (Münch & Barr, 2001)

EBPR has been developed to remove phosphorus from wastewater and is frequently preferred in wastewater treatment plants with eutrophication problems. As can be seen in Figure 3, the system consists of various stages (aerobic tank, aeration tank, settling tank, recycling and effluent). Those steps aim to provide the required environment for the PAOs in the wastewater to make them store P and succeeding removal of phosphorus by these organisms (Münch & Barr, 2001).

The anaerobic tank is where the wastewater is first treated. Here the PAOs present in the wastewater are provided with anaerobic conditions. Firstly, the PAOs use volatile fatty acids (VFAs) as a carbon source, releasing phosphate into the environment (Kristiansen et al., 2013).

Afterwards, a transition is made to the aerobic tank. There, glycogen is utilized to ensure intracellular growth. Glycogen is catabolized to restock the polyphosphate reserves in the following anaerobic phase (Tarayre et al., 2016). In contrast to the anaerobic environment, here polyphosphate is stored intracellularly. Therefore, the intracellular P concentration increases while the P concentration in the medium decreases (Nguyen et al., 2023).

In the third stage, wastewater enters the settling tank. Here, the phosphorus-rich biomass settles to the bottom of the tank and forms an activated sludge (AS) layer. This way, AS goes

to the sludge treatment area or is recycled back into the system. In the third step of the system, it is essential to recycle the sludge that settles to the bottom back into the system. In this stage, the sludge is transferred back to the anaerobic zone, ensuring the continued presence of PAOs in that zone and thus contributing to the EBPR process's continuity.

1.3.1 Factors Affecting EBPR Performance

EBPR is a complex system with many parameters that should be addressed to optimize these systems. Firstly, PAOs need a carbon source in order to grow and survive. These carbon sources can be found in the environment in various forms (sugar, VFAs) (Anders et al., 2023). The carbon/nitrate (C/N) ratio has also been reported to affect the EBPR process (Campo et al., 2020). It has been observed that increasing the sludge retention time (SRT) and anoxic phase can be beneficial to the EBPR process up to a certain level (Oehmen et al., 2005). It has been reported that temperature affects the competition between PAOs and GAOs (Glycogen Accumulating Organisms) (Lopez-Vazquez et al., 2009). The reuptake of VFAs is high at high pH, and PAOs work more efficiently at high pH (Oehmen et al., 2005).

1.4 Flow Cytometry

DAPI is used for the detection and visualization of PAOs in wastewater sludge. This dye binds specifically to DNA, allowing it to be visualized under a fluorescence microscope. When combined with flow cytometry, this dye provides a fast and efficient method for quantitative analysis of populations in wastewater. Each cell provides a different magnitude of absorption, which provides information about the size of the cells and which cells give how much DAPI signal and nucleic acid content (Safford & Bischel, 2019).

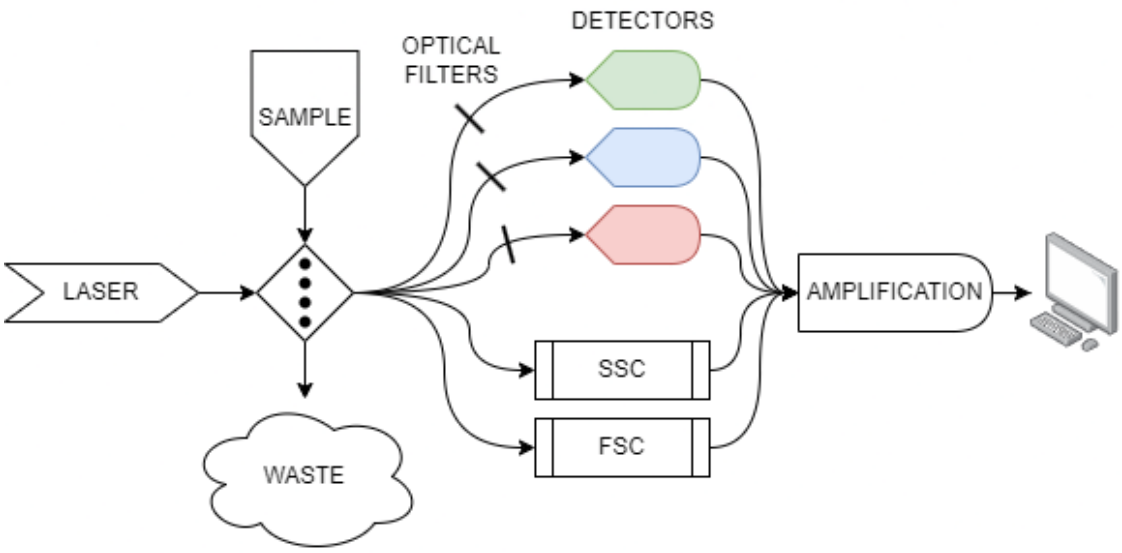


Figure 4. Flow cytometry diagram (Barnett et al., 2008)

2 THE AIMS OF THE THESIS

The aim and scope of the thesis are to investigate the abundance of phosphate-accumulating organisms in different wastewater treatment plants in Estonia, compare microscopic images and quantification of these organisms, and compare these data using flow cytometry. Likewise, to evaluate the differences in these wastewater treatment plants' operational parameters and establish a reliable comparison method.

This thesis's first aim is to characterize these organisms' abundance. In this regard, samples from six different wastewater treatment plants were used. While each plant's treatment process deviated, the samples' contents from these sources varied. This is due to parameters such as the region in which the plant is located, the abundance or absence of industrial and residential areas, the type of plant, and geographical location. In this study, we investigate the effect of these parameters on the abundance of phosphate-accumulating organisms. Therefore, this study aims to provide insight into the performance of these plants and population differences of organisms and to highlight the differences.

Another objective is developing and optimizing a detection method for phosphate-accumulating organisms. DAPI staining, microscopic imaging and flow cytometry are the methods used. It is aimed that these techniques will give accurate results for this goal. The microscope visualization strategy was intended to allow direct visualization of these organisms, and cell counting could be performed in a specific area. On the other hand, flow cytometry was aimed to provide a more efficient and quantitative analysis and would be better in terms of speed.

For this purpose, the method is intended to be used while considering that the content of the samples is diverse, how important the staining protocol is, and how much difference it would make when different usage can affect the detection of PAOs. This study aimed to establish a protocol by using the methods in previous studies in this field and to make the comparison through this protocol.

3 EXPERIMENTAL PART

3.1 MATERIAL AND METHODS

3.1.1 MATERIAL

3.1.1.1 Sample Collection

This study used samples from six wastewater treatment plants in different locations, with different operating principles and conditions, and with various environmental and climatic parameters. Our team members, led by Dr Taavo Tenno from the Institute of Chemistry department, assisted in collecting and delivering the samples. During the selection of the samples, the operating principles of the wastewater treatment plants were considered in advance for proper comparison. Likewise, climate changes were also influential in the sample collection. Therefore, to make our study more accurate, samples from various time periods (11.2022-04.2023) were used to create a more accurate comparison of different wastewater treatment plants.

3.1.1.2 Storing the Samples

In order to ensure the accuracy and integrity of the experiment, time was taken into consideration when the samples were delivered to us because there was little waiting time after the samples were received from the plant (approximately 2 hours). After the samples were taken for the experiments, all samples were stored in the cold room for storage at +4°C. Therefore, the strict working style was used to maintain the validity and accuracy of our findings, resulting in more precise and accurate information.

3.1.2 Preparation for the Methods

We employed specific approaches to prepare and analyze wastewater sludge samples to detect PAOs. Here, protocols for the microscopic detection of PAOs, and detection and quantification by flow cytometry are presented. All methods in these protocols were used the same way in samples from different wastewater treatment plants in Estonia.

3.1.3 Fluorescence Microscopy

3.1.3.1 Sample Preparation for Microscopy

For microscope analysis, we first started the process by centrifuging the sludge sample in the wastewater. The initial volume was 10mL for each sample. The centrifugation step was

performed at 1000xg for 3 minutes at 20 degrees centigrade to remove organic residues and thoroughly separate the liquid and sludge phases. Firstly, 0.5mL of the liquid phase after centrifugation was taken into an empty tube, and the liquid phase was then completely removed.

Table 1. Protocol for centrifuge sample preparation for sludge samples in wastewater

WWTP Sludge Sample	
Sample Number	Microscopy
1 (water)	Liquid phase (0,5 mL) from the first centrifuge + DAPI (25 µL)
3 (PBS)	{ [Solid phase of the first centrifuge (0,8 mL) + PBS (0,8 mL)] + Sonication (15 min 100%) + Centrifuge (1000xg 3 min) } = liquid phase (0,5 mL) + DAPI (25 µL)

The remaining 0.8mL of sludge was suspended in the same amount (0.8mL) of PBS. Sonication was performed at full power for 15 min to optimize the release of intracellular components and to accomplish cell lysis. The remaining 0.8mL of sludge was suspended in the same amount (0.8mL) of PBS. Sonication was performed at full power (100%) for 15 min to optimize the release of intracellular components and to accomplish cell lysis. The sample was then centrifuged, as shown in Table 1. Approximately 0.5mL of the liquid phase was taken into a separate tube.

The pump filtration system was used after the samples were prepared for the microscope. This filtration method ensured that the cells were stained with DAPI on the filter and could be examined under the microscope. Initially, a filter with a pore size of 0.2 µm was carefully placed in the filtration equipment. Before the filtration of the samples, the filter was slightly wetted by adding a few drops of distilled water to ensure proper wetting of the filter surface. The pump was switched on to allow distilled water to pass through the filter.

After carefully dropping the prepared samples onto the filter, the pump was started, and the filtration operation was completed. This ensured that the particles of interest remained on the filter as the sample passed through the filter. Next, 25µL of DAPI with a 0.2µg/mL concentration was carefully dripped onto the filter. The staining process took approximately 10 to 15 minutes and was carried out in the darkness. It is necessary to mention that during the

staining step, a dark environment is maintained to minimize the potential interference of light on the DAPI fluorescence.

After staining, the filter was gently removed from the filtration apparatus, transferred to a microscope slide and immersion oil was added to facilitate optimum imaging quality and resolution under the microscope. The cover glass is also applied, and immersion oil is spread. With a pump filtration step followed by DAPI staining, we aimed to concentrate and label PAOs in the wastewater sludge sample on a filter, allowing them to be visualized under a fluorescence microscope. This filtration technique provided a valuable tool to analyze and assess the presence and distribution of PAOs, facilitating further investigation and characterization of these organisms.

3.1.3.2 Microscopy Analysis

A confocal microscope equipped with a 405nm laser and a 63x magnification lens was used for microscopy analysis. The confocal microscope offers high-resolution imaging capabilities, which allow us to perform detailed examinations of the samples. Two different channels were used to distinguish PAOs during the observation specifically. The first channel covered the 407-550 nm range, allowing all organisms to be visualized, while the 551-580 nm channel was intended to be used only for the detection of PAOs.

Table 2. Imaging parameters

Dye	Color	Detector	Range
DAPI	Blue to Yellow	Ch1	407-550nm
DAPI	Blue to Yellow	ChS1	551-580nm

PAOs were expected to show a green to yellow fluorescence signal when excited by the 405nm laser. Imaging was performed using the appropriate filters and settings. PAOs can be easily distinguished from other cells as other cells will appear blue. In Tables 2 and 3, the microscope-related parameters are indicated.

During the microscopic analysis, a systematic imaging approach was used to obtain reliable representative images of the specimens. A total of 15 photographs were taken for each sample using a specific imaging method known as the "z-scan" technique. Z-scan refers to acquiring images by making z-shaped movements across the microscope slide. This minimizes randomness in the microslide, allowing an accurate average cell count to be made utilizing these images.

Two images were taken in each frame while moving in a Z-shape. The first image was taken at 407-550nm, and the second image was taken at 551-580nm, as indicated in Table 2 above. In this way, the first image gave us an overview of the microbial community, and the second image allowed us to focus on the PAOs. After acquiring the images, these images were analyzed using ImageJ, an image processing tool. The total number of cells and the number of PAOs in the images were quantified and characterized in ImageJ.

Table 3. Image properties

Image Size	224,7 µm x 224,7 µm
Pixel size	0,22 µm

3.1.4 ImageJ

Using the visualization method above and employing ImageJ for cell counting, a systematic and standardized approach was adopted to assess the abundance and distribution of cells, particularly PAOs. This enabled reliable quantification and analysis, supporting the study's overall goals to understand better the dynamics and behaviour of PAOs in wastewater samples.

The thresholding function in ImageJ software is of great importance at this stage. Because during the counting of PAOs, these cells were distinguished from other cells. Considering the pixel density for each image, the threshold level was set most accurately and counted accordingly. This way, PAOs were separated from other cells, and the cells in the frame were counted.

3.1.5 Flow Cytometry

3.1.5.1 Sample Preparation for Flow Cytometry

A different protocol was used for flow cytometry analysis compared to microscopy analysis, as seen in Table 4. In the first centrifugation step used to prepare the samples, 1mL of the liquid phase was taken into an empty tube, and 27µL formaldehyde was added along with 25µL DAPI. For the second sample, the steps were similar to the microscope method, but formaldehyde was added with DAPI at the last stage. These samples were then subjected to flow cytometry analysis, where individual cells were measured and characterized according to their DAPI fluorescence signals. Table 4 below shows the sample preparation protocol for flow cytometry.

Table 4. Protocol for flow cytometry sample preparation for sludge samples in wastewater

WWTP Sludge Sample	
Sample Number	Flow Cytometry
2 (water)	Liquid phase (1 mL) from the first centrifuge + DAPI (25 µL) + Formaldehyde (27 µL)
4 (PBS)	{ [Solid phase of the first centrifuge (0,8 mL) + PBS (0,8 mL)] + Sonication (15 min 100%) + Centrifuge (1000xg 3 min) } = liquid phase (0,4 mL) + DAPI (22 µL) + Formaldehyde (12 µL)

3.1.6 Chemical Dosage Requirements

Chemical dosing needs were determined based on load calculations for sediment addition to ensuring effective phosphorus removal. The data used in this analysis were obtained from the Institute of Chemistry, provided by Elisabeth Kaldvee. Table 5 shows the precipitant dosage distribution range (blue and orange) calculated based on the load measurements and indicates the minimum and maximum amounts required for optimum performance. The table's grey dot represents the dosage administered in each WWTP. WWTP B and WWTP F applied a considerably higher precipitant dosage, while WWTP G and WWTP A applied a relatively lower dosage. Such differences in dosage from the findings of our colleague suggest that WWTP C and WWTP A have the capability for enhanced biological phosphorus removal (BioP) potential. Furthermore, Table 2, also obtained from our colleague's research, shows the ratio of biologically bound phosphorus indicated by orange-coloured circles and the rate of phosphorus available in polyphosphate bodies. These facts provide valuable information on the extent of phosphorus removal achieved through biological treatments and emphasize the importance of BioP in WWTPs.

Table 5. Chemical dosage requirement for WWTPs (Elisabeth Kaldvee, 2023)

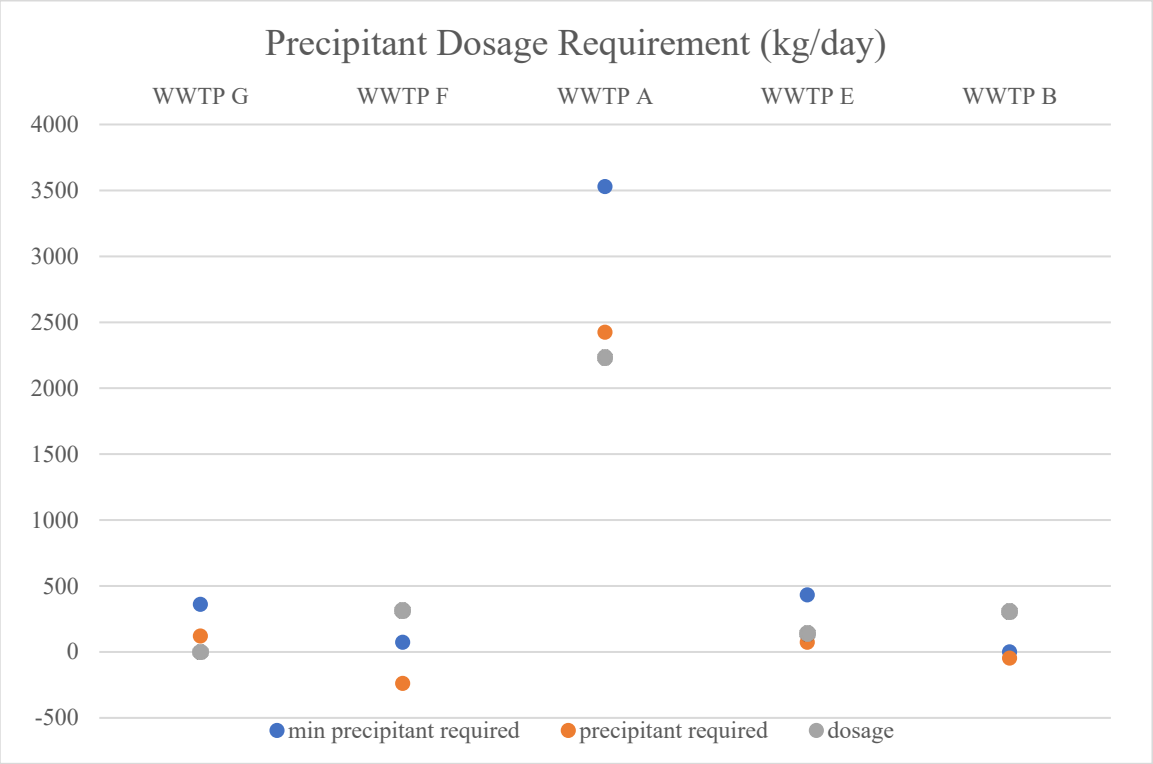
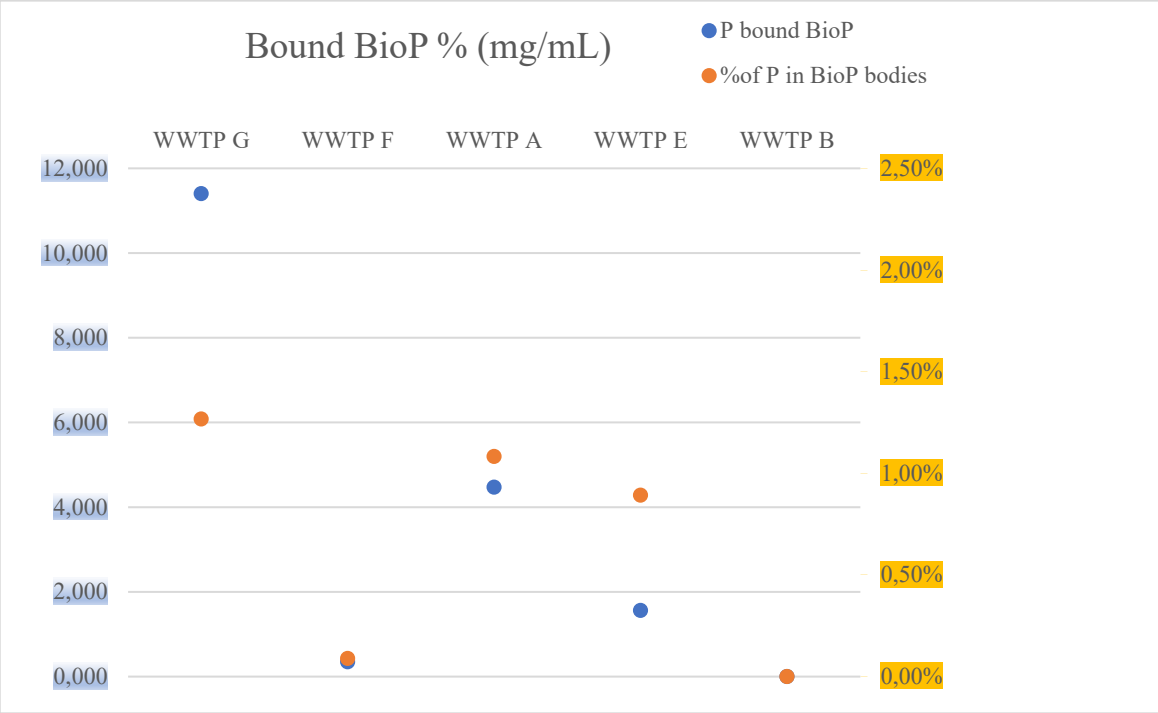


Table 6. Biologically bound BioP concentration and rate of P in BioP bodies (Elisabeth Kaldvee, 2023)



3.2 RESULTS

3.2.1 Microscopy Results

In our studies, sludge samples from wastewater treatment plants located in different regions of Estonia were used and analyzed for bioP abundance, and it was found that these plants have significant differences in PAO abundance.

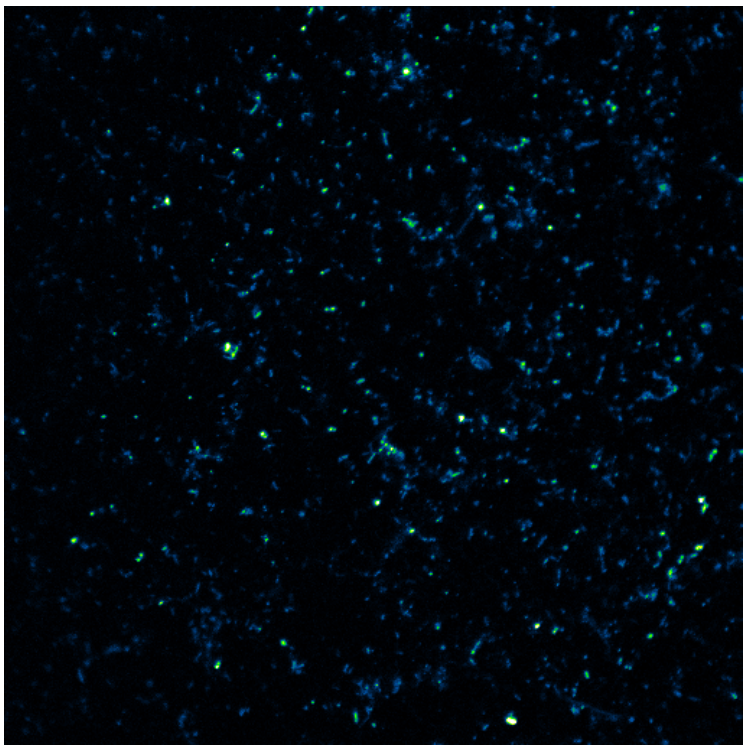


Figure 5. BioP abundance of Plant A

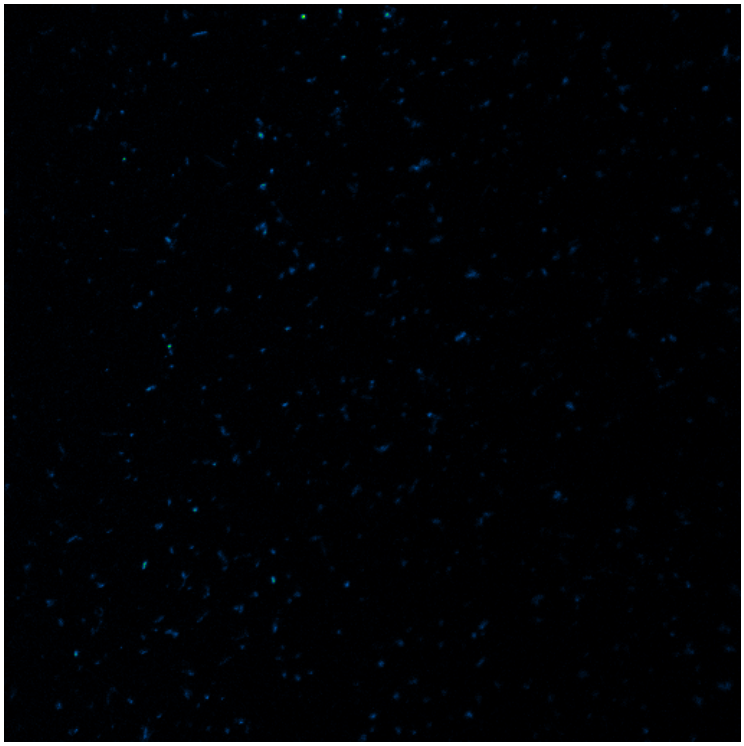


Figure 6. BioP abundance of Plant B

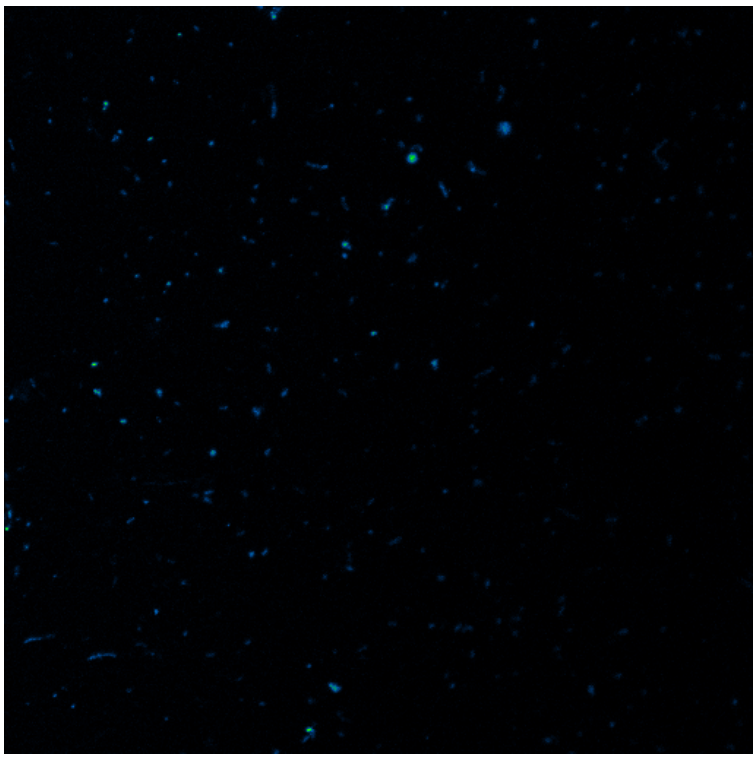


Figure 7. BioP abundance of Plant C

As shown in Figure 5, wastewater treatment plant A has a higher bio P abundance than the other plants. As seen in the illustrations, the colour from green to yellow represents PAOs and can be easily compared from the photographs. These findings showed that the operating parameters, operating conditions, location, amount of industrial and residential areas and treatment process in wastewater treatment plants could affect the growth and reproduction of PAOs. Thus different results in terms of bioP abundance will occur.

Appendix 1 contains additional images to support the main findings and comprehensively demonstrate the microscopy analysis. As can be seen from the images, bioP is more abundant in some regions. Therefore, ten or more photographs were taken to make our findings more accurate and precise.

3.2.2 ImageJ Cell Count

Quantitative investigation of microscopy images using ImageJ indicated meaningful differences in results from different WWTPs, revealing distinguishable characteristics in the PAOs. These results also show that the abundance of PAOs in each treatment plant varies because each plant's conditions and operating parameters are different.

Table 5. ImageJ cell count of plant A (2023.04.06)

PLANT A IMAGE	Poly-P Count	Total Cell Count	Poly-P / Total Count (%)
IMAGE 1	89	1015	8,768473
IMAGE 2	192	1108	17,32852
IMAGE 3	199	1047	19,00669
IMAGE 4	212	1189	17,83011
IMAGE 5	251	1265	19,8419
IMAGE 6	202	1313	15,38462
IMAGE 7	218	1172	18,60068
IMAGE 8	225	1330	16,91729
IMAGE 9	212	1224	17,32026
IMAGE 10	257	1481	17,35314
TOTAL->	2057	12144	16,93841
Standart Deviation	43,763	133,551	2,929

Table 6. Cell count of plant B (2023.04.06)

PLANT B IMAGE	Poly-P Count	Total Cell Count	Poly-P / Total Count (%)
IMAGE 1	8	346	2,312139
IMAGE 2	11	206	5,339806
IMAGE 3	4	101	3,960396
IMAGE 4	37	728	5,082418
IMAGE 5	6	298	2,013423
IMAGE 6	10	225	4,444444
IMAGE 7	2	136	1,470588
IMAGE 8	12	266	4,511278
IMAGE 9	6	155	3,870968
IMAGE 10	11	302	3,642384
TOTAL->	107	2763	3,872602
Standart Deviation	9,306	168,175	1,251

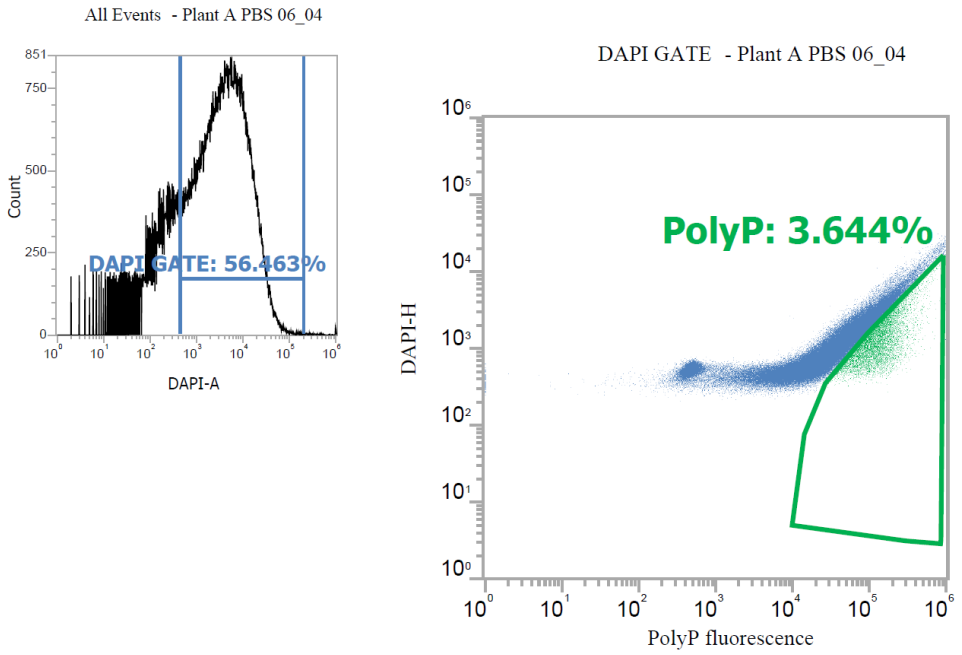
Table 7. Cell count of plant D (2023.04.06)

PLANT D IMAGE	Poly-P Count	Total Cell Count	Poly-P / Total Count (%)
IMAGE 1	22	3117	0,705807
IMAGE 2	37	4163	0,888782
IMAGE 3	30	3583	0,837287
IMAGE 4	23	4152	0,55395
IMAGE 5	50	3633	1,376273
IMAGE 6	41	4007	1,023209
IMAGE 7	35	3110	1,125402
IMAGE 8	130	3681	3,531649
IMAGE 9	49	2689	1,822239
IMAGE 10	19	3306	0,574713
TOTAL->	436	35441	1,230214
Standart Deviation	30,562	464,086	0,84

As can be seen from the tables below, WWTP A has a higher percentage of bioP than the other plants. Microscopy images supported these findings and showed differences in bioP abundance and PAO population of different WWTPs and that they can be detected with our methods. Likewise, when we look at the flow cytometry data, the data stand in a similar trend to our findings in cell counting.

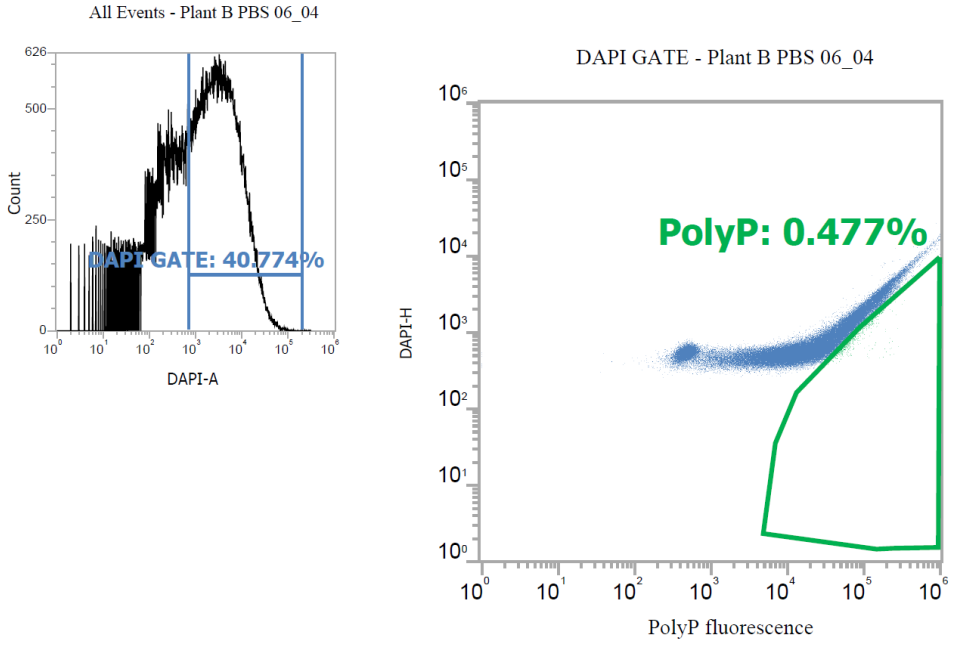
3.2.3 Flow Cytometry Results

As shown in the figures below, the flow cytometry investigation involved the analysis of sludge samples from three different WWTPs. This analysis provided data on the abundance of PAOs in the sludge samples and allowed the characterization of PAOs. All samples were measured by default in the flow cytometry analysis and analyzed accordingly.



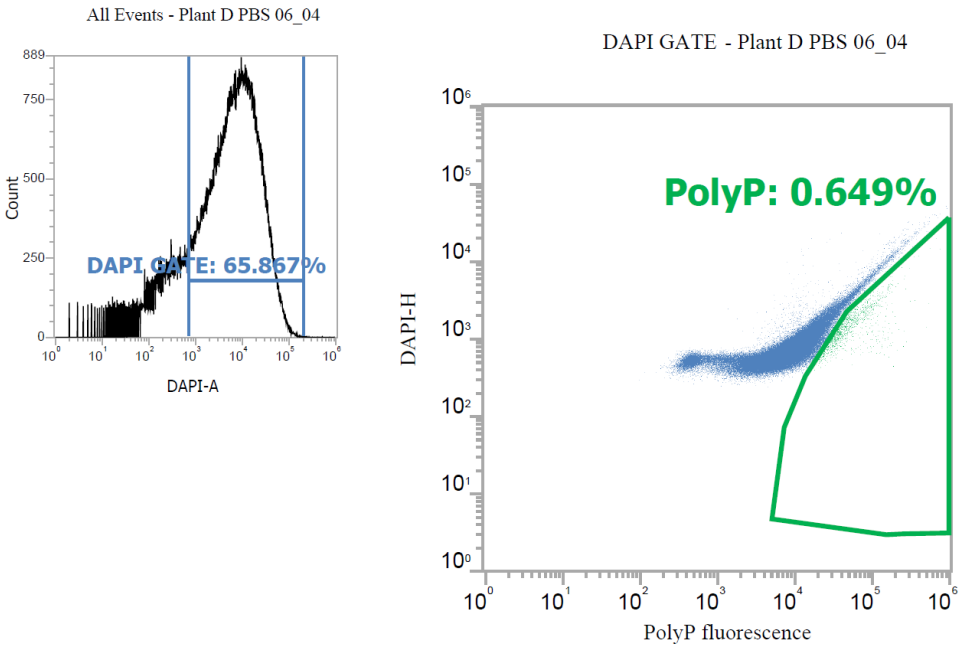
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	336,745	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		190,135	56.463	56.463
PolyP	PolyP	VL3-H	VL1-H	6,929	2.058	3.644

Figure 8. Flow cytometry data of Plant A



Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	299,529	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		122,130	40.774	40.774
PolyP	PolyP	VL3-H	VL1-H	582	0.194	0.477

Figure 9. Flow cytometry data of Plant B



Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	280,194	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		184,555	65.867	65.867
PolyP	PolyP	VL3-H	VL1-H	1,197	0.427	0.649

Figure 10. Flow cytometry data of Plant D

As can be seen in the figure, DAPI gating was correctly performed first, and the noise at the beginning of the graph was not included in the result. Since each sample had different noise proportions, specific gating was performed for each sample individually for the accuracy of the data. For the gating of Poly-P bodies, we used the gating method used in previous studies. In this analysis, we used such an approach to characterize PAOs according to their fluorescence properties.

When we compared the flow cytometry data with our previous microscopy imaging results, we found trends aligned with our expectations. WWTP A has a higher percentage of PolyPs than the other plants, as shown in the Poly-P graph. This finding is consistent with the microscope images. On the other hand, plants B and C have relatively lower percentages, and when compared with the microscope data, it is observed that the results are in the same trend.

3.2.4 Comparing Microscopy and Flow Cytometry Data

Based on the data obtained from the results of our experiments, calculations were made to achieve the cell concentrations in the first sample taken, taking into account all dilution factors. Standard deviations were applied to the data obtained from the microscope images and listed in the table. The data obtained from the liquid and sludge portions of the sewage sludges used in our experiments are presented in tables. However, calculations could not be performed on the images obtained from the liquid parts under the microscope and, therefore, could not be included in the table. Only the flow cytometry analyses and calculations of the samples taken from the liquid part are shown in the table. Similarly, no calculation could be made from the microscope images of WWTPs E and F, and only the flow cytometry results are shown in the table.

Table 8. Calculations for flow cytometry

Sample Number	WWTP	Flow Cytometry Data					
		Total Count	DAPI Gate	Poly-P Count	Poly-P % Rate	Poly-P Bodies per/mL	Total Cells per/mL
1	Aerobic PBS	250466	160976	2151	1,336	23338	1746590
2	Aerobic Water	159284	78798	1067	1,354	11577	854958
3	Anaerobic PBS	214473	136128	194	0,143	2105	1476989
4	Anaerobic Water	339723	146240	3083	2,108	33451	1586704
5	A PBS 11.04	231524	112588	711	0,632	7714	1221580
6	A PBS 06.04	336745	190135	6929	3,644	75180	2062965
7	A Water 11.04	96616	53831	211	0,392	2289	584066
8	A Water 06.04	134062	65219	1526	2,340	16557	707626
9	B PBS 11.04	317452	131444	790	0,601	8572	1426167
10	B PBS 06.04	299529	122130	582	0,477	6315	1325111
11	B Water 11.04	93414	52877	213	0,403	2311	573715
12	B Water 06.04	119064	60093	140	0,233	1519	652009
13	E PBS	113801	74048	254	0,343	2756	803421
14	F PBS	89454	48744	843	1,729	9147	528872
15	C PBS 06.04	209995	88554	195	0,220	2116	960811
16	C Water 06.04	183377	75702	901	1,190	9776	821367
17	D PBS 06.04	280194	184555	1197	0,649	12987	2002422
18	D Water 06.04	211285	86168	632	0,733	6857	934923

Table 9. Calculations for microscopy

		Microscopy Data				
Sample	WWTP	Standart Deviation	SD % Rate	Poly-P / To-tal Cells	Poly-P Bodies per/mL	Total Cells per/mL
1	Aerobic PBS	27,504	2,353	6,112	1579733	19693252
3	Anaerobic PBS	12,653	6,425	4,445	385363	6605251
5	A PBS 11.04	15,284	2,816	8,966	696716	5920809
6	A PBS 06.04	43,763	2,929	16,938	5249614	23613236
9	B PBS 11.04	17,975	5,211	7,095	405780	4357482
10	B PBS 06.04	9,306	1,251	3,873	273072	5372478
15	C PBS 06.04	7,497	1,703	7,948	518071	4966091
17	D PBS 06.04	30,562	0,846	1,230	1112704	68912771

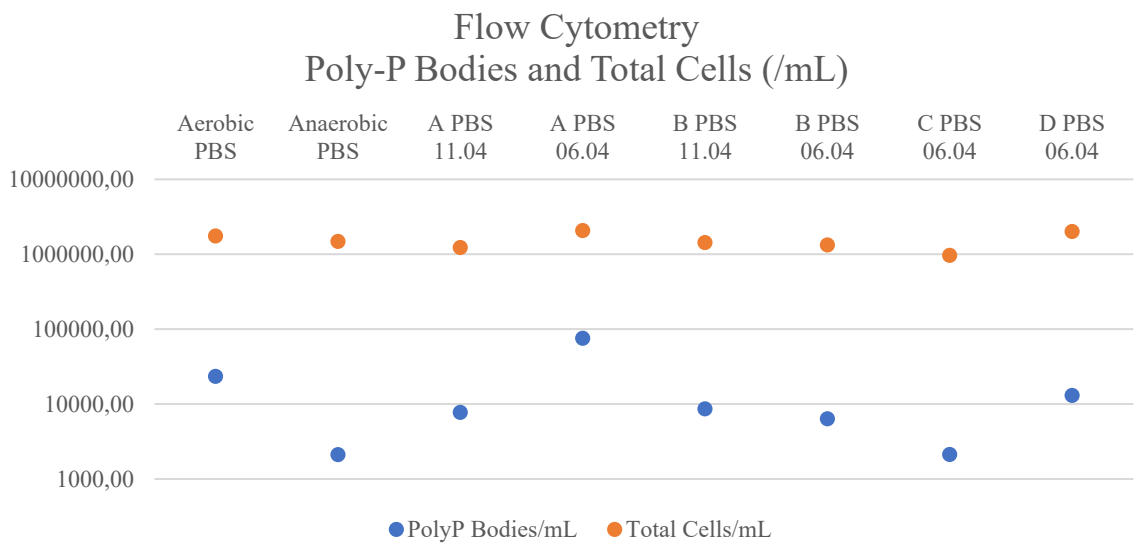


Figure 11. Flow cytometry Poly-P bodies and total cells per mL

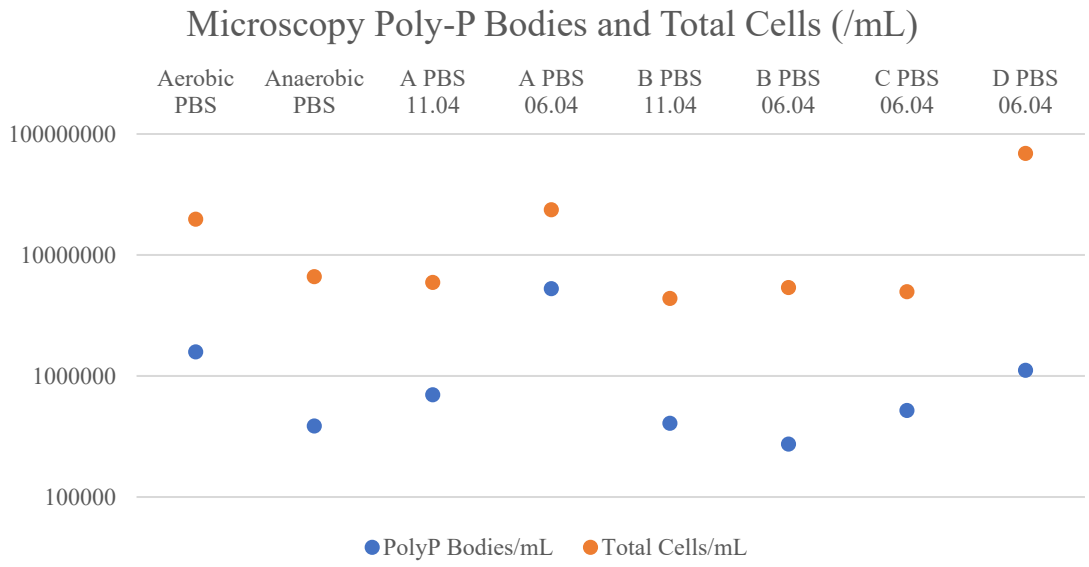


Figure 12. Microscopy Poly-P bodies and total cells per mL

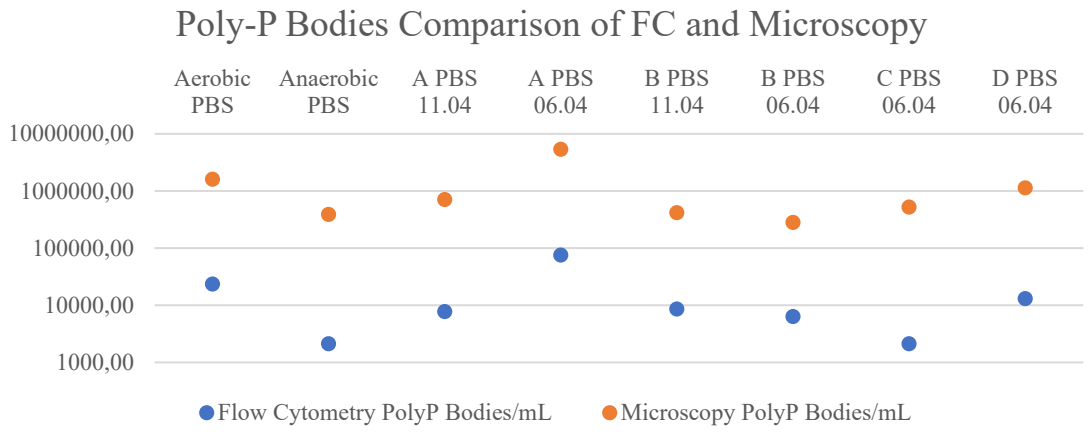


Figure 13. Comparison between FC and microscopy in terms of Poly-P bodies/mL

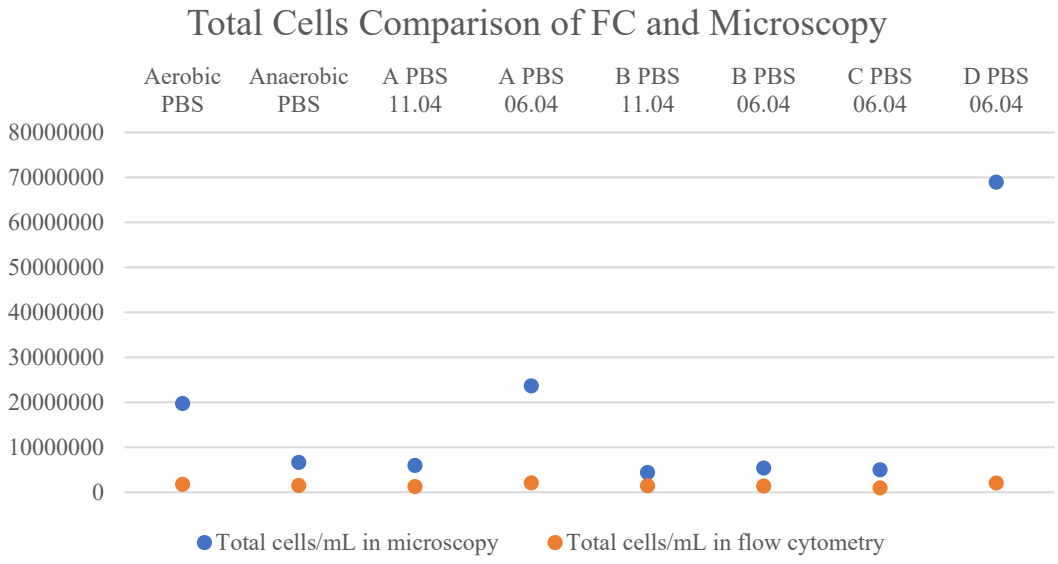


Figure 14. Comparison of total cell count in FC and microscopy

3.3 DISCUSSION

Given the content of the samples and the requirement to start experiments quickly, the time interval between obtaining the sample and obtaining it is essential. We tried to be quick during the experiment to avoid losing the Poly-P held in the samples. Therefore, the preparation of the material is vital for this study.

It is acknowledged how vital the centrifugation and sonication steps are in the sample preparation process. However, it should be noted that the speed, duration and temperature of the centrifugation, as well as the intensity and duration of sonication, can directly influence the distribution of PAOs in the sample and, thus, the measurement step. Although we used parameters developed according to existing protocols throughout the experimental period, slight variations in the steps during the experiments may have affected the results. In studies without positive controls, it was mentioned that the detection of PAOs may be challenging (Tarayre et al., 2017).

Studies have shown that the concentration of DAPI plays a critical role in the microscopic examination of Poly-P granules. It has been reported that 1µg/mL of DAPI stain may not be sufficient to visualise Poly-P granules. However, when DAPI at a concentration of 10µg/mL is used, Poly-P granules can be easily visualised (Terashima et al., 2020). Similarly, some studies have shown that 5µg/mL is sufficient (Hung et al., 2002). We have not tested the effect of using higher or lower concentrations of DAPI on the visualisation of Poly-P granules under the microscope, but most studies agree that 10µg/mL is sufficient. However, some studies have stated that up to 50µg/mL concentration can be achieved (Mesquita et al., 2014). It was also stressed that the staining time should be kept longer if low concentrations were needed (Serafim et al., 2002).

There is a potential area of concern in the filter pump where we stained our sample with DAPI. Although efforts were made to consistently and accurately follow the staining protocol and minimize light interference, it must be acknowledged that achieving perfect homogeneity across the entire filter is not easy. Deviations in dye intensity and homogeneity during the staining procedure can impact our results when conducting microscope analysis and can compromise the consistency and trustworthiness of the results. Similarly, the importance of the staining time with DAPI has been pointed out. In some studies, 3-hour (Saia et al., 2017), 1-hour (Hung et al., 2002), and 30-minute (Terashima et al., 2020) staining times were reported to be preferred, but we used a staining time of 10-15 minutes.

Likewise, when imaging via a microscope, diverse parameters played an active role throughout the imaging. In order to obtain clear and focused imaging, it was necessary to fine-tune the focus. Therefore, it took much work to obtain a consistent and idealized image of all samples, and the images may exhibit inconsistencies in resolution, which can directly affect the overall results of the experiment.

We manually adjusted the threshold to distinguish PAOs from other cells during our cell counting process using ImageJ. However, efforts were made to preserve consistency when thresholding, the consistency of the results may have been affected because we ultimately did it manually. Therefore, it may have caused some uncertainty or potential error in the results.

We have performed our experiments to obtain quantitative results in flow cytometry. It was also noted that tetracycline can be used to detect PAOs when using flow cytometry (Tarayre et al., 2016). However, the consistency of our experiments needed to be clarified because we needed a sharply defined positive control. The necessity of standardised methods, especially flow cytometry, was emphasised (Safford & Bischel, 2019). Likewise, based on existing methodologies and literature, gating has been done manually. Thus, the results may have resulted in differences or uncertainties due to manual adjustment.

SUMMARY

This thesis investigated the abundance and characterization of PAOs, and microscopy and flow cytometry methods were used. Samples from six different wastewater plants in Estonia were studied. The study includes how the EBPR process affects the performance of wastewater treatment plants and the identification and characterization of PAOs in wastewater sludge.

Through microscopy analysis, it was found that the wastewater treatment plants demonstrated distinct bioP abundances, with some WWTPs having high levels of bioP abundance and others having deficient levels of bioP abundance. Imaging was conducted using the DAPI staining approach in the confocal microscope, and data on the presence and abundance of yellow fluorescent bodies were obtained. Correspondingly, cell counting was performed using the images obtained from the microscope in ImageJ software, and the number of PAOs in the initial wastewater sludge was tried to be acquired.

Furthermore, the flow cytometry analyses provided a comprehensive perspective of the experiments and were compared with the microscopy observations. This allowed us to obtain data showing how the wastewater treatment plants we have examined differ in their PAO populations.

Although progress has been made in terms of research, some challenges have been encountered. All steps were optimized to ensure consistent results at each stage of the experiments. Furthermore, the lack of a positive control group made it difficult to identify PAOs accurately. Likewise, manually specifying thresholds in ImageJ software caused difficulties in the experiment's progress and the data's accuracy.

Finally, this thesis uses microscopy and flow cytometry methods to deliver information on the abundance and characterization of PAOs in different WWTPs in Estonia. Based on the findings, the differences in PAOs in terms of abundance are highlighted and shed light on the factors influencing the whole process. This research provides a better understanding of the EBPR process in wastewater treatment plants and allows comparisons of these plants and biological monitoring of the process.

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Appendix 1: Microscopy Images

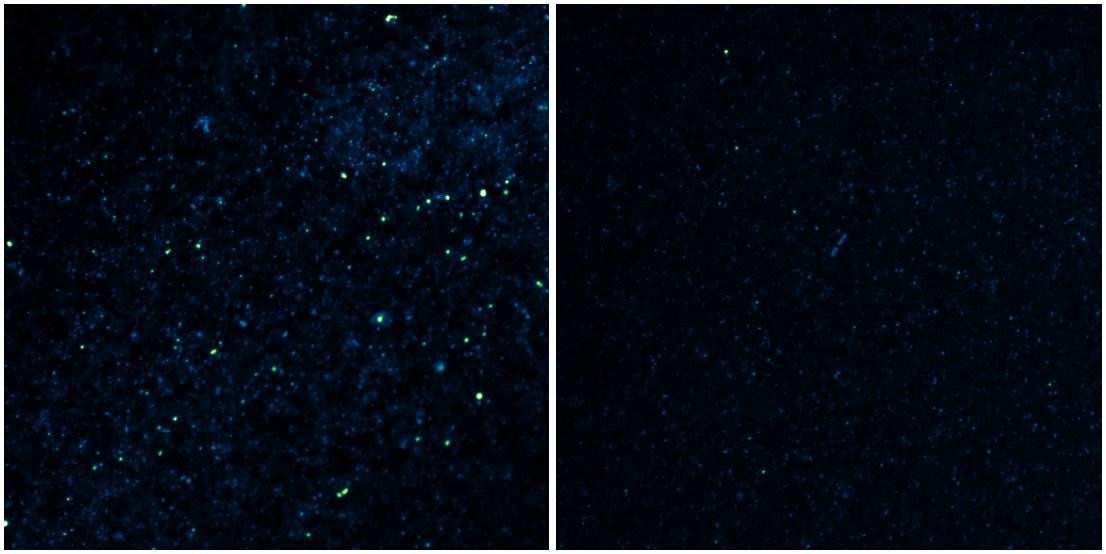


Figure S1. Aerobic WWTP PBS (left) and Anaerobic WWTP PBS (right)

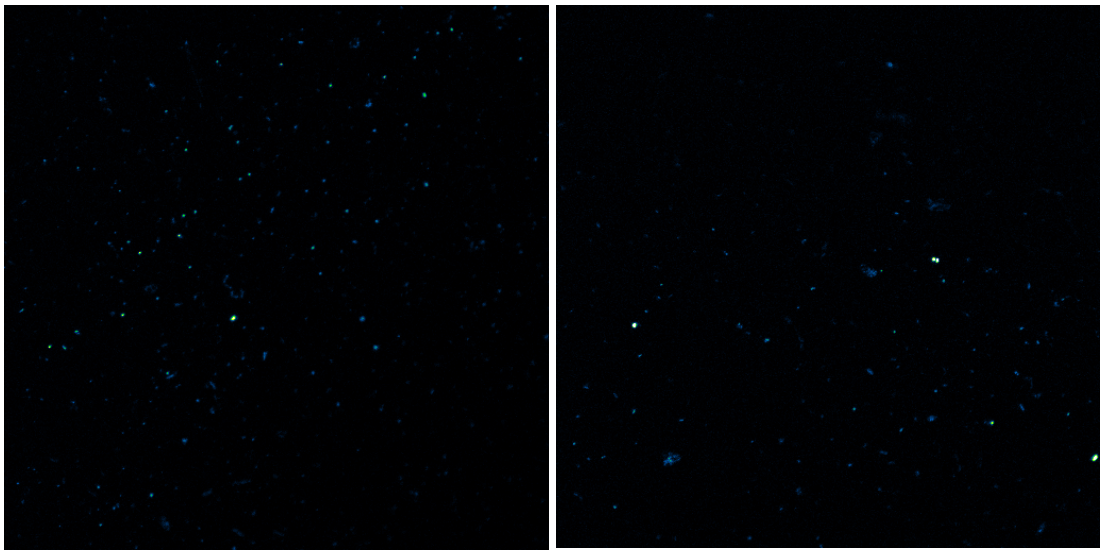


Figure S2. WWTP A PBS (left) and WWTP B (right)

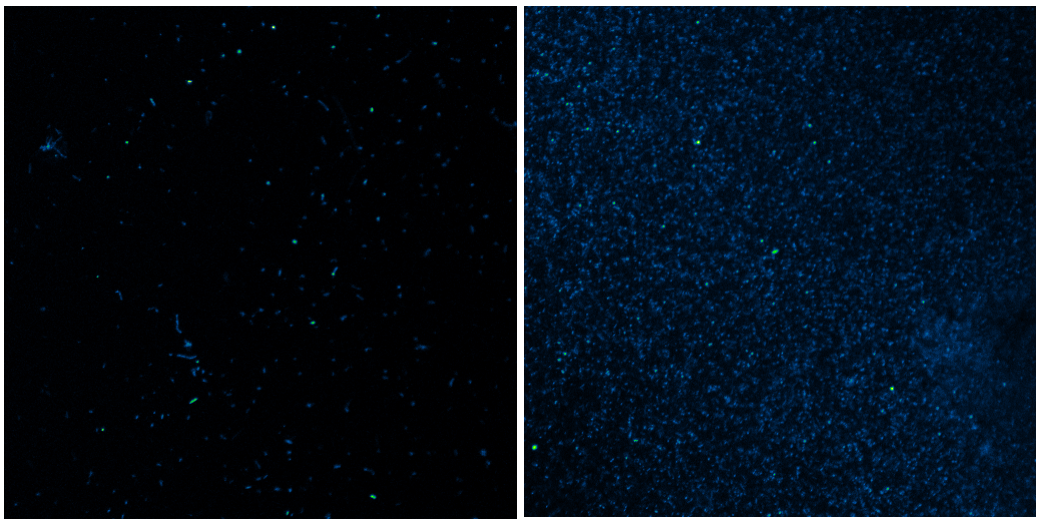


Figure S3. WWTP C (left) and WWTP D

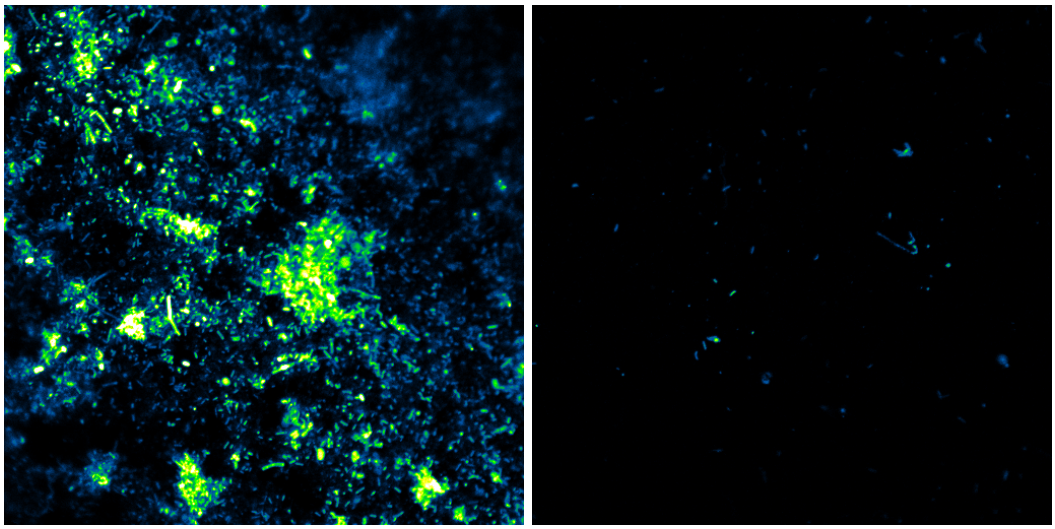
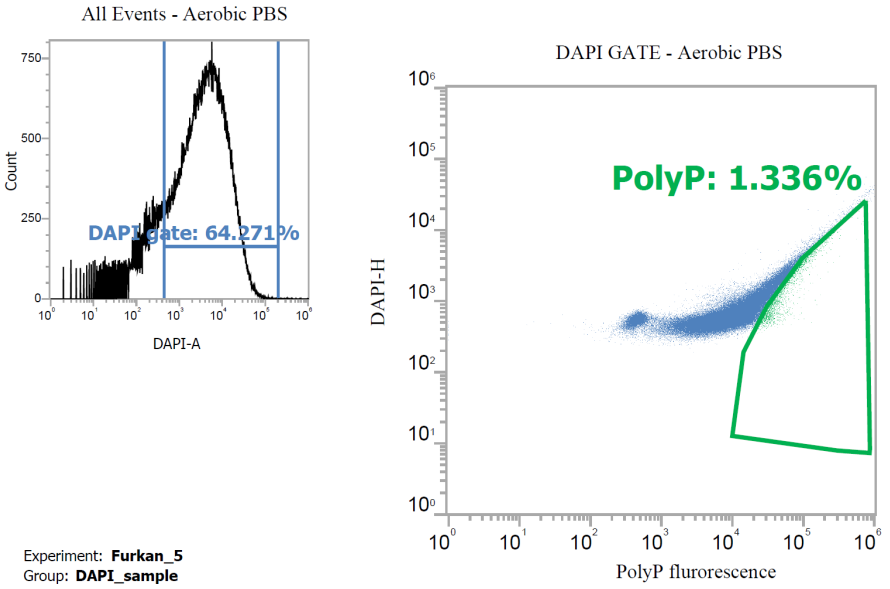


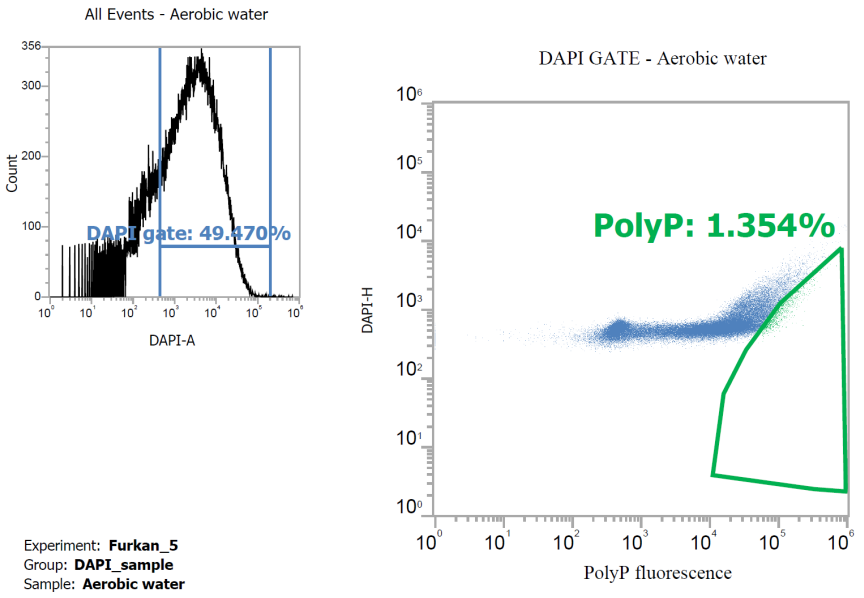
Figure S4. WWTP E (left) and WWTP (F)

Appendix 2: Flow cytometry Data



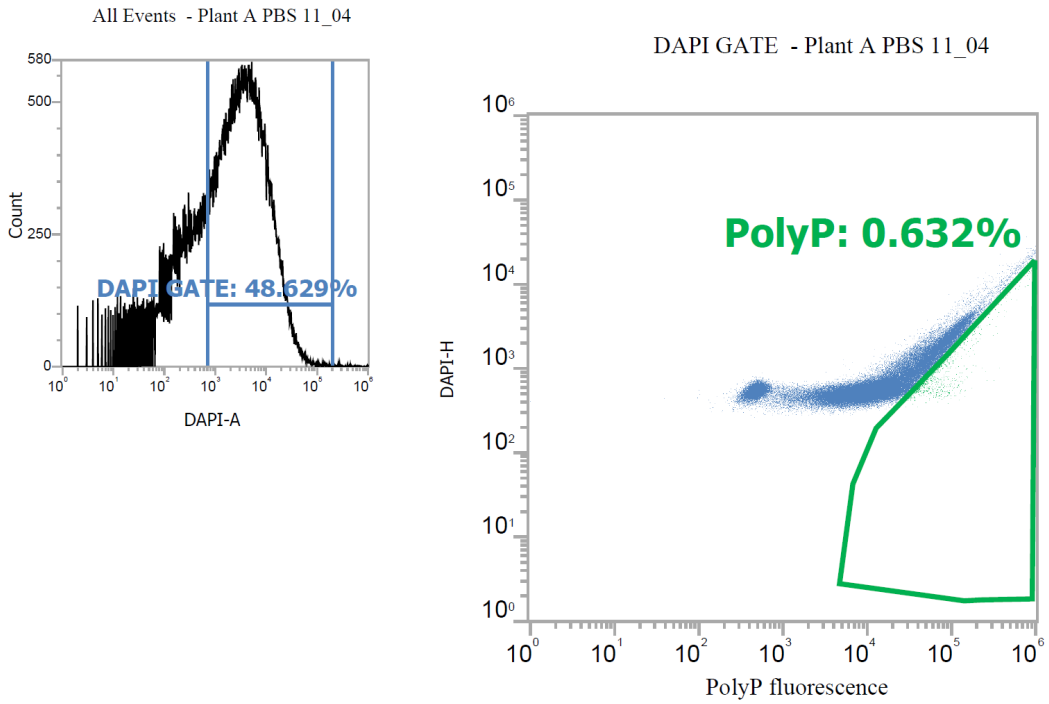
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	250,466	100.000	100.000
DAPI gate	DAPI gate	VL1-A		160,976	64.271	64.271
PolyP	PolyP	VL3-H	VL1-H	2,151	0.859	1.336

Figure S5. Aerobic PBS flow cytometry data



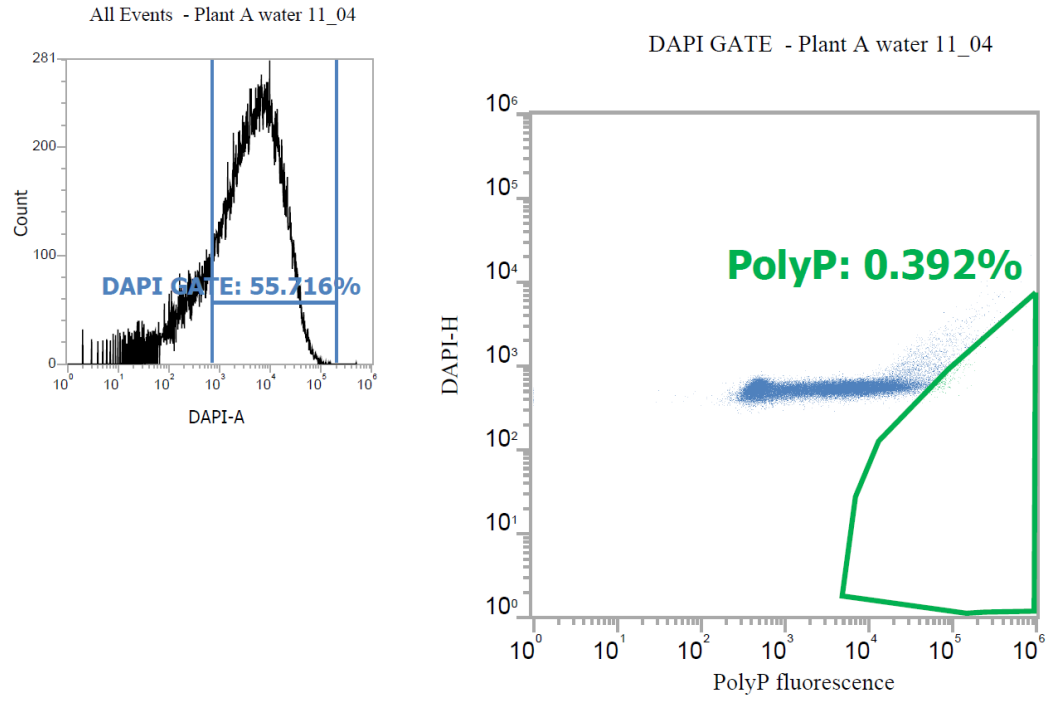
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	159,284	100.000	100.000
DAPI gate	DAPI gate	VL1-A		78,798	49.470	49.470
PolyP	PolyP	VL3-H	VL1-H	1,067	0.670	1.354

Figure S6. Aerobic water flow cytometry data



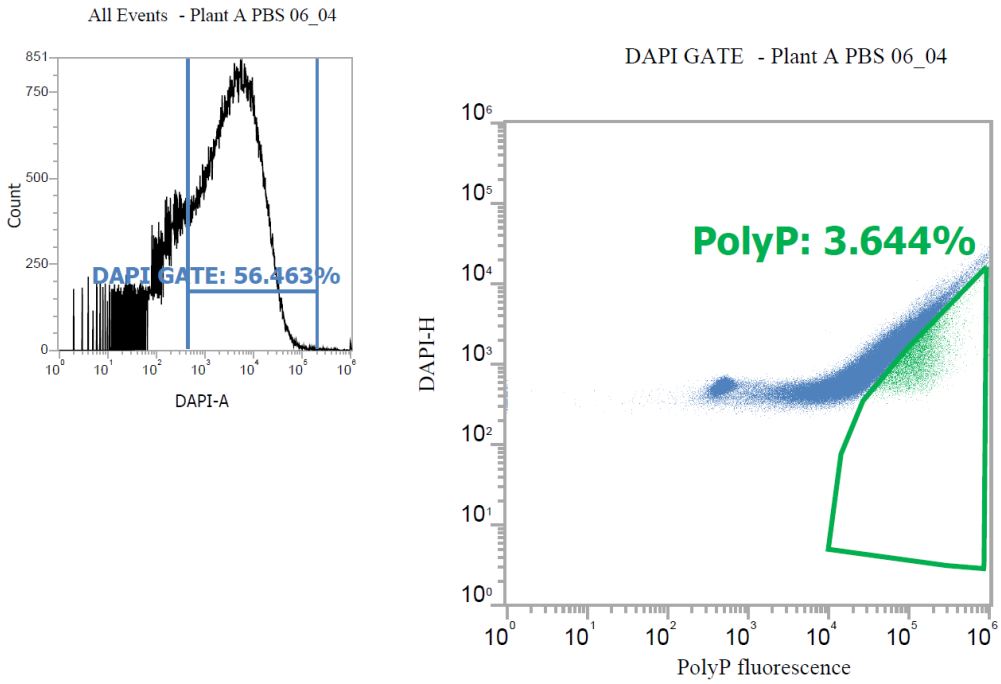
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	231,524	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		112,588	48.629	48.629
PolyP	PolyP	VL3-H	VL1-H	711	0.307	0.632

Figure S7. Plant A PBS (2023.04.11) flow cytometry data



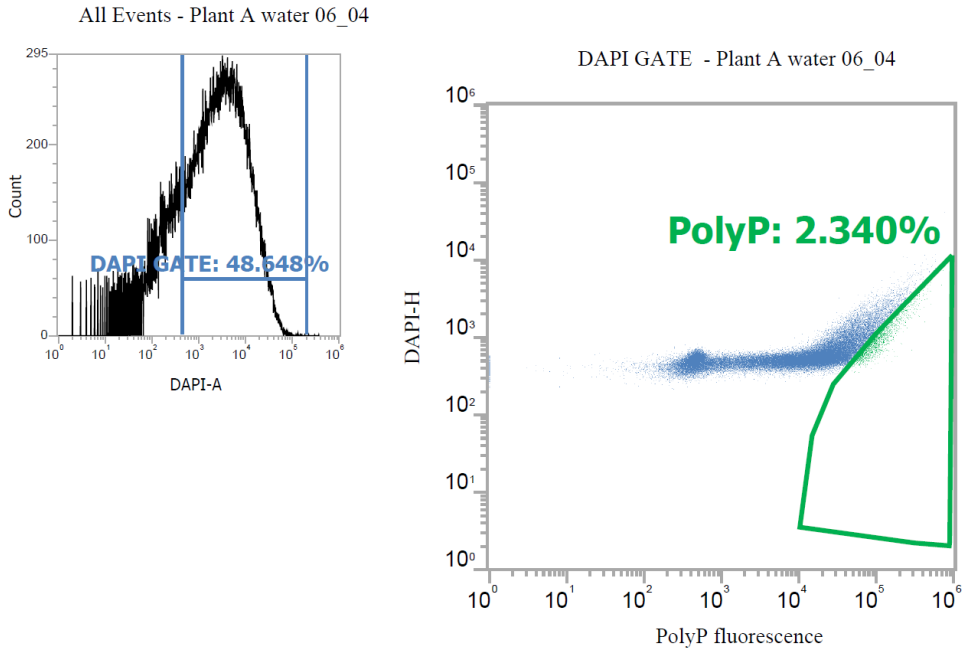
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	96,616	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		53,831	55.716	55.716
PolyP	PolyP	VL3-H	VL1-H	211	0.218	0.392

Figure S8. Plant A water (2023.04.11) flow cytometry data



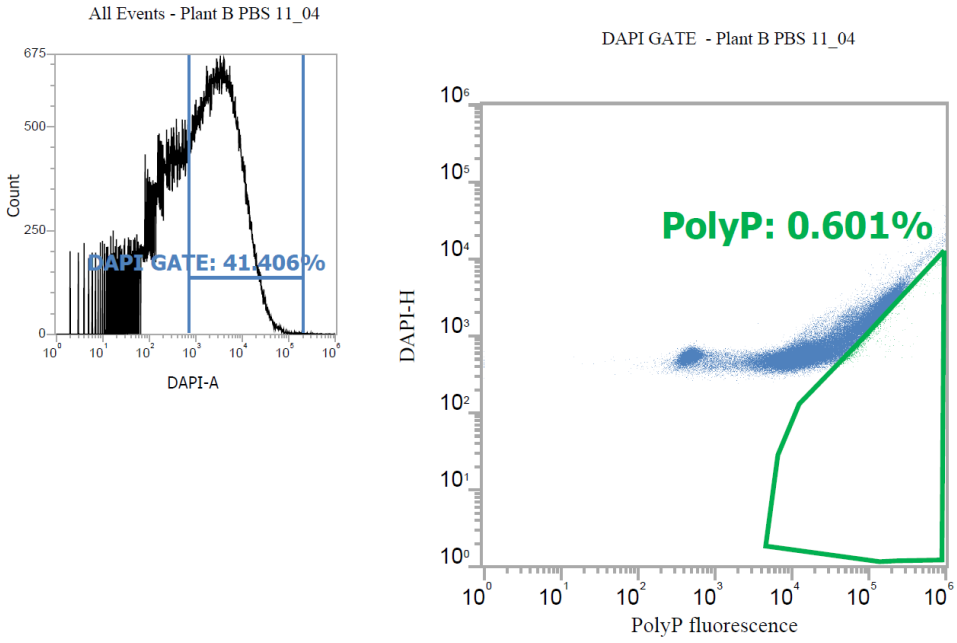
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	336,745	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		190,135	56.463	56.463
PolyP	PolyP	VL3-H	VL1-H	6,929	2.058	3.644

Figure S9. Plant A PBS (2023.04.06) flow cytometry data



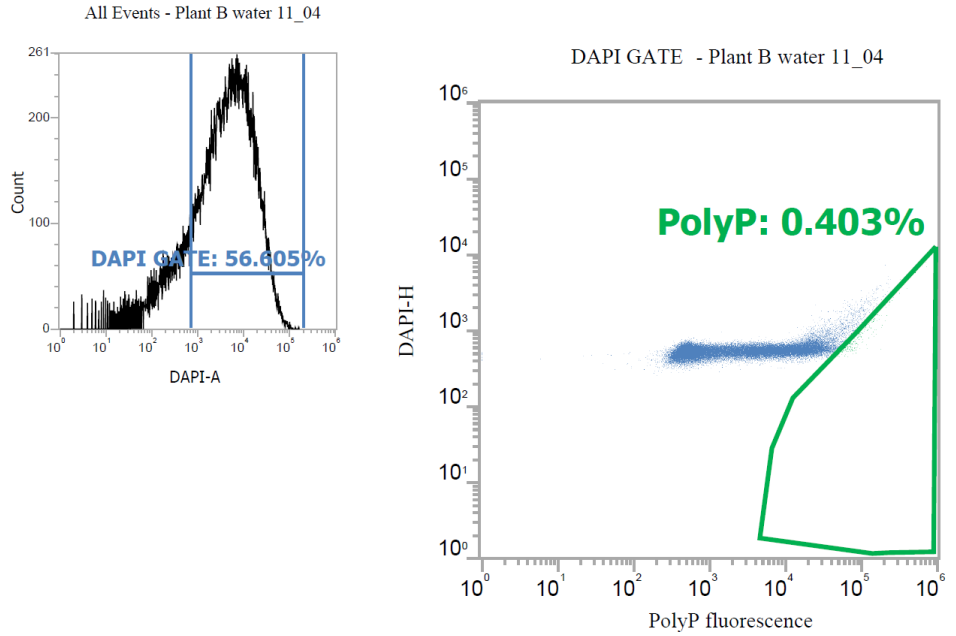
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	134,062	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		65,219	48.648	48.648
PolyP	PolyP	VL3-H	VL1-H	1,526	1.138	2.340

Figure S10. Plant A water (2023.04.06) flow cytometry data



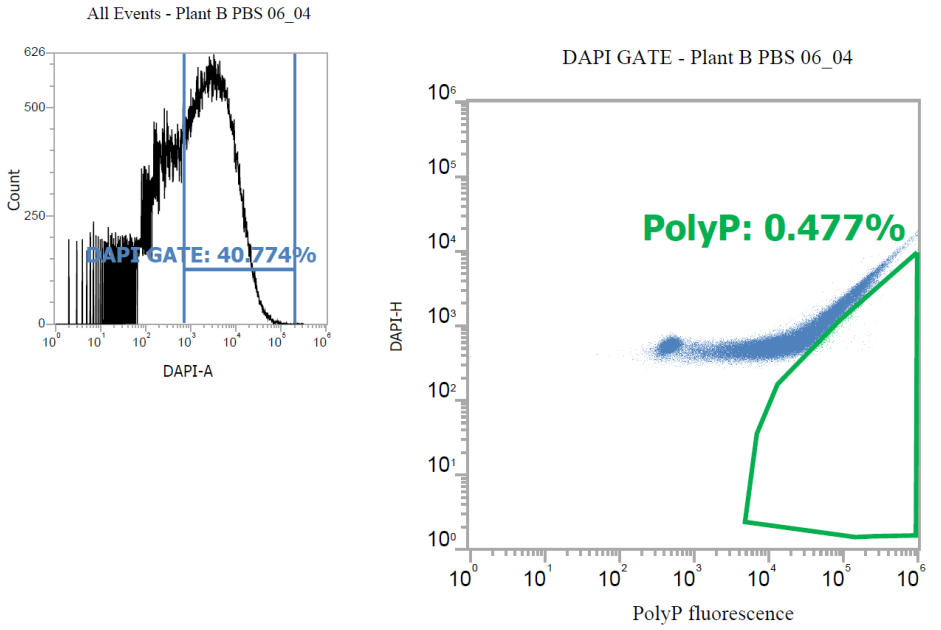
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	317,452	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		131,444	41.406	41.406
PolyP	PolyP	VL3-H	VL1-H	790	0.249	0.601

Figure S11. Plant B PBS (2023.04.11) flow cytometry data



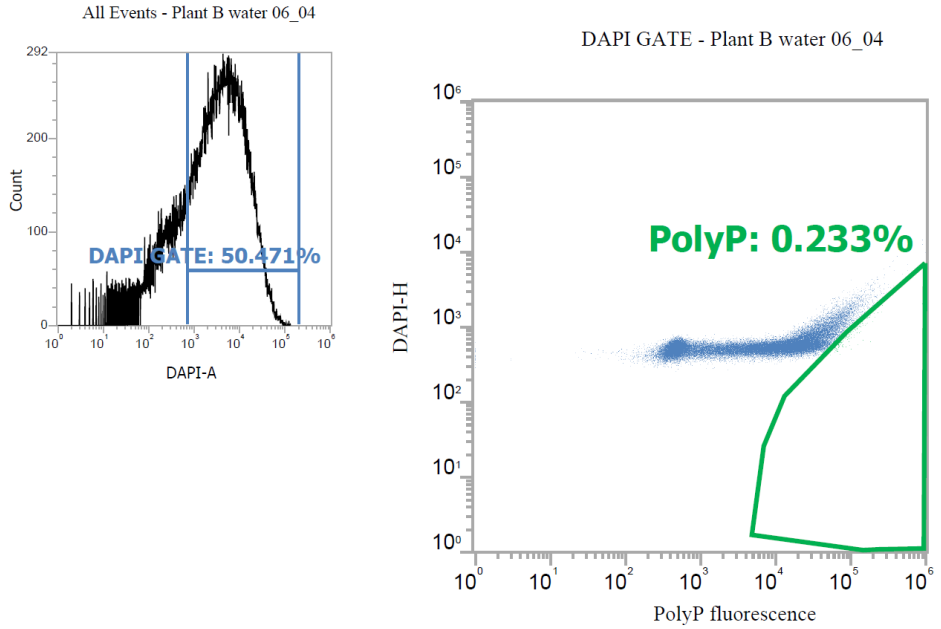
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	93,414	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		52,877	56.605	56.605
PolyP	PolyP	VL3-H	VL1-H	213	0.228	0.403

Figure S12. Plant B water (2023.04.11) flow cytometry data



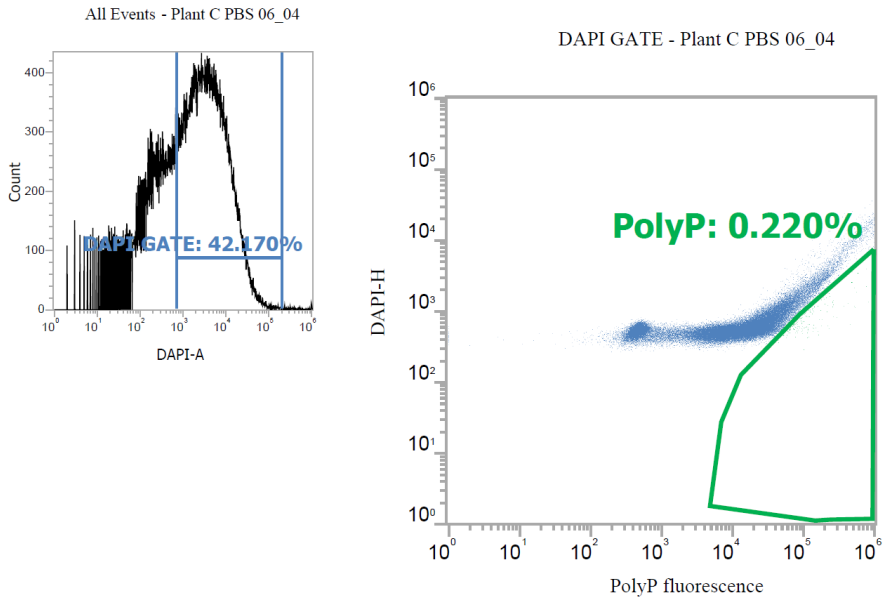
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	299,529	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		122,130	40.774	40.774
PolyP	PolyP	VL3-H	VL1-H	582	0.194	0.477

Figure S13. Plant B PBS (2023.04.06) flow cytometry data



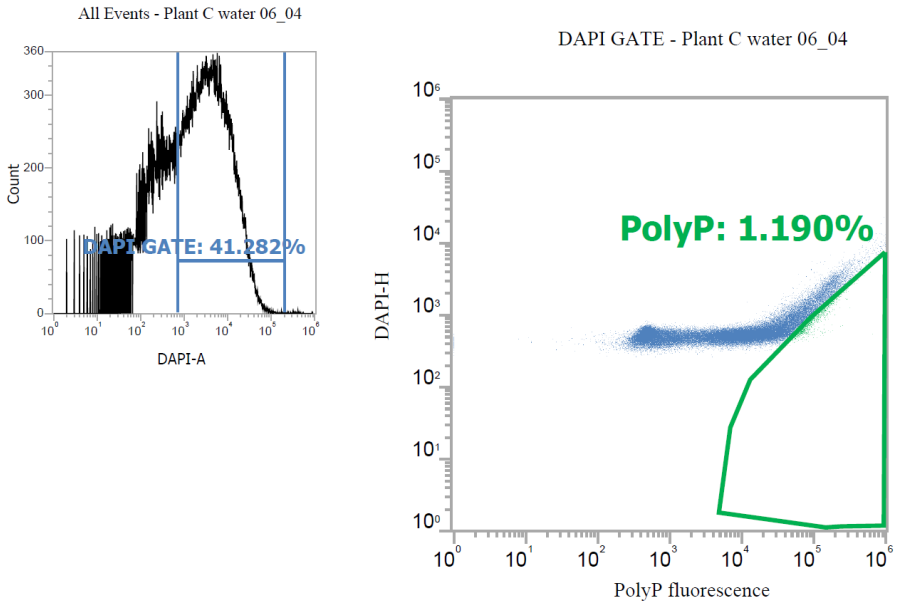
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	119,064	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		60,093	50.471	50.471
PolyP	PolyP	VL3-H	VL1-H	140	0.118	0.233

Figure S14. Plant B water (2023.04.06) flow cytometry data



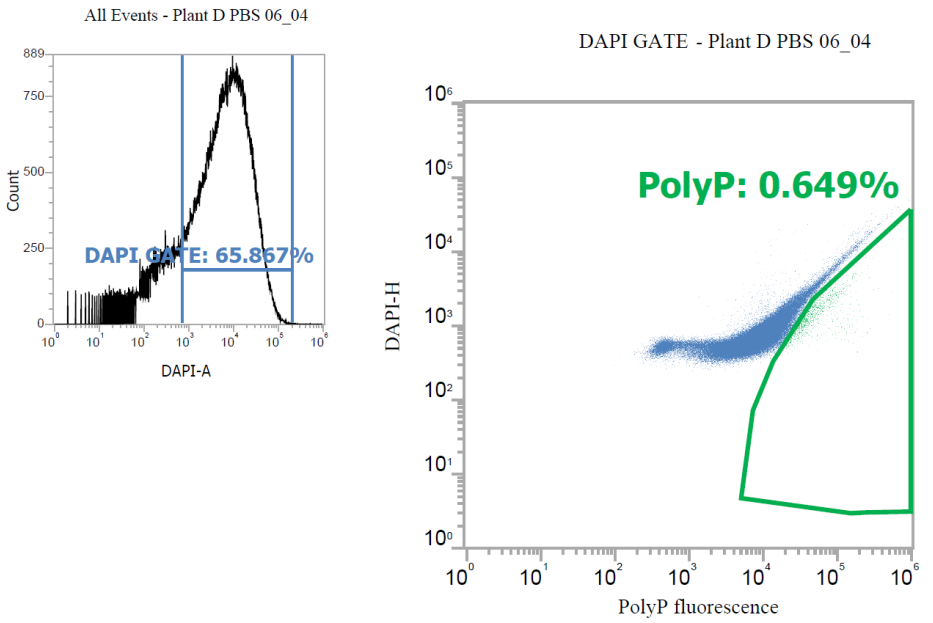
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	209,995	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		88,554	42.170	42.170
PolyP	PolyP	VL3-H	VL1-H	195	0.093	0.220

Figure S15. Plant C PBS (2023.04.06) flow cytometry data



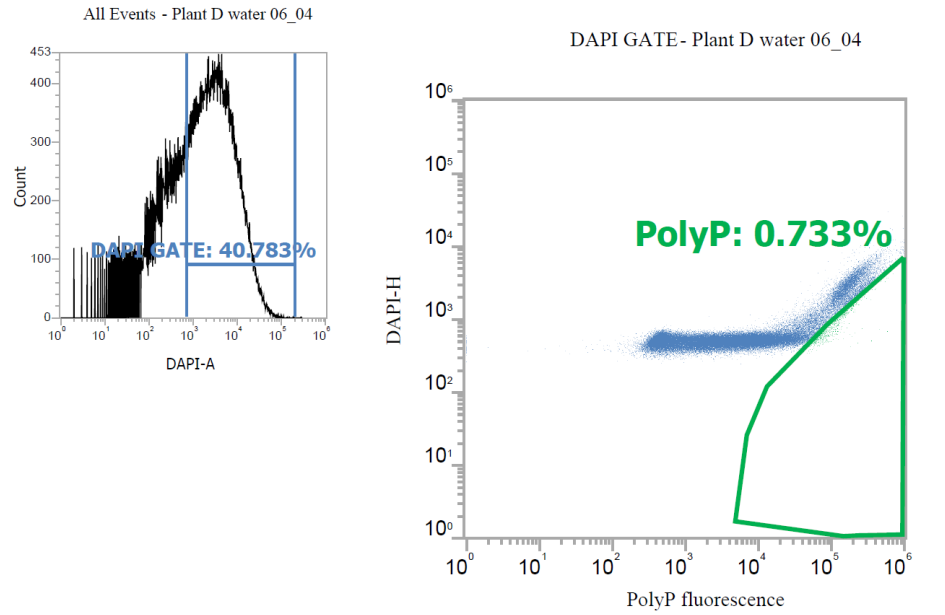
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	183,377	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		75,702	41.282	41.282
PolyP	PolyP	VL3-H	VL1-H	901	0.491	1.190

Figure S16. Plant C water (2023.04.06) flow cytometry data



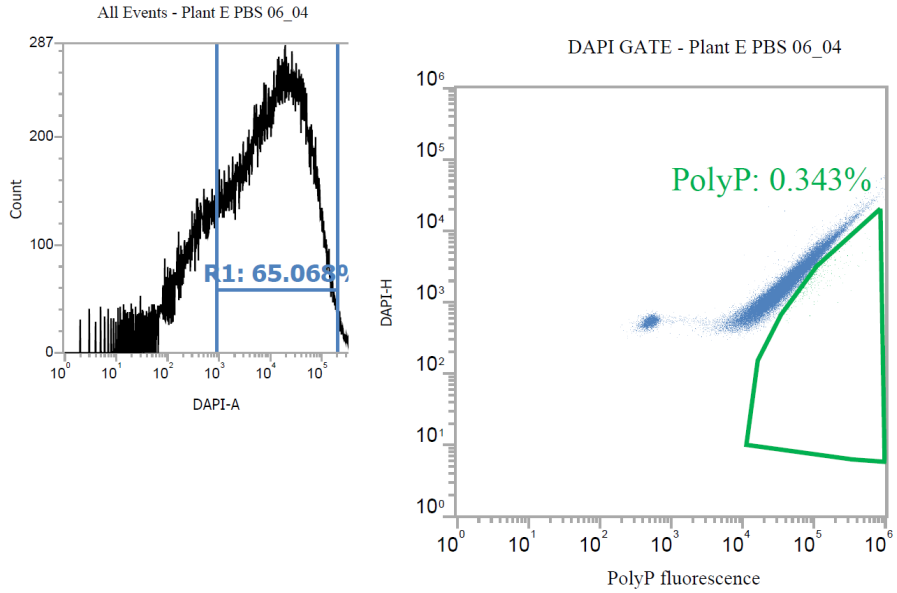
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	280,194	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		184,555	65.867	65.867
PolyP	PolyP	VL3-H	VL1-H	1,197	0.427	0.649

Figure S17. Plant D PBS (2023.04.06) flow cytometry data



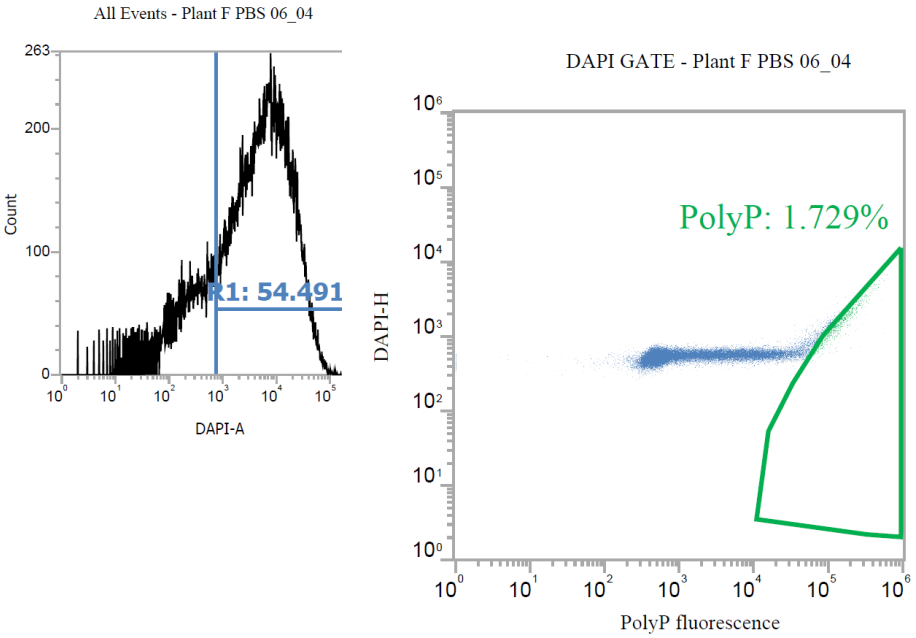
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	211,285	100.000	100.000
DAPI GATE	DAPI GATE	VL1-A		86,168	40.783	40.783
PolyP	PolyP	VL3-H	VL1-H	632	0.299	0.733

Figure S18. Plant D water (2023.04.06) flow cytometry data



Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	113,801	100.000	100.000
R1	R1	VL1-A		74,048	65.068	65.068
PolyP	PolyP	VL3-H	VL1-H	254	0.223	0.343

Figure S19. Plant E PBS (2023.04.06) flow cytometry data



Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	89,454	100.000	100.000
R1	R1	VL1-A		48,744	54.491	54.491
PolyP	PolyP	VL3-H	VL1-H	843	0.942	1.729

Figure S20. Plant F PBS (2023.04.06) flow cytometry data

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24/05/2023