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Leonor Rato Azevedo de Matos

OPTIMIZATION OF PHOSPHATE ACCUMULATING BACTERIAL STRAINS FOR PHOSPHORUS OBTAINMENT IN RESIDUAL WATERS

Dissertação no âmbito do Mestrado em Bioquímica, orientada pela Professora Doutora Paula Maria Vasconcelos Morais e pelo Professor Doutor Harald Weigand, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Resumo

O fósforo é um elemento essencial, que está presente em todos os seres vivos. Paradoxalmente, este elemento é ao mesmo tempo responsável por um tipo de poluição aquática devido a causas antropogénicas, e está em risco de escassez no futuro. As lamas ativadas, usadas em estações de tratamento de águas residuais, são ricas em fósforo e alguns países Europeus têm vindo a implementar legislações que fazem com que seja obrigatória a recuperação de fósforo nas estações de tratamento de águas.

O objetivo deste trabalho é estudar a eficácia de bioaumentação de lamas ativadas numa estação de tratamento de águas residuais de escala laboratorial, para o melhoramento do processo de remoção de fósforo utilizando estirpes de bactérias nativas ou geneticamente modificadas, capazes de remover fósforo de águas residuais, nomeadamente as estipes *Acinetobacter johnsonii* 5bvlmeb2 e *Escherichia coli* BL21_pET30a_*ppk1*, respetivamente.

Nas experiências de bioaumentação, realizada numa estação de tratamento de águas a escala laboratorial, a quantificação diária do fósforo presente no efluente, durante 5 dias, mostrou que a concentração média de fósforo na água, comparada com o controlo, foi reduzida em mais de metade quando se bioaumentou com *E. coli* BL21_pET30a_*ppk1* ao mesmo tempo que a acumulação de polifosfato nas células aumentou substancialmente. Estes resultados indicam que a bioaumentação de lamas ativadas com esta estirpe modificada poderá potencialmente melhorar o desempenho da obtenção biológica de fósforo a partir de águas residuais no futuro e, por isso, mais estudos deverão ser realizados com a estirpe.

Palavras-chave Acumulação de fósforo, lamas ativadas, bioaumentação, gene *ppk1*, economia circular

Abstract

Phosphorus is an essential element that is found in every living entity. Paradoxically, not only is it responsible for aquatic eutrophication, due to anthropogenic causes, but is also at risk of shortage in the future. The activated sludge, produced, during wastewater treatment, is rich in this element and European countries have been implementing legislations making nutrient recovery, i.e. phosphorus, from wastewater facilities mandatory.

The focus of this thesis is on studying native and genetically modified bacterial strains, such as *Acinetobacter johnsonii* 5bvlmeb2 and *Escherichia coli* BL21_pET30a_*ppk1*, respectively, which were show to be capable of removing phosphorus from wastewater. These will be used to bioaugment activated sludge in a laboratory-scale wastewater treatment plant. for the purpose of understanding if the bioaugmentation is efficient or not in improving the phosphorus removal process.

Daily phosphorus quantification of the effluent water from the 5-day bioaugmentation experiments, performed in a laboratory-scale wastewater treatment, showed that the average residual phosphorus concentration was reduced by more than half in comparison to the control when using *E. coli* BL21_pET30a_*ppk1*. It was also shown that the polyphosphate uptake increased substantially. These results indicate that activated sludge bioaugmentation using this modified strain could potentially improve biological phosphorus removal in the future and, for that, more studies should be conducted with more depth.

Key-words Phosphorus uptake, activated sludge, bioaugmentation, *ppk1* gene, circular economy

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Abbreviations

P - Phosphorus

PolyP - Polyphosphate

PAO-Phosphorus-accumulating organisms

PR- Phosphate rock

WWTPs - Wastewater treatment plants

AS - Activated sludge

ATP - Adenosine triphosphate

GTP - Guanosine 5'-Triphosphate

NaOH - Sodium hydroxide

$(NH4)_2S_2O_8$ – Ammonium persulfate

H_2SO_4 - Sulfuric acid

HCl -Hydrochloric acid

CFU - Colony-forming unit

DO- Dissolved Oxygen

BOD- Biological Oxygen Demand

DNA - Deoxyribonucleic acid

ppk gene - Polyphosphate kinase gene

***ppx* gene** - Exopolyphosphatase gene

CRM- Critical Raw Material

CE- Circular Economy

EU- European Union

TSA - Tryptone Soya Agar

TSB- Tryptone Soya Broth

LB- Luria Broth

LAM - Leeds *Acinetobacter* Medium

O.D. 600 - Optical Density at 600 nm

PHA - Polyhydroxyalkanoates

PCR - Polymerase Chain Reaction

Tm – Melting Temperature

bp – Base pairs

TAE – Tris-acetate-EDTA

GFP – Green Fluorescente Protein

PH_3- Phosphine

EBPR- Enhanced Biological Phosphorus Removal

DNA- Deoxyribonucleic Acid

RNA- Ribonucleic Acid

IPTG- Isopropyl β- d-1-thiogalactopyranoside

VFA- Volatile Fatty Acids

EC- European Comission

1. Introduction

1.1. Phosphorus

In 1669, the German alchemist Henning Brandt, in pursuit of a method that could turn metals into gold, discovered white phosphorus (P_4), an elemental form of phosphorus (P), by heating and distilling urine. This method was used for more than a century to produce P until new sources such as bones were found (Ashley et al., 2011). The name phosphorus comes from Greek and translates to "light-bringer" since P_4 is capable of emitting light when in contact with oxygen. P occurs in many allotropic forms and the most common are white, red, and black, with the latter being the least reactive of the three (Wisniak, 2005).

P is an essential non-metallic element to all lifeforms on Earth and plays an important role in numerous biological processes. In the form of phosphate (PO_4^{3-}), phosphorus is present in DNA, RNA, phospholipids, ATP, and GTP (Alewell et al., 2020). Although it is one of the most abundant elements in Nature, the majority of P occurs in the form of minerals such as apatite. Rocks containing this mineral are called phosphate rock (PR) and are limited to specific geographical locations (Figure 1) (Wisniak, 2005).

P has many uses, since it was first discovered, for example: in medicine, military warfare, and agriculture as fertilizers. The latter may appear paradox since P is abundant in Nature but plants can only uptake P when it is in a soluble inorganic form (orthophosphate or reactive phosphorus, meaning it only contains one phosphate unit). During the 18^{th} century, Europe experienced a decline in soil quality which lead to a rise in famine. After it was discovered that P was an essential, non-replaceable element for crop growth, there was a mass production of P fertilizers to increase food production and alleviate hunger (Schroder et al., 2010). With the increase in food production, mankind could no longer rely on the natural concentrations of orthophosphate present in the soil to meet the current needs, and it was forced to depend on the P produced from PR mining. Nowadays, the majority of mined P is used in the food industry to produce fertilizers, the rest is used for other industrial purposes (Ashley et al., 2011).

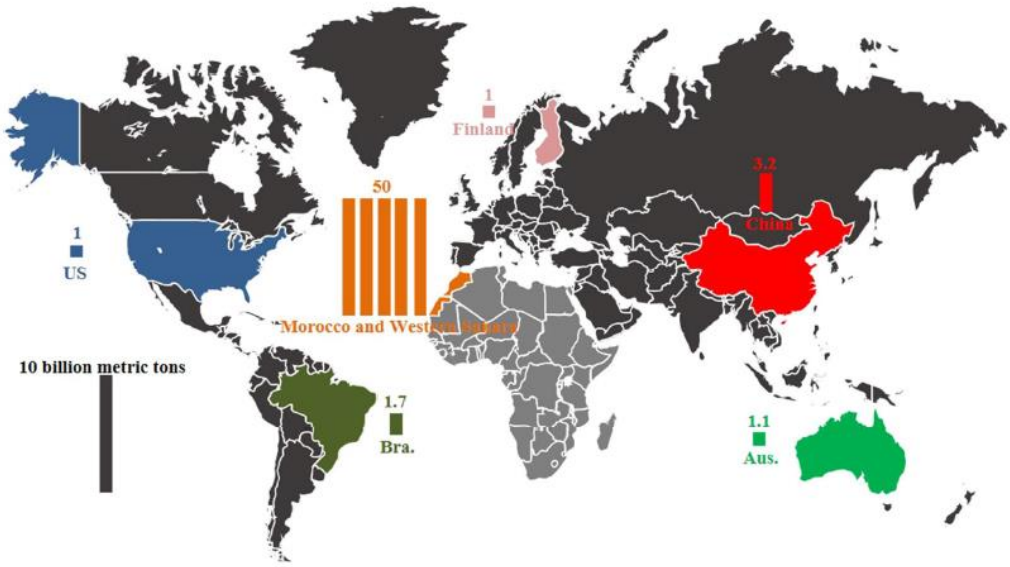

Figure 1. Locations of PR reserves in the world (Meng, 2019).

In the natural cycle, after weathering of parent mineral phases, P is released into soils and waterbodies. In the lithosphere, plants and microorganisms will uptake P and through the food chain, it will find its way to animals. The animals release P back into the environment by excretion and decomposition. Once P reaches the ocean, new calcium phosphate sediments will start to form, and after a period of more than 10 million years, these sediments will become a part of the lithosphere once again (Figure 2) (Liu & Chen, 2008; Meng et al., 2019). Because PR takes millions of years to form it is considered to be a finite resource. This cycle is set apart from other biogeochemical cycles since there is no P flow from aquatic to terrestrial environments as P. This is due to the fact that, although P possesses a gaseous form, phosphine (PH_3), its presence in the atmosphere is not significant to be relevant to the cycle (Fu & Zhang, 2020). Also, unlike other cycles, the reactions that occur in it are mostly based on hydrolysis and although there is more than one possible oxidized state for P, the only one that is found freely in the environment is the most oxidized form (+5 oxidation state) This means that when referring to the P cycle the terms P and PO_4^{3-} are interchangeable (Jahnke, 1992; Jupp et al., 2021).

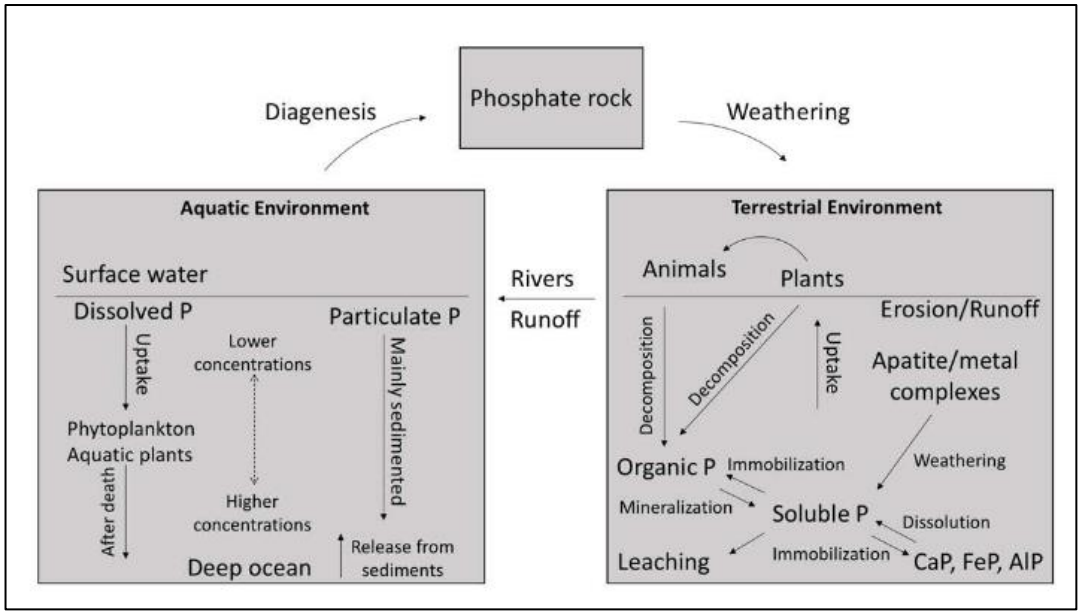

Figure 2. Natural P cycle in the environment (Daneshgar 2018).

1.2. Development of Sanitary Practices

In the course of history, mankind has always desired to have access to clean water and maintaining its quality. Wastewater, which is described by Sonune & Ghate (2004) as "a combination of liquid or water-carried waste" removed from various sources, has always existed. There has not always been concern over the way it was handled, disposed of, and/or treated but disposal practices have advanced, drastically, since the appearance of the first humans (Lofrano & Brown, 2010).

During the European Middle Ages, it was common practice to discharge domestic waste directly into the streets. A small portion of this waste would later be used as fodder and fertilizer, by farmers. The accumulation of sewage soon became unbearable due to the smell and aesthetic and the construction of road pavement only served to make it harder for waste to disperse (Laughlin, 1999). In the modern era, due to a rise in population growth and the insufferable hygienic daily conditions people experienced, epidemics started sweeping Europe and so the French King at the time ordered the construction of cesspools, which were underground tanks designed to contain domestic sewage. This failed to solve the pollution problem since cesspools were seldomly emptied and would overspill thus contaminating nearby water supplies (Lofrano & Brown, 2010). The Industrial Era prompted a massive influx of citizens to European cities, in the 18th

century, this soon led to a public health crisis. A high population density in a region will create high quantities of excrement and manure and since the wastewater management, at the time, was not prepared to accommodate these changes, waterborne diseases were easily spreadable, such as cholera. Although the flushing toilet had gained popularity, in which the waste would go into the recently developed sewage systems and not a cesspool, the sewerage was nevertheless being discharged into the river untreated and the sewers themselves would regularly overflow from the amount of waste going through them daily. This facilitated the contamination of freshwater compartments. For these reasons, it was no longer possible to ignore the consequences of poor sanitation. Furthermore, there was also a need to design new water treatment practices before the discharge in waterbodies. The previously used method of dilution was not adequate for the amount of waste produced (Davenport et al., 2019; Feo et al., 2014; Laughlin, 1999).

The 19th century marked the beginning of what would be called the Sanitary Revolution, during which diseases and water pollution were prevalent in Europe. The treatment procedure relied on filtration and settlement in a septic tank (Ranade & Bhandari, 2014). Paradoxically, although sanitary advances coupled with industrialization helped to improve the standard of living and public health, in general, it also had and continues to have adverse effects on the environment, in particular on the global P cycle.

1.3. Anthropogenic Phosphorus Cycle

Before the development of the agriculture industry and its intensive practices, farmers relied on the, then unknown, intrinsic soil properties; organic waste, namely human excrement and manure (natural fertilizers); and other ancient farming techniques, such as burning (Ashley et al., 2011). For soils to be able to handle this transition it was necessary to continuously supply them with large amounts of synthetic fertilizers, so the nutrients available would never be completely consumed. Synthetic fertilizers are man-made and, compared to natural fertilizers, are easily dissolved allowing nutrients to be instantly available for use. As aforementioned, P is an essential nutrient needed for plant growth, hence the rise in synthetic P fertilizer usage to maintain soil quality and food production. This fertilizer is produced by adding either phosphoric acid or sulfuric acid to the minerals extracted from PR mines (Tripathi et al., 2020).

Due to the excessive P fertilizer usage over natural ones, the growth of the livestock industry as well as the increase in human and animal waste products and the advancements in sanitary measures, the natural P cycle (Figure 3) suffered some disturbances (Wang et al., 2018; Liu & Chen, 2008).

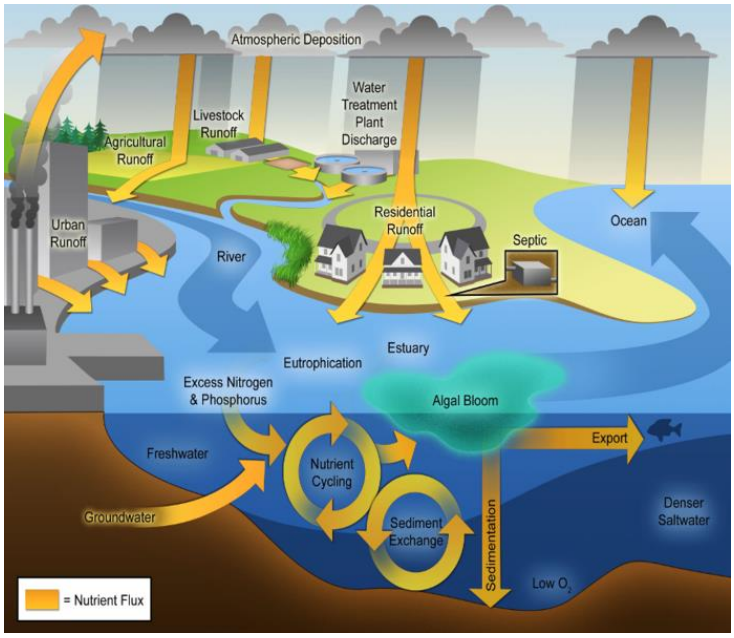

Figure 3. Flow and sources of nutrients that cause eutrophication (Dr. Hans W. Paerl).

The anthropogenic P cycle poses several concerns, two of the most pressing being PR depletion and the P surplus in water reservoirs, causing a type of aquatic pollution named eutrophication (Dorofeev et al., 2020). Whilst eutrophication is a natural aging phenomenon that every aquatic system goes through until its disappearance, the time it takes to set and the extent of the damage depends on numerous factors, such as the degree of nutrient accumulation, the rate of organic matter production by aquatic life and the dissolved oxygen (DO) concentration, all present in the water. Once there is an increase in nutrients, like phosphorus, in the water, algae and aquatic plants will grow unrestrictedly. The excessive growth and following decomposition of algae on the surface will lead to a decrease in the DO available and an increase in ammonia production, which in turn will endanger and kill fish and other aquatic beings because of the creation of anoxic zones. The eutrophication process used to take several centuries to be completed but anthropogenic factors have accelerated it at an alarming rate and make water unsuitable for drinking, fishing, irrigation, tourism, etc., since its quality will be poor (Munn et al., 2018).

There is a need to control P concentration in water bodies to reduce pollution levels and the environmental and economic damages that it causes, given both water and P are vital, non-renewable, resources. For this reason, P is one of the polluters that is analyzed and removed in wastewater treatment plants (WWTPs) (Greeson, 1969).

1.4. Wastewater treatment process

Wastewater treatments happen in a specialized facility called WWTP and are executed with the intention of maintaining public health and providing clean water to the community. Today, with the emergence of new pollutants which persist in water even after going through WWTPs, there was a need for new technologies that would be able to remove them. These are called advanced treatment methods and can be added to the standard treatment procedure, depending on the characteristics of the contaminant. The basis of wastewater treatment (Figure 4) has remained the same and comprises 4 main treatment phases, prior to the discharge or reuse (Drinan & Whiting, 2000).

When wastewater first enters the facility, it is subjected to a preliminary and primary treatment. In these, large and small residues are separated from the water and the biological oxygen demand (BOD) is lowered. BOD represents the oxygen concentration consumed by organisms when they break down organic matter, the higher the BOD value the higher the pollution present in the water (Peirce et al., 1998). Sludge starts to be produced, which is the mixture of residual waste, and the microorganisms present in it that possess relevant biological properties for water purification. Once the sludge enters the secondary, or biological, it will be aerated and thereby converted to the so-called activated sludge (AS). In this step, organic matter is converted into ATP, carbon dioxide and water, through biodegradation processes. There will also be a substantial reduction of BOD at the end of the process. As the AS settles, the water may go through a last phase of treatment, prior to discharge, the disinfection step (Sharma, S. K., & Sanghi, 2012; Sonune & Ghate, 2004).

As mentioned previously, P is considered a pollutant and therefore its concentration must be monitored during the treatment. The majority of P is found in wastewater in either the form of orthophosphate or PolyP ((see abbreviation list and check throughout)), and a small percentage in the form of organic compounds (Ruzhitskaya & Gogina, 2017). P removal in a WWTP can be achieved through more than one method,

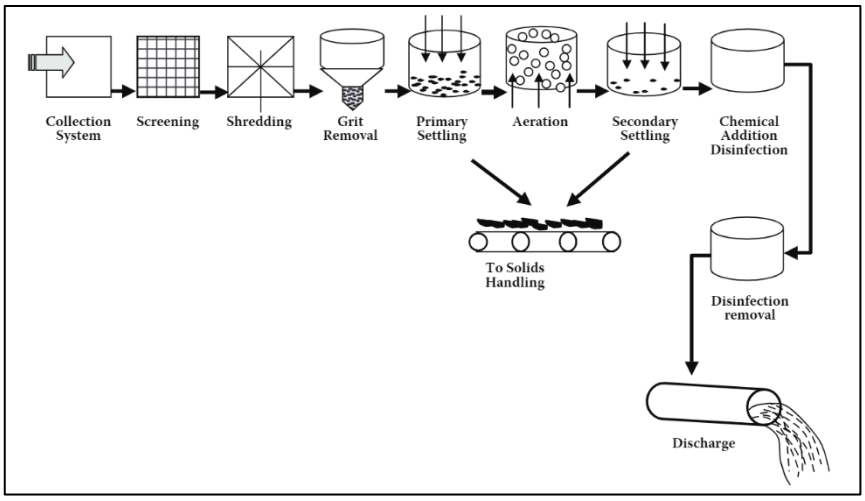

Figure 4. Schematic representation of wastewater treatment steps normally present in a WWTP (Drinan & Whiting, 2000).

such as physical, chemical and biological.

1.4.1 Enhanced Biological Phosphorus Removal

Enhanced Biological P Removal (EBPR) is a biological method that relies on the ability that microorganisms found in AS have to uptake and store P in the form of polyP, beyond the concentration required to fulfil their metabolic needs. PolyP is a linear polymer consisting of inorganic orthophosphates bound together and it can be used as an energy source for the cell (Hirota et al., 2010). The process of uptaking P in excess is referred to as P-luxury uptake and is carried out by P-Accumulating Organisms (PAO) (Izadi et al., 2020; Khoshmanesh et al., 2002).

The method consists of an alternation of anaerobiotic and aerobiotic phases. Although the precise composition of the AS microbiome is not fully known to this day, since most of the organisms present are non-cultivable, there is a standard mechanism the microbial community performs during EBPR, presented in Figure 5 as well as the presence of key genes and enzymes for P reduction (Kulaev et al., 2005). Normally, in the anaerobic phase, fermentative bacteria produce volatile fatty acids (VFAs) that will be consumed by PAOs, as carbon sources. At the same time, polyP, stored in the cells, is

hydrolyzed and released into the water as orthophosphate, glycogen is also degraded. The energy provided from these reactions will allow polyhydroxyalkanoates (PHAs) to be formed from VFAs and stored (Schaum, 2018). In the aerobic phase, PHA are metabolized and the energy released will be greater than the one used in PHA formation (Bunce et al., 2018). This allows for a larger amount of P to be uptaken in this phase than it was previously released in the anaerobic phase, meaning there will be a reduction of the final P concentration in the effluent (Dorofeev et al., 2020; Seviour et al. , 2003)

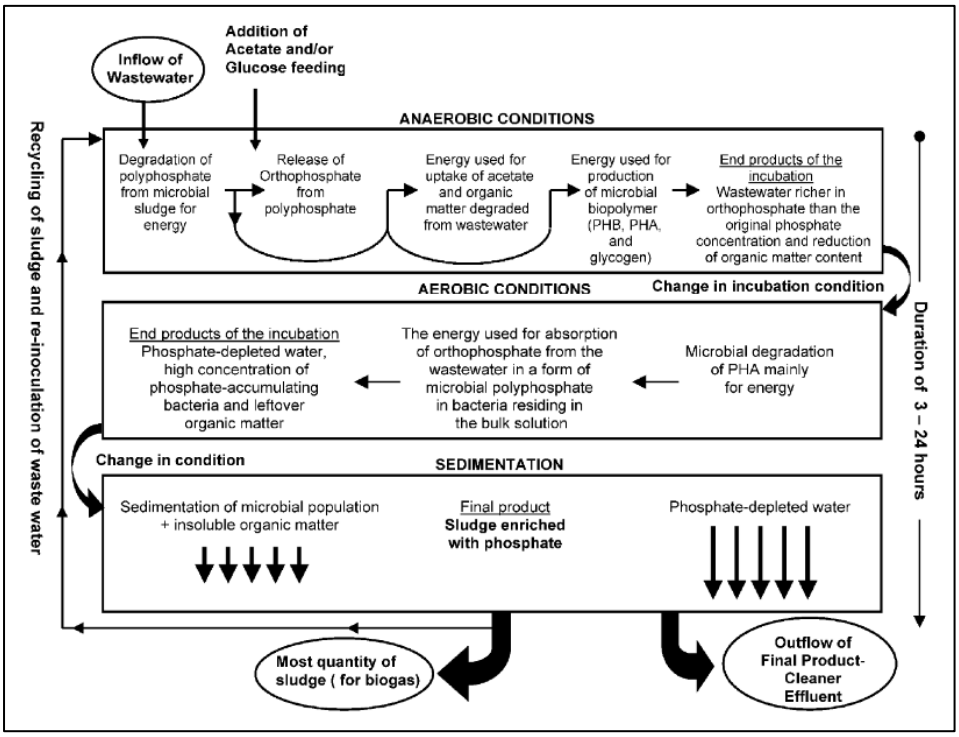

Figure 5. Schematic representation of the EBPR process (Luz & Bashan, 2004).

1.4.2. Bacterial polyphosphate metabolism

As stated previously, P is essential to all organisms and it can be accumulated intercellularly as polyP . More information is known about how polyP enzymes function and what is the polymer's purpose, beyond the established ones, in prokaryotes than in the other two domains (Jiménez et al., 2017; Wang et al., 2018).

Bacteria are known to produce, store and degrade polyP and more than one enzyme has been described in literature linked to their polyP metabolism, some of which are shown in Figure 6. The enzymes PPK1 (polyP kinase 1) and PPK2 utilize ATP and GDP, respectively, to either breakdown or synthetize the polymer and are highly conserved in both eukaryotes and prokaryotes. While PPK1 favors the synthesis of polyP,

PPK2 like PPX (exopolyphosphatase) favor the degradation reaction. In *E. coli*, the *ppx* and *ppk* genes are found in the same operon but the genus is not capable of uptaking P in large amounts, even though it possesses the essential genes for it to occur (Brown & Kornberg, 2008; Wang et al., 2018).

Given that *Acinetobacter* spp. strains have the ability to accumulate high quantities of P in their cells and one of the first genus to be discovered in AS and characterized as PAO, it was believed that they played a major role in the EBPR process in WWTPs (Bunce et al., 2018; Zhang et al, 2019). It was later discovered that the *Acinetobacter* genus only accounts for a small portion of the AS microbiome, which includes not only bacteria but other groups of organisms but this does not discard the genus' potential to improve P removal in the future (Crocetti et al., 2000; Tarayre et al., 2016).

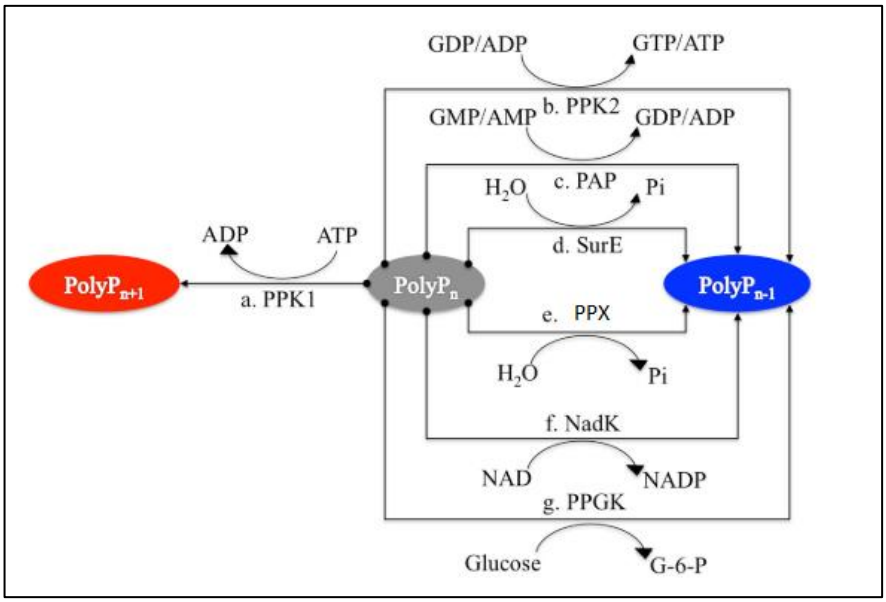

Figure 6. Enzymes involved in bacterial polyp metabolism (Adapted from Wang et al., 2018).

1.5. Phosphorus in a Circular Economy

At the start of the Industrial Revolution, the Earth's resources appeared limitless and that mindset contributed to the implementation of a linear economy, which is still seen today and continues to cause great damage to our ecosystem. This socio-economical behavior follows the "take-make-use-dispose" model, where raw materials are used to create a product and as soon as it is no longer usable, it is discarded as waste (Laumann, 2018).

Industrialization demands continuous mass production and since the European Union (EU) depends, to a large extent, on raw material import, the European Commission (EC) submitted a list of critical raw materials (CRM), under the European Raw Material Initiative (RMI), in 2008. This initiative is meant to devise strategies to alleviate supply dependency and to begin a transition from a linear economy to a new economic model that considers materials' availability and the impact their current life cycles have on the environment, the circular economy (CE) model (Kirchherr et al., 2018). The CRM list is updated according to the selection criteria used to define "critical", that is to be economically relevant and have a high-risk supply (Smol, 2019).

In the last decade, both PR and P have been added to the CRM list, since Europe does not possess any significant PR mines, the only one being located in Finland, and relies solely on imports to meet its P needs. Besides the fact that all P is imported, it is not being efficiently handled. As P is also an irreplaceable nutrient connected to food security and is extracted from a finite resource, there is a need to find new P sources.

Today, besides preventing the spread of diseases and removing or lowering the number of pollutants present in water, there is also a focus on recovery technologies in WWTPs, to lessen the burden on the environment. AS is considered waste and for that reason must be handled accordingly, after its purpose is fulfilled. Although it is considered waste it contains nutrients needed for agriculture, in addition to other hazardous elements. There is more than one option to manage AS and it depends on its final purpose, whether it will be recycled or simply disposed of (Figure 7). In Portugal, AS is mostly used for agriculture, in which it will be applied directly on soils, if it undergoes proper treatment and follows the guidelines established by the legislation Decreto Nº 276/2009 of October 2nd . This is important, since AS does not only contain the nutrients needed for crop growth but also potentially harmful substances and, before its use began being regulated, it was applied in soils indiscriminately, just as human and animal waste had been in the past. In Germany, prior to the approval of the updated "Sewage Sludge Ordinance", a quarter of all sludge produced in WWTPs was disposed of by applying on soils. This legislation aims to completely eliminate the use of AS in soils as fertilizer and take advantage of the fact that AS is rich in P and recover it from WWTPs, transition to the CE model (Günther et al., 2018; Neczaj & Grosser, 2018).

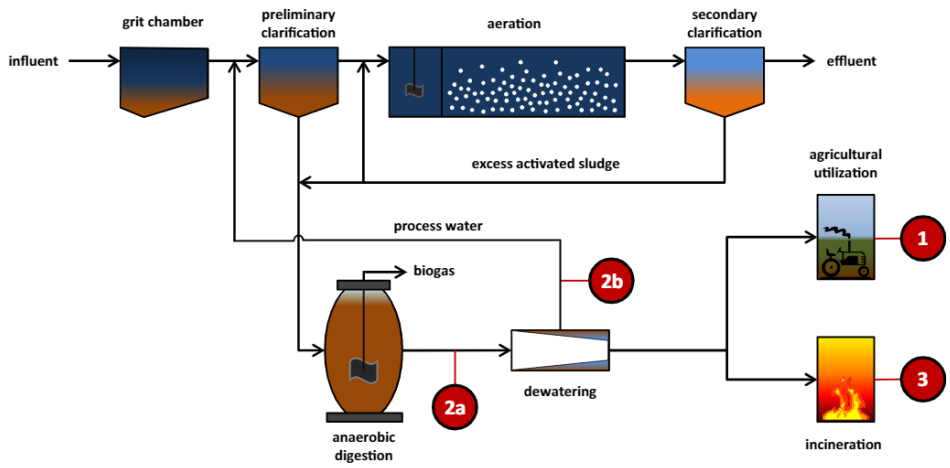

Figure 7. Sources for P recovery during the wastewater treatment. 1- AS usage in soils without previous treatment; 2- P recovery from AS prior to dewatering process (a) and posteriorly (b); 3- P recovery from AS ash (Schoumans et al., 2015).

2. Objective

A previous project, in the Laboratory of Microbiology in the University of Coimbra, studied the capacity of strains isolated from the sludge of a Portuguese WWTP to accumulate P from residual waters in the form of polyP. The presence of the *ppk1* gene in the strains, capable of producing and accumulating polyP, was also verified. In order to overexpress this gene and study the strains' P accumulating profile, the gene *ppk1* was amplified from those strains and expressed in *E. coli* BL21 strains.

This work focuses on the two strains used in the previous project that were shown to accumulate polyP under laboratory-scale conditions: *Acinetobacter johnsonii* 5bvlmeb2 and the *E. coli* BL21 strain containing the *ppk1* gene from the former as a construction. These strains will be studied for their ability to stabilize the biological treatment stage of a laboratory-scale WWTP, with bioaugmentation experiments. Additionally, the efficacy of P removal from the wastewater and polyP accumulation in the biomass will also be followed.

3. Methods and Materials

3.1. Preliminary work

Strains isolated from AS of a Portuguese WWTP (Table 1) were analyzed for potential PAO characteristics. The experiments were performed under aerobic conditions at the laboratory-scale with the intent of developing bioprocesses that would optimize P removal from residual waters, be cost-effective and reduce sludge production, simultaneously.

All the *Acinetobacter* spp. strains studied showed the capacity to uptake P in high amounts but it was the strains from the species *A. johnsonii* that performed best. In order to understand the mechanisms used by these strains for P removal, they were tested for the presence of the *ppk1* gene (Figure 8). The *ppk1* gene from each *A. johnsonii* strain was amplified by PCR with the primers: MA16 CATGCCATGGCTATGGATAATTTTCAGCATTCA and MA17 GCGGGATCCTTAAATTTTGAGTTGCTTCTG. Afterwards, each gene was cloned into a pET30a plasmid and transformed into *E. coli* BL21 strains. The P accumulation potential of the modified strains was shown to be greater than the native strains (Almeida & Morais, unpublished).

Table 1. Strains isolated from AS of a WWTP in Portugal and their respective modified strains (Adapted from Almeida & Morais, unpublished).

Strain name	**Relevant characteristics**
A. guillouiae 7	Isolated from the AS of a WWTP receiving effluents from urban and industrial tannery areas of central Portugal
A. guillouiae 4X	
A. johnsonii 2	
A. johnsonii 2P	
A. johnsonii 5bvlmeb2	
MCA80	*E. coli* BL21 containing plasmid pET30a
MCA81	*E. coli* BL21 containing plasmid pMCA1
MCA82	*E. coli* BL21 containing plasmid pMCA2
MCA83	*E. coli* BL21 containing plasmid pMCA3
Plasmids	**Relevant characteristics**
pET30a	pET30a (empty)
pMCA1	pET30a_Aj2_ppk1; containing *ppk1* from *A. johnsonii* 2 strain

pMCA2	pET30a_Aj2P_ppk1; containing *ppk1* from *A. johnsonii* 2P strain
pMCA3	pET30a_Ajmeb2_ppk1; containing *ppk1* from *A. johnsonii* 5bvlmeb2 strain

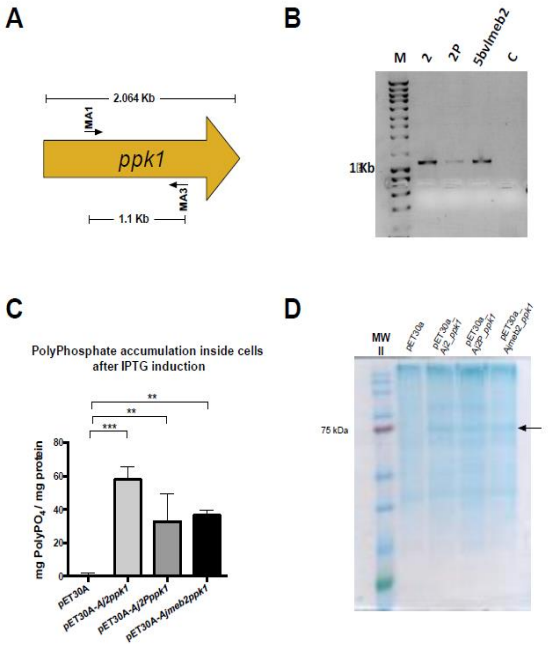

Figure 8. A- Schematic representation of the *ppk1* gene and primers used in cloning experiments. B - Presence of the *ppk1* gene in the isolated strains. C - PolyP accumulation after IPTG induction of modified strains. D- SDS-PAGE of modified strains expressing PPK1 protein (Almeida & Morais, unpublished).

3.2. Laboratory-scale phosphorus removal assays

3.2.1. Bacterial growth for inoculation of synthetic wastewater

The same strains used previously in bioaugmentation experiments were used for the inoculation of synthetic residual water with different K2HPO4 concentrations.

Acinetobacter johnsonii 5bvlmeb2 was grown on TSA plates at 37 °C. To grow the pre-inoculates, bacterial mass was transferred to 100 mL of TSB medium. After 24 hours of growth, the OD_{600} was read to determine the necessary volume to inoculate 400 mL of TSB with a final OD_{600} of 0.1. The pellet acquired after centrifuging for 15 minutes at 10.000 rpm was stored at 4 °C.

E. coli BL21_pET30a_*ppk1* was grown on LB plates at 37 °C. To grow the pre-inoculates, bacterial mass was transferred to 100 mL of LB medium with 100 µl of kanamycin (50 mg/mL). The OD_{600} was read to determine the necessary volume to inoculate 400 mL of LB with an OD_{600} of 0.1 and after 7 hours of growth, the pellet acquired after centrifuging for 15 minutes at 10.000 rpm was stored at 4 °C.

3.2.2. Synthetic residual water preparation

Distilled water was autoclaved at 121 °C, for 20 minutes. The ingredients from Table 2 were stored in a sterile compartment depending on the K_2HPO_4 concentration desired and added to the sterile water on the day of start of the experiment. By not autoclaving the synthetic residual water we ensure that there will not be a decrease in the P concentration present since high temperatures can lead to P precipitation (Wang et al., 2018).

3.2.3. Phosphorus Uptake Assay

To obtain the strains' P uptake profile, bacterial suspensions of each strain were inoculated into 100 mL of synthetic residual water, prepared previously. The OD_{600} value was adjusted to 0.5 and 100 µL of IPTG - Isopropyl β- d-1-thiogalactopyranoside - (0.5 M) (Nzytech) were added at the start of the experiment at room temperature and 130 rpm. For *E. coli* BL21_pET30a_*ppk1*, the experiment was also performed at its optimum temperature of 37 °C. Residual water without inoculation was used as a negative control.

3.2.4. Phosphorus and polyphosphate quantification

Samples collected at 0, 3, 6 24, 27, 30, 48, 72, and 96 hours of the WWTP were centrifuged at 10.000 rpm for 15 minutes. The pellet was used for polyP extraction and quantification. The protocol, which does not consider iron interference, is described in Aravind *et al*., (2015). Briefly, a known volume of NaOH is added to the pellet overnight in a shaker. This step is necessary to burst the cells and release the polyP therein accumulated. After centrifugation, the pellet is weighted and the supernatant is divided into 2 parts: one for direct P quantification and the other for hydrolyzation. In the hydrolyzation step, the same volume of HCl is added to the supernatant followed by incubation for 10 minutes at 100 °C. This method only allows for the quantification of the hydrolyzed phosphorus and the orthophosphate (non-hydrolyzed phosphorus). After the samples are prepared, the polyP is quantified using the molybdenum-blue method

(International Organization for Standardization, 2004) and the result is expressed in mgP/g. The P concentration in the supernatant is also quantified with the molybdenum-blue method, although directly and the result is expressed in mgP/L.

3.2.5. Evaluation of PPK1 protein's heterologous expression

Sterile distilled water (500 μL) was added to the samples for the freeze/thaw cell lysis method. Briefly, after vortexing the samples, these were frozen at -80 °C for 15 minutes and then boiled at 100 °C also for 15 minutes. This cycle was repeated two more times. When the last cycle was completed, the samples were centrifuged and the supernatant was kept. The supernatants were used for quantification using the Bradford method and the protein mass was adjusted for SDS-PAGE.

In order to visualize the PPK1 protein expression, the supernatants (10 μg of protein), were run by SDS-PAGE in a 12 % acrylamide gel for 90 minutes at 180V in running buffer (5 mM Tris-HCl pH 8.8, 0.2 M glycine, 3.5 mM sodium dodecyl sulfate (SDS)). Afterwards, the gel was stained with Coomassie blue previously preheated at 50 ºC for 30 minutes at room temperature and then left in a destaining solution for 24 hours.

3.3. Phosphorus removal in a laboratory-scale wastewater treatment plant

EBPR can be improved through biotechnological methods such as the construction of genetically modified organisms capable of removing pollutants in the water (Dorofeev et al., 2020). Based on the results obtained previously, regarding the capacity of *A. johnsonii* 5bvlmeb2 and *E. coli* BL2 1_pET30a_*ppk1* to remove P from residual waters, they were chosen for two types of experiments, both in the same equipment: bioaugmentation using AS from Giessen's WWTP, in Germany and upscaling.

Upscaling P removal from small-scale experiments to an EBPR laboratory-scale will allow us to determine whether the results seen previously can be reproduced in a scale similar to a real wastewater treatment. Bioaugmentation is described as the addition of microorganisms capable of removing difficult pollutants from the water, in a cost-efficient and eco-friendly way (Nzila et al., 2016). In this case, it is performed to

understand if the chosen strains are capable of surviving alongside the AS's microbiome and if so, if they can improve the overall P removal process.

3.3.1. Equipment

The experiments were performed in a behrotest® KLD 4 N/SR (behrLabor-Technik GmbH, Germany) and consisted of (i) a control, where only AS was used, (ii) an inoculated variant of AS the with the 2 strains separately and (iii) an inoculated variant with only the native strains (*A. johnsonii* 5bvlmeb2).

The behrotest® KLD 4 N/SR (behrLabor-Technik GmbH, Germany) laboratory-scale WWTP used during the bioaugmentation experiments was designed to simulate the biological phosphorus removal process, with its cycle of anaerobiosis/aerobiosis (Figure 9). The equipment has 3 main tanks for the anaerobic, aerobic, and settlement stages, 2 storage tanks for influent and effluent and 3 pumps.

Prior to the start of the experiment, the tanks were filled with a total of 11 L of AS, collected from Giessen's WWTP. Afterwards, 35 L of synthetic wastewater (OECD, 2010) (Table 2) were added daily to the influent tank and a pump began transferring it to the aeration tank. As the volume in the anaerobic tank rose, both the sludge and wastewater proceeded to the aeration tank. At the same time, as the volume increased, the content also flowed towards the settlement tank. The sludge then settled settle at the bottom of the settlement tank and returned to the first tank through a pump. The water, now free of phosphorus, was stored in the effluent tank. This cycle was repeated as many times as desired as long as there was wastewater available in the influent tank.

In the anaerobic tank, microorganisms present in the AS are supposed to uptake organic nutrients and utilize the accumulated polyP as a source of energy. While in the aeration tank, the microorganisms accumulate P from the water by using the organic nutrients stored in the anaerobic step, as a source of energy. The oxygen concentration is regulated by a sensor and set to the optimal value (2 mg/L). The experiments were run without temperature control, i.e. at room temperature. In the settlement tank, AS, now rich in P, deposits at the bottom due to gravity and precipitated and is ready to be returned to the anaerobic tank to release its P, through a pump that connects the two tanks. The water, now phosphorus-free, is stored in the effluent tank.

For each experiment, water is added daily to the effluent tank and the pumps' flux is adjusted depending on the consistency of the sludge (Table 3).

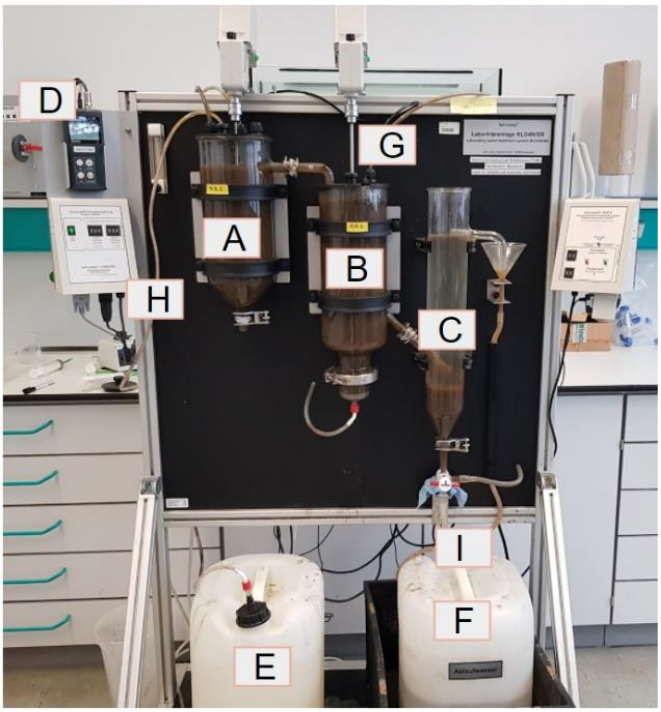

Figure 9. Behrotest™ laboratory-scale WWTP. A - Anaerobic tank (4,2 L); B - Aerobic tank (5 L); C - Settlement tank (1,8 L); D - DO regulator/ thermometer; E- Synthetic wastewater tank; F - Clean water tank; G - Nitrate recirculation pump; H - Synthetic wastewater pump; I - Settlement recirculation pump.

Table 2. Synthetic residual water medium composition (OECD, 2010)

Ingredients	**(mg/L)**
Peptone	160
Meat extract	110
Urea	30
K_2HPO_4	28
NaCl	7
$CaCl_2*2H_2O$	4
$MgSO_4*7H_2O$	2

Table 3. Conditions of each experiment performed in the behrotest® KLD 4 N/SR

Experiments	**Medium flux (L/h)**	**Nitrate recirculation flux (L/h) ((don't get the meaning of this))**	**Settlement recirculation flux (L/h)**
AS from Giessen's WWTP	1.5	2.9	1.5
Bioaugmentation of AS with *E. coli* BL21_pET30a_*ppk1*	1.21	1.21	1,7
Bioaugmentation of AS with *A. johnsonii* 5bvlmeb2	1.1	2.4	3.4
A. johnsonii 5bvlmeb2	1.1	2.4	3.4

3.3.2 Batch-scale bacterial growth for inoculation in bioaugmentation experiments

The wild strain *Acinetobacter johnsonii* 5bvlmeb2 and the respective modified strain: *E. coli* BL21_pET30a_*ppk1* were chosen for the bioaugmentation experiments due to their high accumulation capabilities identified in a previous study by Almeida & Morais (unpublished).

Acinetobacter johnsonii 5bvlmeb2 was grown on TSA plates at 30 °C. To grow the pre-inoculum, bacterial cells were transferred to 100 mL of TSB medium and after 24 hours of growth, in batch conditions, the OD_{600} was determined to inoculate 11L of TSB with an initial OD_{600} of 0.1. The pellet, acquired after centrifuging the growth for 15 minutes at 10.000 rpm, was stored at 4 °C.

E. coli BL21_pET30a_*ppk1* was grown on TSA plates, at 30 °C. To grow the pre-inoculum, bacterial cells were transferred to 100 mL of TSB medium in batch conditions. After 24 hours, the OD_{600} was determined to inoculate TSB with an initial OD_{600} of 0.1. Before the bioaugmentation experiment, 2 mg/L of lactose (an analogous of IPTG) was added to the inoculum alongside AS, for 3 hours, to induce the strain MCA83. The pellet, acquired after centrifuging the growth for 15 minutes at 10.000 rpm, was stored at 4 °C.

3.3.3. Sampling to follow P removal and uptake

Samples of each experiment were taken daily, i.e. at 0, 24, 48, 72, and 96 hours. The samples were collected from the anaerobic tank the aerobic tank and the effluent. Afterwards they were be centrifuged and both the biological fraction (pellet) and the supernatant were used for determination of polyP concentration inside the cells and P concentration in the water, respectively. Duplicates were stored at -20 °C, for posterior analysis such as CFU determination; DNA extraction and quantification and amplification of the *ppk1* gene.

3.3.3.1. Polyphosphate extraction and quantification

The method for polyP extraction and for the P and polyP quantification is, in part, the same as the one used in Laboratory-scale phosphorus removal assays. Since Giessen's WWTP uses a high amounts of iron during P removal, in the bioaugmentation experiment using *E. coli* BL21_pET30a_*ppk1*, the preparation for polyP quantification was performed with a second method, although the cell lysis method, for polyp release, at the beginning, remained the same as in Aravind *et al*., (2015).

The second protocol which considers iron interference, is from the U.S. Environmental Protection Agency (EPA, USA). Following the addition of NaOH to the pellet, the supernatant obtained from centrifugation is diluted to 50 mL, if necessary, and the dilution is added to 1 mL of 5.5M of H_2SO_4 and 0.4g of $(NH4)_2S_2O_8$. If there is iron in the sample, 5 mL of sodium bisulfite is added to the mix and placed at high temperatures for half an hour.

3.3.3.2. Phosphorus removal efficiency

The daily phosphorus removal from the wastewater was calculated considering the concentration in the supernatant at the start of the experiment and the effluent concentration of the next day. The initial amount of phosphorous in the system results from the sludge inoculation process and the concentration present in the synthetic residual water added daily (influent).

3.4. Biological parameters

3.4.1. Microorganisms enumeration by Colony-Forming Units

The control and the pellets of the bioaugmented samples were weighted and added to 1 mL of sterile water. From this, ten-fold dilutions were prepared and 100 µL from the sludge suspensions was used to inoculate TSA medium, in triplicate. After 24 hours of growth, at 37 °C, the number of colonies were counted and the values of CFU (Colony-Forming units) per dry weight (g) were calculated.

Since the apparatus was not sterile, in the case of the experiment with only the native strain, two different media were prepared to determine which one is better suited for the growth and CFU calculation of *Acinetobacter* spp.: TSA and LAM with vancomycin (10 mg/L), cefsulodin (15 mg/L), and cephradine (50mg/L) (Jawad et al., 1994). The latter was prepared as stated in Jawad et al., 1994. The CFU values were calculated per volume (mL).

3.4.2. DNA extraction and quantification

DNA was extracted from the samples by using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek), according to the manufacturer's instructions. DNA from *A. johnsonii* 5bvlmeb2 was extracted through the boiling method. Afterwards, each DNA sample was added to a Thermo Scientific NanoDrop™ 2000c Spectrophotometer to measure the concentrations (ng/µL) and purity ratios.

3.4.3. Amplification of *ppk1* gene

PCR was accomplished in a MyCycler™ Thermal Cycler (BioRad). For the purpose of choosing the most suitable Taq DNA Polymerase, two different protocols were used for the reaction. In the first, the samples were prepared by mixing 6.25 µL of NZYTaq II 2x Green Master Mix (Nzytech); 1 µL of each primer (10 µM); 3 µL of DNA template and the necessary volume of sterile miliQ water to bring the final volume to 30 µL. In the second, the samples were prepared by mixing 5 µL of Reaction buffer 10×; 2,5 µL of $MgCl_2$ (50 mM); 2 µL of dNTPs mix; 2 µL of each primer (10 µM); 1 µL of Supreme NZYTaqII (Nzytech); 2 µL of DNA template and the necessary volume of sterile miliQ water to bring the final volume to 50 µL. DNA extracted from *A. johnsonii* 5bvlmeb2 was used as a positive control.

The primer set used in the PCR was previously designed by Almeida & Morais (unpublished) by using the *ppk1* gene of *A. johnsonii* XBB1 as reference (accession number in NCBI: NZ_CP010350.1: 86315-88378).

The reaction started with an initial denaturation, at 95 °C, for 5 minutes, followed by 30 cycles compromising of: denaturation, at 95 °C; annealing, at 52 °C, and extension, at 72 °C. A final extension occurred, at 72 °C, for 7 minutes. PCR products were run in 1% agarose gel with 1X TAE buffer, at 80 mV, for 1 hour. Due to the ethidium bromide present in the gel it was possible to observe the bands with Imager® ChemiDoc™ XRS System (BioRad).

The PCR amplicons from the positive control and one sample, which showed a band in the with the same weight as the control, were cut from the gel and purified using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek), according to the manufacturer's instructions. To prepare samples for sequencing, 10 µL of purified product and 3 µL of the forward primer were added to a sterile Eppendorf and submitted to Stabvida Lda. (Caparica, Portugal). The results were analyzed by using MEGAX software together with the BLAST program from National Center for Biotechnology Information (NCBI).

Table 4. Information about the set of primers used for PCR amplification of *ppk1* gene.

	Primer Identification	**Sequence (5'-3')**	**Anneals to**	**Tm (°C)**	**Organism**	**Amplicon size**
MA1	MA1_ppk_fwd	CTGTTTCCAGGGATGAAAGC	*ppk1*	53.8	*A. johnsonii*	1 Kbp
MA3	MA3_ppk_rev	CCACAATCGAACGCACACG		57.2		

3.5. Statistical Analysis

Statistical analysis was performed in Microsoft® Excel®. The statistical difference of the CFU/mL values between the bioaugmented samples and the control were evaluated through a one-way ANOVA, with a significance level of 0.05. Since the difference was significant ($p < 0.05$), another test was also performed: two different t-tests for independent means, with a significance level of 0.05. The statistical difference between the P decrease in the effluent during the two bioaugmentation experiments were compared to the control, through t-tests for independent means, with a significance level of 0.05. Finally, the statistical difference between the CFU/mL values from the *A. johnsonii* 5bvlmeb2 upscale P uptake experiment, in two different mediums were analyzed through a t-test for independent means, with a significance level of 0.05.

4. Results and discussion

4.1. Laboratory-scale phosphorus removal assays

4.1.1. Phosphorus Uptake Assay

In Almeida & Morais (unpublished), the native strains tested were inoculated in residual waters ranging from high to low concentrations of dissolved P, at room temperature. After analyzing the strains' P removal results, *A. johnsonii* 5bvlmeb2 was shown to remove nearly all the P present in low-P waters, up to 2 mgP/L, and in P-rich waters, it was able to remove almost 40% of it, meaning 8 mgP/L, during the 24th and 48th hour of the experiments. It is important to note that the average P concentration present in wastewaters is between 5 and 10 mg P/L and it should be lowered to at least 1 mg P/L before being released into the environment (Dorofeev et al., 2020).

The P removal experiments from Almeida & Morais (unpublished) were reproduced in this project, where synthetic residual waters with 3 different concentrations were used, although in this case they were not supplemented with 1 % sodium acetate. After sampling, both the pellet and the supernatant were used for polyP and P quantification, respectively. *A. johnsonii* 5bvlmeb2 showed a similar removal pattern to Almeida & Morais (unpublished), since there was a decrease in the P concentration at the 3rd hour of the experiment, due to bacterial uptake. After this, the P concentration started increasing meaning *A. johnsonii* 5bvlmeb2 released P into the water but at the 48th hour a decrease was seen again. The P removal values were not as high as in Almeida & Morais (unpublished), with the lowest being 2 mgP/L and the highest, 4.7 mgP/L (Figure 10). As mentioned above, the water used was not supplemented with sodium acetate, which acted as an added carbon source for the P uptake experiments in Almeida & Morais (unpublished) (Seviour et al., 2003).

Figure 10 also showed that the initial P concentration in the water appeared to be correlated with the time at which the best removal value occurred. Inoculating *A. johnsonii* 5bvlmeb2 in initial P concentrations of 5.5; 10 and 30 mgP/L produced the best removal values at the 48th, 24th and 3rd hour, respectively. Polyp quantification was performed from the pellets of these 3 samples showing that as the initial P concentration increases so does the polyp concentration in the cell, i.e. 0.27; 0.61 and 0.81 mg polyP/g.

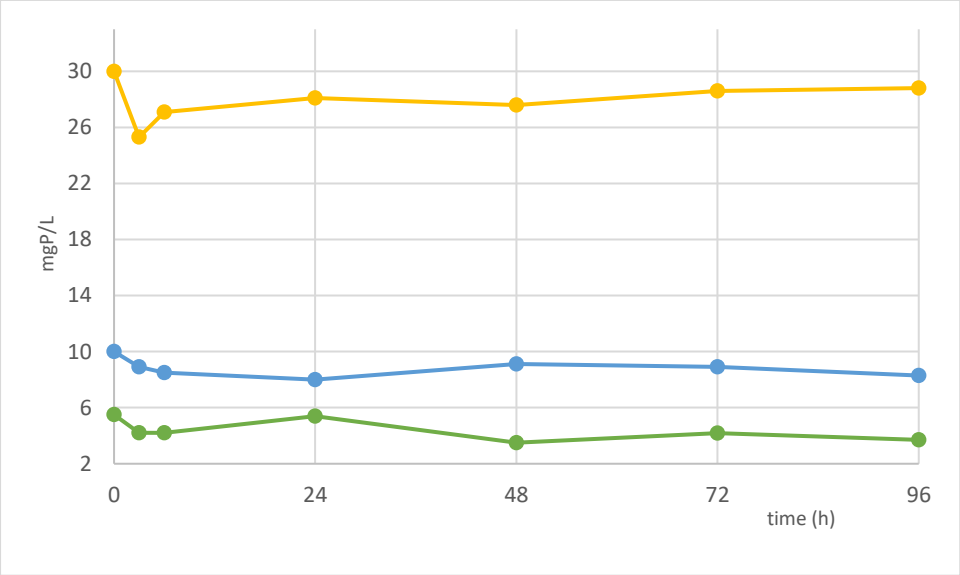

Figure 10. P removal by *A. johnsonii* 5bvlmeb2, in synthetic residual waters with different concentrations, at room temperature and 120 rpm, for 5 days.

In the case of the selected modified strain, *E. coli* BL21_pET30a_*ppk1*, the only data available was from its P accumulating capacity at the optimal growth temperature for *E. coli* (37 °C) and in LB medium supplemented with 0.25 g/L of KH2PO4. Since those polyP accumulation values will not be reflected in a WWTP scenario, *E. coli* BL21_pET30a_*ppk1* was inoculated in synthetic residual water (Table 2) both at room temperature and at its optimal growth temperature. The time frame of this experiment was only for 24 hours and the P concentration in the water was much higher than in the experiment with *A. johnsonii* 5bvlmeb2. This was performed in order to determine how much P would the strain removes from P- rich waters. *E. coli* BL21_pET30a_*ppk1* was able to remove P more efficiently and quicker at 37 °C, with 10 mg P/L removed at 4 hours. Since E. coli grows slower at lower temperatures, it is expected more time will be needed for the strain to start removing P from the water. This is shown in Figure 11, where in the experiment at room-temperature, compared to the one at 37 °C, it takes 24 hours for P uptake to start. In this case, not only it takes longer for P removal but it also removes less than in optimal conditions, being able to remove 6.5 mgP/L.

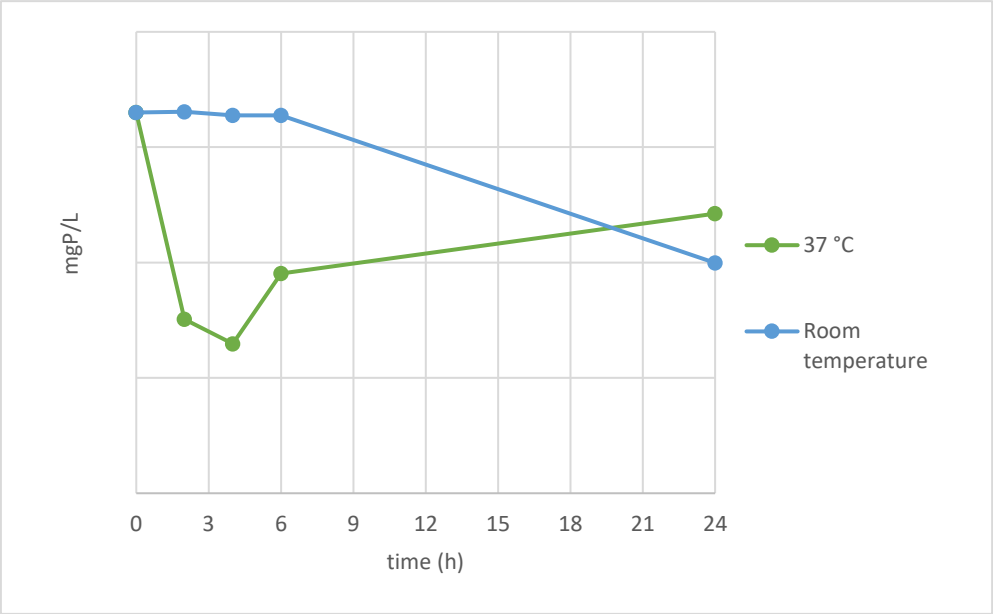

Figure 11. P removal by induced *E. coli BL21_pET30a*_ppk1, in synthetic residual water with a high P concentration, at different temperatures, at 120 rpm, for 1 day.

4.1.2. Evaluation of PPK1 heterologous expression

To determine if the P removal values seen in the previous experiment, using *E. coli* BL21_pET30a_*ppk1*, was due to the presence of the protein PPK1, produced by the expression of *ppk1* gene present in the strain, an SDS-PAGE was performed. Total protein was extracted from the strain pre-IPTG induction with an O.D.600 of 2.6 and 6 hours after induction. Additionally, total protein was extracted from the same strain also 6 hours post-induction but that had been induced at a lower O.D.600, of 0.3. This was done to understand how the initial O.D.600, at which the strain is induced, influences PPK1 expression. An SDS-PAGE was run using 10 µg of total protein from each sample. Figure 12 shows 3 bands around 75 kDa, corresponding to the molecular weight that Almeida & Morais (unpublished) had predicted for the PPK1 protein, based on the gene's sequence. Inducing the strain at an initial O.D.600 of 0.3, rather than at a later cell density, appears to lead to the best overexpression results.

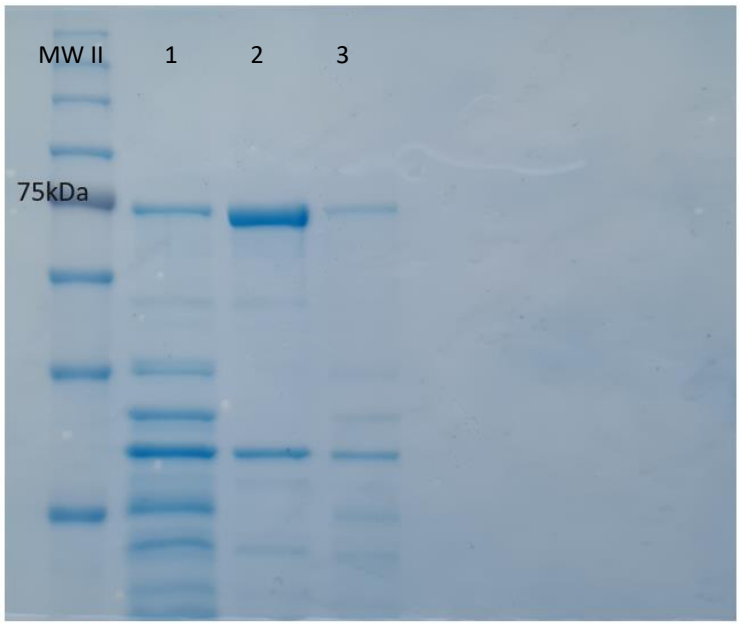

Figure 12. SDS-PAGE of comparison of PPK1 expression *E. coli* BL21_pET30a_ppk1: pre-induced,, with an O.D.600 of 2.6 (1); 6 hours after *E. coli* BL21_pET30a_ppk1 induction, with cell density (O.D.600) of 0.3 at the moment of induction (2); 6 hours after *E. coli* BL21_pET30a_ppk1 induction, with cell density (O.D.600) of 2.6 at the moment of induction (3). MWII – NZYColour Protein Marker II. For the experiment, 10 µg total protein of each sample was used;

4.2. Laboratory-scale wastewater treatment station

4.2.1. Phosphorus removal efficiency and polyphosphate accumulation in the cells

The following experiments aimed to confirm whether the P removal observed so far on a small-scale could be reproduced on a larger-scale. The larger-scale used is comparable to EBPR in a WWTP and not just under aerobic conditions. *A. johnsonii* 5bvlmeb2 and *E. coli* BL21_pET30a_*ppk1* were both used in bioaugmentation experiments with AS from Giessen's WWTP and *A. johnsonii* 5bvlmeb2 was also used in an upscale experiment. Although the bioaugmentation control was not performed at the same time it serves as a reference for the normal P removal and polyP accumulation values seen in Giessen's AS's native microbiome.

During the first 24 hours of every bioaugmentation and upscale experiment, except for the bioaugmentation control, there was a substantial amount of P present initially in the machine. This was due to the nature of the inoculation step, where the

medium used for the inoculate growth was also added to the behrotest® KLD 4 N/SR. At the beginning of every experiment, there was no separation between the anaerobic, aerobic and settlement tanks, since the P concentration was equal in them. This meant the supernatant from the 0 hour represented the whole and not just the aerobic tank, from which the samples would be taken from, from that moment onwards. To calculate the P concentration that would pass through the machine in the first 24 hours it was necessary to know the additional P concentration in the machine due to the inoculation process mentioned above. After the calculation, this value was added to the know P concentration of the influent. After 24 hours, the P concentration of the influent was the sole concentration to be considered for the P removal daily percentages and since the concentration is constant, the removal percentages after the first 24 hours can be compared to each other.

The values seen by upscaling P removal with *A. johnsonii* 5bvlmeb2 during the first 48 hours are similar to lab-scale tests, but for the last 2 days of the experiment, an increase in the P concentration of the effluent was seen after a first decrease. This could indicate cell lysis and re-release of P previously accumulated. This pattern of uptake and release was also observed on the aerobic upscale experiment performed by Almeida & Morais (unpublished), although with higher uptake values since the residual water used was bio-stimulated with 1% sodium acetate, since it meant and additional source of energy was available for the strain to utilize (Hrenović, 2001). The latter was not the case here.

In the bioaugmentation experiment with *E. coli* BL21_pET30a_*ppk1* it was not possible to induce the strain with IPTG due to the fact that 35L of synthetic residual water were used daily and the IPTG volume required would be costly. Therefore lactose, which is an analogous compound to IPTG, was chosen as an inducer and was only added once.

To determine if there was a decrease in average P concentration in the effluent, both the bioaugmentation experiments were compared to the control through t-tests for two independent means performed at a significance level of 0.05, where the values from the first 24 hours of the bioaugmented experiments were excluded. The data was normally distributed and there was homogeneity of variance as assessed by Levene's test of equality of variances. AS bioaugmented with *A. johnsonii* 5bvlmeb2 ($M = 0.91$, $SD = 0.77$) compared to the control ($M = 0.98$, $SD = 0.62$) showed no statistically significant difference between the two, $t(5) = 0.139$, $p = 0.447$. When comparing the control to the

results from AS bioaugmented with *E. coli* BL21_pET30a_*ppk1* (M = 0.47, SD = 0.81), there was no statistical difference between them, $t(5) = 0.952$, $p = 0.385$.). Despite this fact, it can be seen in Table 5 and Figure 13 that the average of P present in the effluent, during the entirety of the experiments, is less than twice the average value in the control. More data will be needed to confirm that bioaugmentation with *E. coli* BL21_pET30a_*ppk1* improves AS's P removal capacity.

Table 5. Daily P removal efficiency of the control experiment using AS from Giessen's WWTP; upscale with *A. johnsonii* 5bvlmeb2 and two bioaugmentation experiments with A. johnsonii 5bvmeb2 and *E. coli* BL21_pET30a_*ppk1*.

P removal experiment	**Time frame (h)**	**P concentration in the influent (mg/L)**	**P concentration in the effluent (mg/L)**	**P removal efficiency (%)**
Using AS from Giessen's WWTP	0 - 24	5	0.39	92
	24-48	5	1.52	70
	48-72	5	1.21	76
	72-96	5	0.51	90
P removal Upscale with *A. johnsonii* 5bvlmeb2	0 - 24	17.3	13.5	22
	24-48	5	1.44	71
	48-72	5	7.34	-
	72-96	5	6.62	-
Bioaugmentation of AS with *A. johnsonii* 5bvlmeb2	0 - 24	33.5	21.59	36
	24-48	5	0.20	96
	48-72	5	1.73	65
	72-96	5	0.80	84
Bioaugmentation of AS with *E. coli* BL21_pET30a_*ppk1*	0 - 24	34.7	12.3	65
	24-48	5	1.40	72
	48-72	5	0.01	100
	72-96	5	0.02	100

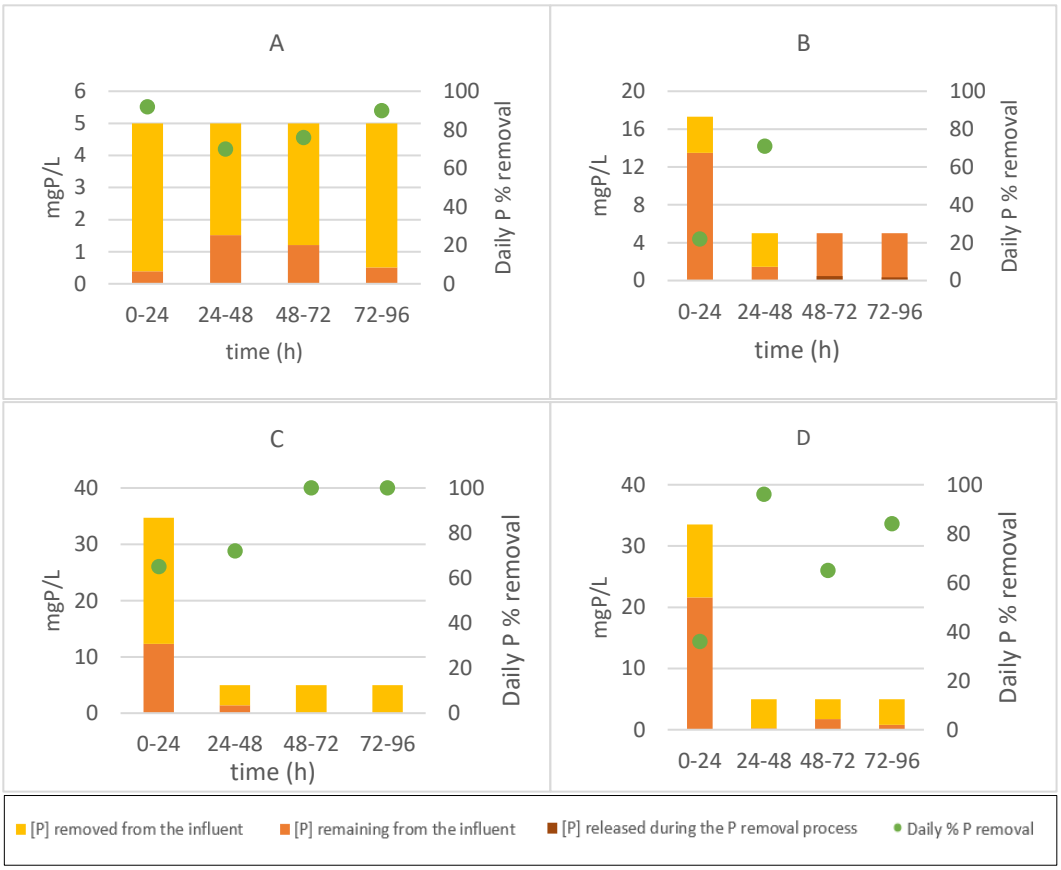

Figure 13. Daily P removal of four experiments in terms of concentration and percentage removed. A- AS from Giessen's WWTP (bioaugmentation control); B- Upscale of P removal with *A. johnsonii* 5bvlmeb2 with distilled water; C- Bioaugmentation of AS with induced *E. coli* BL21_pET30a_*ppk1*; D- Bioaugmentation of AS with *A. johnsonii* 5bvlmeb2.

The samples collected from the aerobic tank were centrifuged and the pellet was used for polyP quantification in the cells and the polyP uptake values of the experiments mentioned in Table 5 can be seen in Figure 14. Compared to the control, both the bioaugmentation experiment with the native strain, *A. johnsonii* 5bvlmeb2, and the modified strain, *E. coli* BL21_pET30a_*ppk1*, showed a momentary increase in polyP in the AS biomass. This coincides with the P removal from the synthetic residual water. Out of the 4 experiments shown, the bioaugmentation with *E. coli* BL21_pET30a_*ppk1* showed the highest increase in intracellular polyP concentration. At 48 hours, the polyP accumulation value in the biomass was 3 times higher than in the control. This combined with fact that the average P concentration in the effluent was lower than in the control shows the promising potential this strain has for P removal.

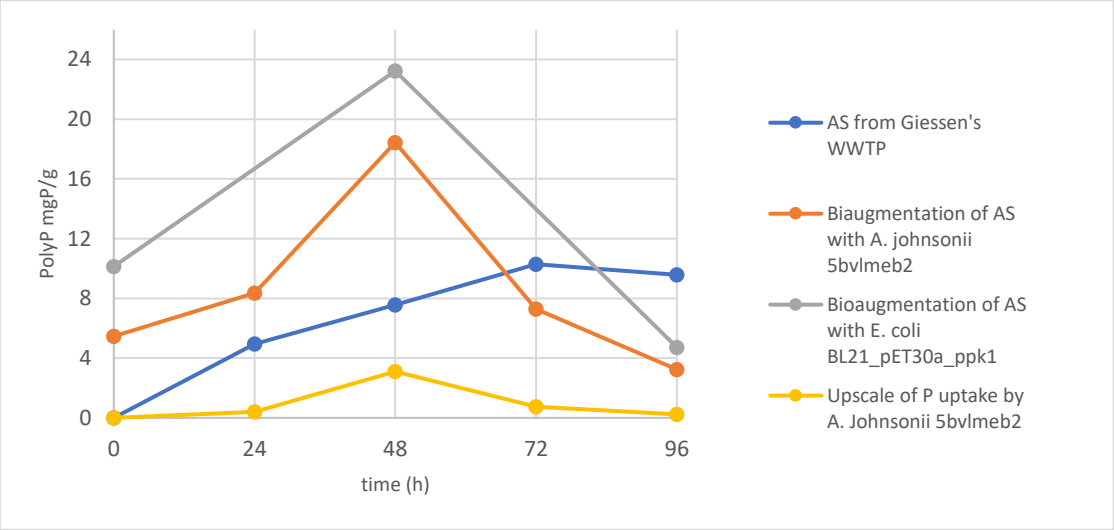

Figure 14. PolyP accumulation in the cells in the aerobic tank of a Behrotest™ laboratory-scale WWTP: Blue- AS from Giessen's WWTP (bioaugmentation control); Orange - Bioaugmentation experiment where the *Acinetobacter johnsonii* 5bvlmeb2 strain was inoculated in 11L of AS; Yellow - Upscale experiment where *Acinetobacter johnsonii* 5bvlmeb2 strain was inoculated in 11L of distilled water; Grey- Bioaugmentation experiment where induced *E. coli* BL21_pET30a_*ppk1* was inoculated in 11L of AS.

4.2.2. CFUs

4.2.2.1. Bioaugmentation experiments

As mentioned before, bioaugmentation is the addition of microorganisms to an already existing microbiome. To be considered a success, the strain used for bioaugmentation must meet certain criteria, such as be able to grow in the presence of the indigenous microbiome and endure environmental changes. Although even if it is able to survive it is not guaranteed that it will influence nutrient removal for the better (Raper et al., 2018).

Consequently, it is expected that in bioaugmentation experiments the CFU value will increase in comparison to the control at least fort sometime after the inoculation.

To determine whether the CFU values in the bioaugmentation experiments were higher than in the control, a One-Way ANOVA test was performed at a significance level of 0.05. The results showed that, indeed, there was a statistically significant difference among the groups ($F(2,12) = 3.998, p = 0.046$). Given this, post-hoc tests were performed. Specifically, t-tests for two independent means at a significance level of 0.05 were performed. The CFU values from the three experiments were normally distributed

and there was homogeneity of variance as assessed by Levene's test of equality of variances. CFU counts from the bioaugmentation experiment with *A. johnsonii* 5bvlmeb2 (M = 9.62, SD = 0.98) differed significantly from the control (M = 8.2, SD = 1.03); $t(8) = -2.13$, $p = 0.032$. The same was also observed when comparing the control to AS bioaugmented with *E. coli* BL21_pET30a_*ppk1* (M = 9.7, SD =0.65), $t(8) = -2.66$, $p = 0.015$.

The results show that there was a significant increase in the CFUs number in both bioaugmentation experiments compared to the control. Although, the daily CFU values continuously decrease (Figure 15).

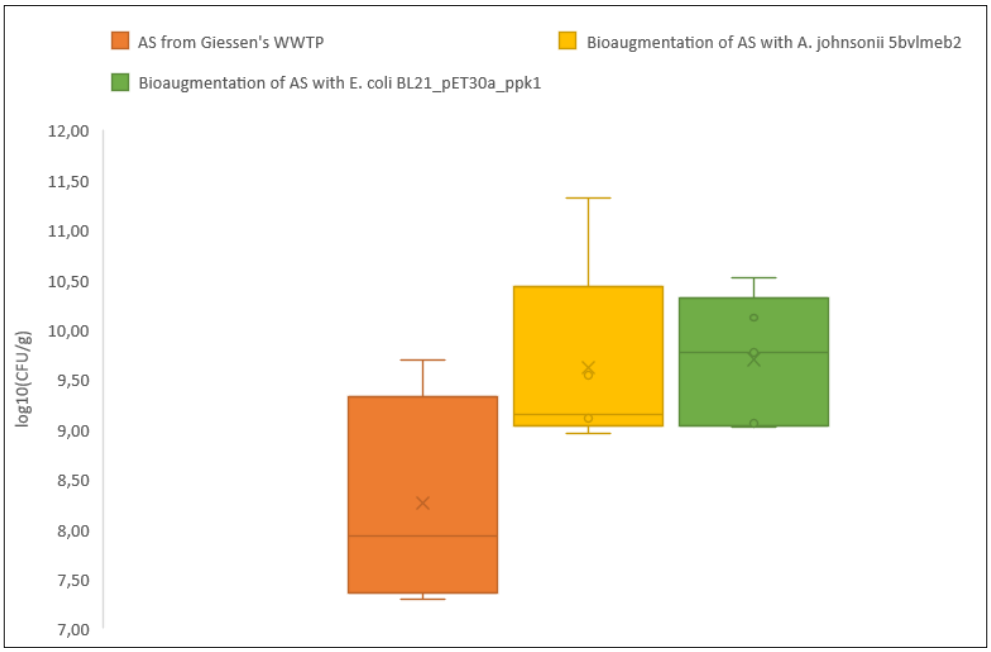

Figure 15. Average of CFU/g of AS and bioaugmentation experiments using *A. johnsonii* 5bvmeb2 and *E. coli* BL21_pET30a_*ppk1* for 5 days.

4.2.2.2. Uptake experiments

Since the laboratory-scale WWTP used was not sterile, to obtain the most accurate CFU result from the upscaling of *A. johnsonii* 5bvlmeb P removal experiment, suspensions were spread plated in two different media (Figure 16).

The CFU values from both mediums were normally distributed and there was homogeneity of variance as assessed by Levene's test of equality of variances. Therefore, a t-test for two independent means was performed with a significance level of 0.05. The results showed there was no significant difference between CFU values using TSA (M = 9.5, SD = 1.63) and using LAM with antibiotics (M = 9.32, SD =1.58), $t(7) = 0.17$, $p =$

0.8, between plaquing *A. johnsonii* 5bvlmeb in TSA or LAM with antibiotics. Which shows that the LAM medium, although being a specific medium for *Acinetobacter* species, mainly for virulent ones, is not suitable for this particular strain, since it allows other non-selective strains to grow in it.

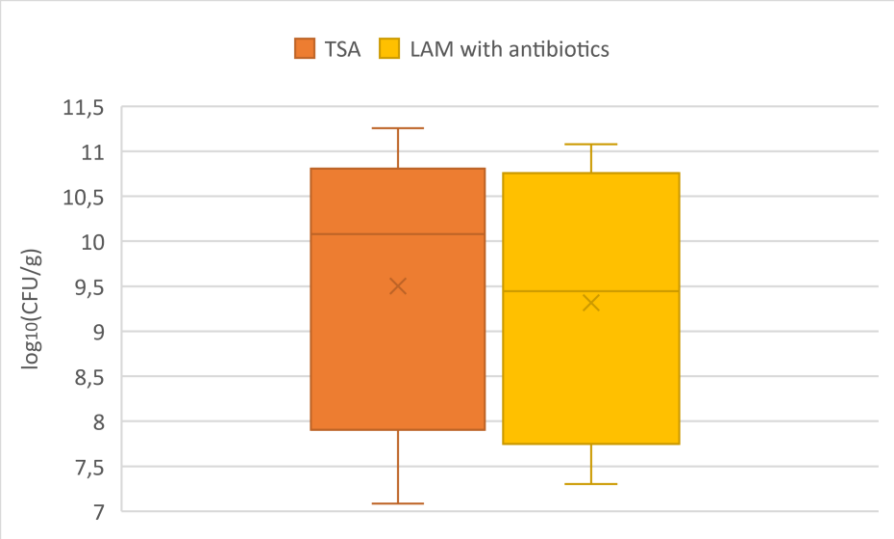

Figure 16. Average of CFU/g from each day of the 5 day- upscale experiment with *A. johnsonii* 5bvlmeb2 using two different mediums: TSA and LAM with antibiotics.

4.2.3. Quantification and purity of DNA samples

DNA from the daily samples of the two bioaugmentation experiments and the control collected from the aeration tank was extracted and the concentration and purity ratios are shown in Table 6. Since soils contain humic substances that can interfere with DNA quantification, besides the ratio for protein contamination, A (260/280), the humic substances contamination ratio, A (260/230), was also considered (Yeates et al., 1998).

On average, the extraction method yielded high concentrations of DNA, and no protein contamination. The low 260/230 ratios indicate contamination by humic substances which could mean that the DNA extraction method would need to be repeated if the PCR bands show no amplification.

Table 6. DNA quantification and purity ratios values of the samples from the P removal experiments performed on a behrotest® KLD 4 N/SR (behrLabor-Technik GmbH, Germany).

P removal experiment	**Time of sampling (h)**	**[DNA] ng/µL**	**A (260/280)**	**A (260/230)**
Using AS from Giessen's WWTP	0	149.7	1.86	0.02
	24	48.4	1.87	2.09
	48	49.4	1.85	1.55
	72	131.8	1.87	0.71
	96	95.6	1.86	2.12
Bioaugmentation of AS with *A. johnsonii* 5bvlmeb2	0	159.5	1.85	1.33
	24	91.4	1.87	2.15
	48	160.9	1.87	2.26
	72	124.4	1.87	2.07
	96	77.3	1.87	2,.18
Bioaugmentation of AS with *E. coli* BL21_pET30a_*ppk1*	0	145,5	1.86	1.49
	24	72.6	1.87	1.56
	48	88.4	1.85	0.65
	72	117.6	1.85	1.18
	96	145.6	1.87	1.49

4.2.4 PCR for *ppk1* detection and sequencing

To correlate the P removal values with the presence of the *ppk1* gene in the biomass during the bioaugmentation experiments, a PCR was performed. The PCR

products were visualized in agarose gel electrophoresis (Figure 17). The gene of interest has 1 Kb and is present in all experiments, including the control, which was not unexpected. The gene *ppk1* was found in *A. johnsonii* 5bvlmeb2 and other *A. johnsonii* strains, isolated from AS of a Portuguese WWTP, with these primers by Almeida & Morais (unpublished). Therefore, it is not surprising to discover the presence of this gene in a sample of AS from Giessen's WWTP.

The bands of the positive control and two other samples, all with the same molecular weight of 1 KB, were sent for sequencing analysis. One of the samples contained impurities, but it was possible to compare the sample from AS bioaugmentation with *E. coli* BL21_pET30a_*ppk1*, at the 24th hour, to *Acinetobacter johnsonii* XBB1's *ppk1* gene sequence, which had been used by Almeida & Morais (unpublished) for primer construction. The percentage identity between them was of 95.

The lack of bands in two of the samples in the gel (Figure 17-2;17-8) was due an error during the PCR process.

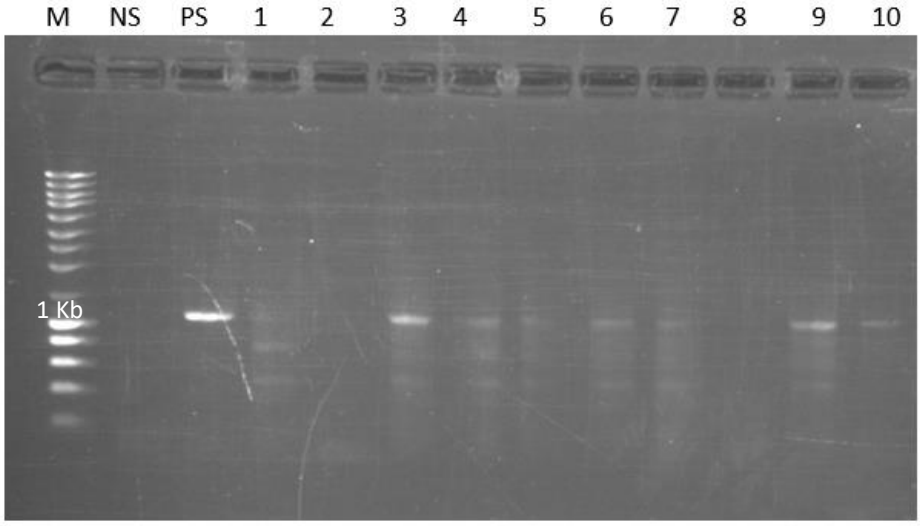

Figure 17. PCR screening for the *ppk1* gene, during the bioaugmentation experiments, present in both *A. johnsonii* 5bvlmeb2 and *E. coli* BL21_pET30a_*ppk1*: M- Marker; NS- Negative control; PS- Positive control; 1, 2, 3- Bioaugmentation experiment with *E. coli* BL21_pET30a_*ppk1* at 0, 72 and 96 hours, respectively; 4, 5, 6, 7- Bioaugmentation experiment with *A. johnsonii* 5bvlmeb2 at 0, 24, 72 and 96 hours, respectively; 8, 9, 10- AS from Giessen's WWTP at 48, 72 and 96 hours, respectively.

5. Conclusions and future work

Wastewater bioaugmentation, focusing on P recovery, will potentially become a topic of extreme relevance in the future. The European CE transition approach is due to many factors, such as economic, political and environmental. As the population grows rapidly, having a reliable access to a supply of clean water and reducing pollution levels is becoming imperative. one way to address these concerns is to strengthen and improving wastewater treatment procedures to meet those needs. Although economic barriers still pose a challenge for the CE model transition, studying economically-efficient methods to, for example, successfully recover nutrients from AS and also obtain usable water will be crucial to shorten the transition period and alleviate the burden caused by non-renewable PR mining quicker (Mannina et al., 2021).

When comparing the two bioaugmentation experiments it is clear that AS bioaugmented with *E. coli* BL21_pET30a_*ppk1* was able to remove P faster, and it also had a 100% P removal for two consecutive days. This shows the potential this strain has to improve the EBPR process in the future.

The information available in the literature on AS bioaugmentation using native strains is still sparse and even less is known on the use of genetically modified microorganisms (Hirota et al., 2010).

In this project, it was shown that bioaugmentation, using induced *E. coli* BL21_pET30a_*ppk1* (11L of growth) on AS, lowered the average dissolved P concentration in the effluent during a 5-day experiment, although it was not possible to demonstrate the stabilization of this modified strain in the sludge. In the future, it would be of interest to tag the strain's *ppk1* gene with a green fluorescent protein (gfp) as it would allow for the visualization of both the PPK1 protein expression and cell viability throughout the experiments as McLaughlin et al. (2006) carried out in their bioaugmentation experiment to remove 4-chlorophenol from water. This could possibly also answer the question why there is a maximum polyP uptake and P removal around 48 hours after the start of the experiments. Furthermore, for future studies daily inoculations should be performed during the bioaugmentation experiments to compare to the results obtained from a single initial inoculation.

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