Cranfield University

Robert Colston

# Enhanced bio-minerals production using catalysts to accelerate resource recovery in wastewater treatment plants

School of Water, Energy and Environment
Research Degree

PhD

Academic Year: 2019-2023

Supervisors: Ana Soares & Tom Stephenson

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

**Abstract**

The biomineralisation mechanisms of five known bio-struvite producing microbes have been established and their ability to recover said biomineral from synthetic solutions and sludge dewatering liquors has been trialled. There is a lack of evidence and knowledge how these microbes perform in open culture conditions and the impact encapsulating media has on their ability to remove and recover orthophosphate as bio-struvite. In this PhD thesis, these microorganisms (*Brevibacterium antiquum, Bacillus pumilus, Halobacterium salinarum, Idiomarina loihiensis, Myxococus xanthus*) were investigated initially, this was streamlined into investigating encapsulated cultures of *B. antiquum* and *B. pumilus* in wastewaters under open culture conditions. The inoculation of all five microbes in source-separated urine in open culture conditions showed growth rates as high as 0.18 1/h and high nucleic acid proportions >80% within 24 hours of incubation. An orthophosphate removal of up to 70% was achieved by *B. antiquum* inoculations and was increased to 100% when magnesium was increased to a 1:1, P:Mg. Encapsulated cultures of *B. pumilus* were incubated B4.1 growth media, the removal of orthophosphate and chemical oxygen demand was equal to suspended cell inoculations of *B. pumilus*. In pure culture and open culture sludge dewatering liquors, encapsulated cultures of *B. pumilus* and *B. antiquum*, removed 55% and 70% of the initial orthophosphate over 24 hours respectively. The minimal difference in orthophosphate removal between pure and open culture conditions indicates that encapsulation provided an environmental advantage to the selected microbes to out compete the native species within the open culture sludge dewatering liquors. Suspended cell inoculations into open culture sludge dewatering liquors did not remove any more orthophosphate than non-inoculated controls. In continuous reactors fed by open culture sludge dewatering liquors orthophosphate removal for both encapsulated microbes averaged between 20% and 30%, at phosphorus loading rates of 0.4 kg $P/m^3$.d and 0.6 kg $P/m^3$.d. Supplementing a carbon source to the equivalent of 150 mg sCOD/L and increasing

the ratio of P:Mg to 1:1.5, achieved an orthophosphate removal of 96% on average by encapsulated *B. antiquum*. Bio-struvite recovered from all open culture wastewaters was euhedral, prismatic and tabular and was typically coated in a secondary abiotic calcium phosphate. Micropollutant analysis showed the recovered minerals were below international heavy metal limits and were absent from faecal coliforms, pharmaceuticals and other micropollutants for fertilisers. Potential end users and consumers from the public and industry showed a strong willingness to use and eat produce grown from recycling derived fertilisers. There remains to be optimisation of the biomineralisation technique to improve the efficiency of recovery and streamline the operational set up, however the data collected in this PhD strongly supports the development of this technique into industry and will satisfy a growing need for circular economies and closing the nutrient loop.

**Keywords**

Biomineralisation, bio-struvite, source-separated urine, open culture conditions, phosphate removal, phosphorus recovery, encapsulation, biocatalyst, *Brevibacterium antiquum*, *Bacillus pumilus*, sludge dewatering liquors, sustainable fertiliser, recycling derived fertilisers, biomineral recovery, end-users, consumers, circular economy, nutrient loop

## Acknowledgements

This PhD thesis would not have been possible without the invaluable input and rationale form my supervisor, Professor Ana Soares. The determination and rigour into developing mine and Cranfield University's research abilities was constant inspiration. Secondly, I would like to thank Professor Tom Stephenson, the guidance and writing support you provided was invaluable, as was all the coffees. I am deeply indebted to my project sponsors, Severn Trent Water and Microvi Biotech, Pete and Ajay's enthusiasm and passion for the wider implications of this project was always refreshing in our catch ups and conferences we attended. Additionally, this endeavour would not have been possible without the additional support and funding by the EPSRC and UKRIC, whose understanding and action after the pandemic (2020-2021) ensured my project, as well as many others across the UK, had the funded extensions needed to complete our research.

I am grateful for the support and guidance of the technical team at Cranfield University, their patience and willingness to help take me through the various analytical procedures required, saved me a huge amount of time and heartache. A special thanks also goes out to my colleagues, academic staff and support team at Cranfield, the lunchtime discussions helped me avoid potential speed bumps and ensured I remained honest.

Last but by no means least, I would like to thank my friends and family. My friends, from home, past universities and sporting clubs who were always interested in what I was doing, or at least were excellent at feigning interest in it. My family were always there (and remain to be) to listen to any grievances or frustrations I might have had at the time and were always willing to provide recommendations. Angie and Matt (Mum and Dad), thank you for your never-ending patience, words of wisdom and being my 3rd and 4th supervisors. Finally, doing this PhD has taught me a lot, that is hopefully explained clearly in the pages that follow. It has also taught me patience is a virtue and I am so glad I did not give up, Tizzi thank you for being a great human, always making me laugh, I appreciate you and our Rubster.

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**List of Abbreviations**

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| ATP | Adenosine triphosphate |
| TRL | Technical readiness level |
| WWTP | Wastwater treatment plant |
| SGDs | Sustainable development goals |
| BCM | Biologically controlled mineralisation |
| BIM | Biologically induced mineralisation |
| GHG | Greenhouse gasses |
| EPS | Extracellular polymeric substances |
| PAOs | Phosphate accumulating organisms |
| EBPR | Enhanced biological phosphorus removal |
| VFAs | Volatile fatty acids |
| PHA | Polyhyrdoxyalkonoate |
| sCOD | soluble Chemical oxygen demand |
| GAOs | Glycogen accumulating organisms |
| SS | Sewage sludge |
| AD | Anaerobic digestion |
| FBR | Fluidised bed reactor |
| CSTR | Continuously stirred tank reactor |
| BOD | Biological oxygen demand |
| PUFAs | polyunsaturated fatty acids |
| EU | European Union |
| SDL | Sludge dewatering liquors |
| TP | Total phosphorus |
| TN | Total nitrogen |
| ICP-MS | Inductively coupled plasma-mass spectrometry |
| SEM-EDS | Scanning electron microscopy-Energy dispersive spectroscopy |
| TCC | Total cell count |
| ICC | Intact cell count |
| LNA | Low nucleic acid |
| HNA | High nucleic acid |
| RNA | Ribonucleic acid |
| UB | Urine batch |
| XRD | X-ray diffraction |
| SI | Saturation index |
| MNE | Microniche engineering™ |
| Bp | *Bacillus pumilus* |
| Ba | *Brevibacterium antiquum* |
| SBRs | Sequencing batch reactors |
| QTOF | Quadrupole time-of-flight mass spectrometer |
| RDFs | Recycling derived fertilisers |
| CAPEX | Capital expenditure |
| OPEX | Operational expenditure |

# 1. Introduction

## 1.1. Background and motivations

Phosphorus (P) is an irreplaceable nutrient, it is required in deoxyribonucleic acid (DNA, $C_{15}H_{31}N_3O_{13}P_2$), ribonucleic acid (RNA, $C_{27}H_{34}N_6O_{22}P_2$), adenosine triphosphate (ATP, $C_{10}H_{16}N_5O_{13}P_3$) and phospholipids, constituting the genetic makeup of all living organisms, providing transport and energy pathways through and across cell membranes and provides the structure to the cell wall membrane.

Phosphorus is a major limiting nutrient in plant growth and therefor food security[1,2]. With population rise and changing diets, dependence on P-rich fertilisers has dramatically increased as unfertilised soils cannot provide enough nutrients to meet the demands of intense farming [3,4]. In Europe, P-demand is dominated by food production and agriculture accounting for up to 91% of the P-demand[5]. The demand for P-rich fertilisers has dominantly been taken up by mined phosphate minerals (4.9 – 9% w/w P content), however this source of P is finite (50-200 years left in reserves) and unevenly distributed across the world (75% sub-Saharan Africa)[6,7]. Rock phosphate is one of twenty critical raw materials designated by the EU commission, so reserves need to be managed, and recovery methods applied[8].

Wastewater Treatment Plants (WWTP) are the main point of anthropogenic P-accumulation i.e., from domestic and industrial sewage. Left unmanaged P can cause a number of issues within WWTP such as abiotic precipitation, scaling and blockages and when discharged into surface waters, increasing the nutrient content prompting algal blooms and eutrophication[1,7,9]. This led to the European Commission writing into legislation May 1991, directive 91/271/EEC setting out codes of practices that needed to be met to reduce the nutrient and contaminant content of urban wastewaters and ensure water reuse when possible. Technologies are in place in WWTP to concentrate and remove P from wastewater, either in sludge or abiotic

precipitation in dewatering liquors, yet P is still lost to the environment for example up 221 kt P was lost through municipal sewage water in 2005 across 27 European Union (EU) member states[5].

Phosphorus recovery methods within WWTP currently concentrate P within sludge which can either be reused directly in land application or concentrated further through incineration and reuse of the P-rich ash[10,11]. Also, P is recovered through abiotic, chemical precipitation of struvite or calcium phosphate in sludge or sludge dewatering liquors[10,12,13]. However, chemical precipitation requires the input of reagents and kinetic changes (such as carbon dioxide stripping) to raise pH to >8.5 and ensure a sufficient concentration of P and magnesium (Mg)[14,15]. Meaning that efficient and economical recovery of P using chemical recovery of struvite can only occur when P is >100 mg P/L, much greater than the average P concentration reaching WWTP each day (3 – 10 mg P/L per day in the UK)[2,16].

Mineralisation of struvite has seen the bulk of research in nutrient recovery, with many commercial processes available for chemical recovery of struvite, other minerals include calcium phosphate and calcium carbonate. Struvite has taken precedence for recovery due to its bulk composition (magnesium ammonium phosphate) being suitable for use as a slow-release fertiliser[17–19], with successful recovery occurring in WWTP across the world including the UK[8].

Issues with chemical struvite recovery and demand for a circular economy in the water sector due to uneconomical production has led to growing research into using microorganisms to mediate the precipitation of struvite using biomineralisation[20–22]. Over several years the ability of selected microorganisms, such as *Brevibacterium antiquum, Bacillus pumilus, Halobacterium salinarum, Idiomarina loihiensis* and *Myxococus xanthus* have been tested in

synthetic media and various sludge dewatering chemistries[20,22–24]. These lab-scale experiments have identified the ideal growth conditions for these microbes, the mechanisms of biomineralisation used by these microbes, and the species of phosphorus utilised. To surmise, these microbes are very well suited to grow in various sludge effluents in principle, with optimum pH between 8 and 9, in oxygenated liquors and have shown that biologically controlled mineralisation is used by *B. antiquum* and *M. xanthus* and biologically induced mineralisation is used by *B. pumilus, H. salinarum* and *I. loihiensis*[22,24,25]. These microorganism have achieved P removal rates of up to 94% P from sludge effluents, recovering 'biostruvite' crystals that are up to 250 μm long, prismatic and tabular (euhedral)[21,22] supporting its case for suitable reuse as a slow release fertilisers[26].

The ability of these microorganisms to recover biostruvite in under open culture conditions and in continuous reactors has yet to be tested and is required to ensure sustainable and long-lived P-recovery. Retaining the biomass of these microorganisms and overcoming competition from native microbes in sludge effluents are causes for concern for the industrialisation of the biomineralisation technique, however inroads have been made using biocatalysts and immobilisation techniques to retain biomass and provide ideal environments for selected microorganism to flourish[27–30].

The coupling of microbial encapsulation and recovery of phosphorus using biomineralisation could be the defining step in making nutrient (P) recovery from wastewater economically viable for WWTP, reducing the need for imported chemical fertilisers by providing a sustainable and 'green', slow-release fertiliser in biostruvite benefiting the circular economy.

### 1.2. Aims and objectives

The main research aim of this study is to progress the use of microorganisms encapsulated by a catalyst for improved phosphorus removal, biomineral synthesis and recovery from

wastewater and urine. Furthermore, this study aims to demonstrate this technology at pilot scale to achieve a Technical Readiness Level (TRL) 6 or higher. The overreaching goal of this research is to establish a sustainable phosphorus removal and recovery process which can provide a practical fertiliser alternative (Figure 1-1).

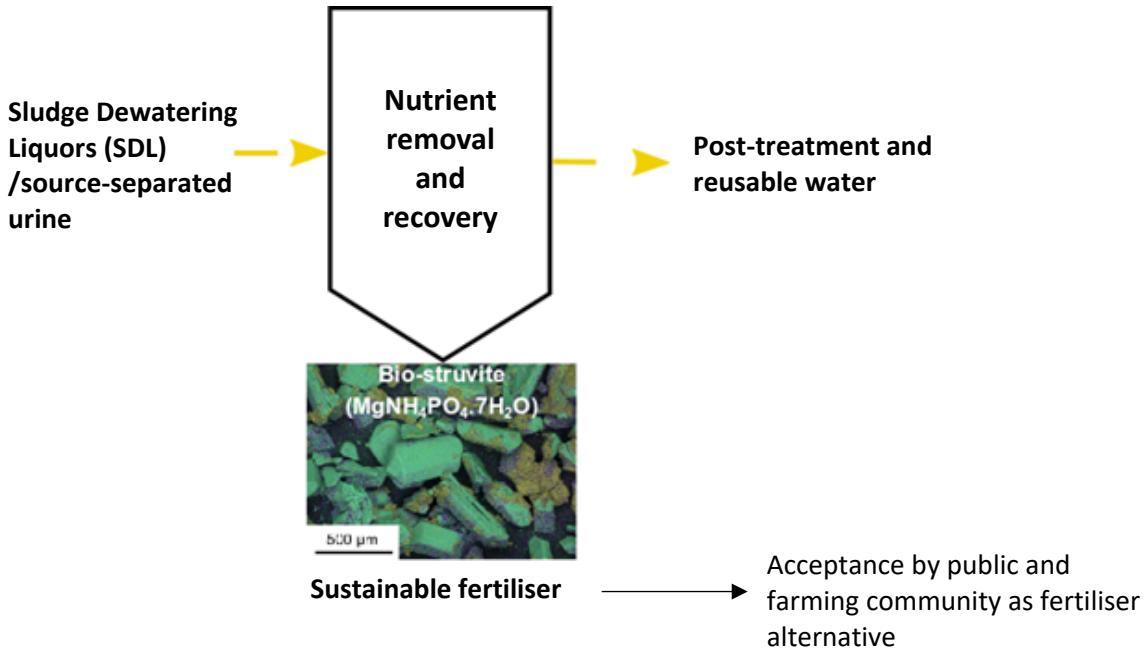


**Figure 1-1 Schematic of thesis aim**

The following objectives will be addressed in the completion of this study.

1. Conduct a comprehensive literature review of the current state of phosphorus removal, struvite recovery, biomineral formation and production in a various wastewater chemistry, and uses for microbial immobilisation, encapsulation and catalysis.
2. Select and evaluate five identified microorganisms which biomineralise struvite through laboratory experiments to assess their growth in; urine and wastewater, without encapsulation.
3. Conduct lab-scale experiments to test the selected microorganisms and encapsulation processes with wastewater (with or without urine) liquors.

4. Develop and assemble a pilot-scale reactor to investigate the capability of encapsulated microorganisms to remove phosphate and recover it as bio-struvite in a continuous reactor.
5. Evaluate the quality of bio-minerals recovered, including the purity of desired nutrients and contaminants such as, heavy metals, pharmaceuticals and faecal coliforms.
6. Conduct market research of potential end-users, address potential barriers, develop an economic business case, and analyse the overall findings from the research.

### 1.3. Thesis plan

Each of these thesis chapters were produced in the format of a journal paper, submitted individually or are in preparation to do so. Figure 1-2 presents the overall structure of the thesis linking aims and objectives with chapter.

Robert Edward Colston carried out all the planning, experimental work and data analysis. All chapters were written by the first author Robert Edward Colston with comments from supervisors Dr Ana Soares and Prof Tom Stephenson. Dr Peter Vale, from Severn Trent Water PLC, and Ajay Nair, from Microvi Biotech, provided industrial supervision. Named authors in chapters 3, 5 and 6 contributed their knowledge and expertise to aid data collection and analysis under the supervision of Robert Edward Colston.

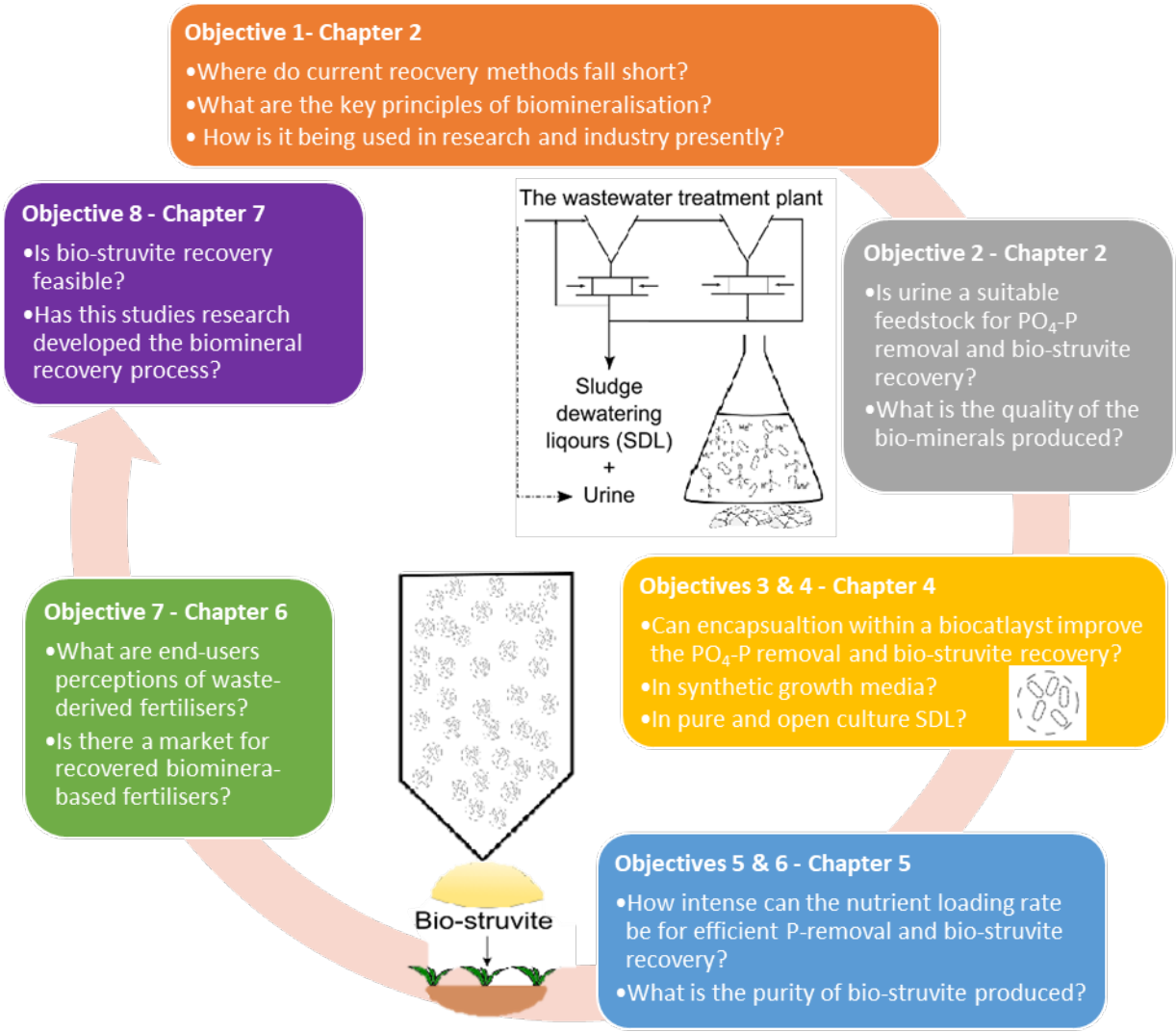


**Figure 1-2 The development plan of thesis objectives and chapters addressing these aims**

Chapter 2 introduces the existing techniques in place for phosphorus recovery, the mechanisms involved for biomineralisation and critically reviews other uses of biomineralisation in the water industry. The findings from this chapter influenced the methodologies of the subsequent chapters 3, 4, 5, 6, 7…. This chapter has been separated and published within 'Resource Recovery from Water: Principles and Application' by *International Water Association Publications* and as 'A review of Biominerals; mechanisms, bioinspired techniques, and application to sustainable development' in *Environmental Science: Water Research & Technology.*

Chapter 3… Examines the ability of 5 selected microorganism (*B. antiquum, B. pumilus, H. salinarum, I. loihiensis* and *M. xanthus*) to grow and recover P as biostruvite using their biomineralisation mechanisms in raw and untreated urine. To highlight further opportunities for resource recovery from waste streams not analysed before and provide evidence for selecting two microorganism for biocatalyst trials. This has been submitted to the journal *Environmental Science & Technology.*

Chapter 4… trialled two selected microbes based on the conclusions from chapter 3 (*B.* antiquum and *B.* pumilus) encapsulated using Microvi Biotech® Mirconiche Engineering™ (MNE™) biocatalyst in sterile and raw sludge dewatering liquors, other immobilisation techniques (alginate beads and hydrogels) and suspended cells in B4.1 synthetic growth media. The outcomes of this paper provided evidence for which microorganism to continue to optimisation experiments and continuous bioreactor trials. This chapter is preparation for *Chemosphere.*

Chapter 5… investigated the delivery of MNE™ biocatalysts supporting *B. antiquum* and *B. pumilus* into continuous reactors using data from batch experiments. All pilots were ran in open conditions and ambient temperatures to make the physical conditions close to full scale WWTP processes. Different loading rates were tested for a period of up to 30 days each, with one loading rate being dosed by additional carbon and magnesium to monitor the impact on orthophosphate removal when no nutrients are limiting. This chapter is preparation for *Water Research.*

Chapter 6… surveyed potential end-users of the biostruvite product (farmers, pubic and manufacturers) to analyse current uses of fertilisers and whether biostruvite can be substituted into their needs, also their perceptions of recovered fertiliser products such as quality and

hazards. Using these responses full characterisation of the biostruvite product was analysed to address any issues and build a case for biostruvite to receive 'end of waste' status. This chapter is preparation for *Science of The Total Environment.*

Chapter 7… discusses the results of this thesis and provides a business case in the form of capital and operational expenditure reports (CAPEX and OPEX) for several scenarios. Comparing the biomineralisation technique with chemical struvite recovery.

Chapter 8… brings together the overall conclusions of the study in relation to the initial objectives.

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# 2. Biomineralisation technologies in wastewater treatment and resource recovery

Robert Colston[a], Peter Vale[b], Ajay Nair[c], Tom Stephenson[a], Ana Soares[a1]

[a]Cranfield Water Science Institute, Cranfield University, Cranfield, MK43 0AL, UK
[b]Technology and Development, Severn Trent Water Ltd., Avon House, St. Martins Road, Coventry, CV3 6PR, UK
[c]Microvi UK Ltd., Sci-Tech Daresbury, Keswick Lane, Daresbury, Warrington WA44FS, UK

[1] Corresponding author: a.soares@cranfield.ac.uk

**Key words:** *Biominerals, biotechnology, resource recovery, circular economy, wastewater*

**Abstract**

There is resistance to the recovery of nutrients and metals due to the energy requirements, reagent demand and, ultimately, costs. Steps need to be made to accelerate the use of 'Green Chemistry' so that ions can be conserved and recovered to meet growing demands and reduce the need for extraction of minerals and metals from the Earth's rock reserves. Biomineralisation techniques can meet the requirements of 'Green Chemistry' by reducing or removing the need for reactants and energy requirements. An example of this is the biomineralisation of struvite to recovery phosphorus at concentrations below 100 mg/L, which is unfeasible for chemical recovery techniques in wastewater treatment plants and provides a valuable mineral fertiliser alternative. Continued research is needed to develop such techniques to an industrial scale however, once implemented can provide valuable resources from waste streams, resource resilience and ensure sustainable development through a circular economy.

## 2.1 Introduction

Unchecked nutrient loss to natural watercourses and uncontrolled nutrient use (over-fertilisation) has well-documented, detrimental impacts to the receiving ecosystems through the process of eutrophication[1]. Additionally, poor legislation and controls on wastewater treatment of industrial wastewater, including mining has led to the pollution of other contaminants, such as heavy metals and radionuclides. Many Nations have recognised the issue of nutrient loss from wastewater treatment plants (WWTP) and have strict discharge effluent parameters e.g. the EU Urban Wastewater Directive requires at least 80% of the phosphorus (P)-load, 70-80% of the nitrogen (N)-load to be removed and, require final effluents with P content less than 2 mg/L[2]. However, this varies globally, in China discharge limits are uniform which leads to issues due to local conditions and differing environmental protection demands which limits the development of efficient wastewater treatment, up to 35% of sludge is left untreated and is discharged into the environment[3].

Traditionally, to reduce the concentration of harmful pollutants/nutrients from wastewater and contaminated water, WWTP utilised coagulants such as iron or aluminium salts which bound the nutrients and organic matter into larger flocs that could be settled and removed and biological nutrient removal. Implementing nutrient recovery at WWTP is growing in popularity to offset the cost of increased removal demands, achieve climate change targets and research into new resource recovery techniques continues to grow[4]. An example of pioneering nutrient recovery is using precipitation reactors and dosing chemicals to encourage the abiotic precipitation of the target nutrient, for example the dosing of magnesium (Mg) with pH and temperature control to precipitate struvite for P recovery[5]. Struvite is a viable fertiliser alternative that can replace mined fertiliser products[6–8]. Over-fertilisation is a large contributor to nutrient pollution to receiving watercourses, China and India have a fertiliser excess of 4.6 and 3.1 million tonnes P respectively, compared to the

mass of P they harvest[9]. Incorporating nutrient recovery at WWTP can ensure that nutrient cycle remains balanced by minimising the need to input external sources. Additionally, recovering nutrients for fertilisers to be used by nations who extract more P from harvests than they deposit (e.g., Sub-Saharan Africa) could be a step towards food security for these Nations, allowing local production of more nutrient-rich produce.

Our understanding of biominerals has improved in the past 20 years. Biomineralisation techniques can and will play a key role in the advancement of 'Green chemistry' and sustainable development[10]. For example by reducing the need for reactants such as ferric dosing, that are both costly and can lead to hazardous waste through their extraction, processing and use, to remove and recover nutrients at WWTP[8,11] or recovering heavy metals from acid mine drainage and contaminated land[12,13]. Furthermore the use of microorganisms to recover nutrients and other ions can produce recovered products that have an economic benefit such as struvite for fertiliser and reduce the demand the Earth's natural resources[14]. This switch could help drive countries to circular economies in addition to achieving the United Nation's sustainable development goals (SDGs) by conserving resources and providing recovered products in decentralised systems to communities that would not be able to afford them normally.

Biomineralisation is a phenomena observable throughout geological history and in the present-day, from fossilised remains of hydroxyapatite (bones and teeth)[15] to benthic phytoplankton and diatoms[15–17]. Our understanding of the mechanisms, inhibitors, and purpose of biomineralisation is crucial for understanding environmental processes and developing remediation techniques: e.g., understanding how ocean acidification will impact oceanic bio-geochemical cycles[18] and remedying challenges faced today such as carbon sequestration through 'ocean fertilisation'[19–21]. Factors impacting biomineralisation includes

supersaturation, temperature, pH, and productivity of the organism such as the rate it can concentrate nutrients or metabolise to create conditions favourable for mineralisation. Nucleation is the first step of biomineralisation, occurring when the ionic activity product exceeds the thermodynamic equilibrium; ionic activity itself is controlled by the super saturation of ions, temperature and pH[22–24] (Figure 2-1). As nucleation progresses, particles aggregate together leading to crystal growth (Figure 2-1). Retention relies on conditions remaining in equilibrium so that dissolution will not occur and is paramount for successful recovery yields.

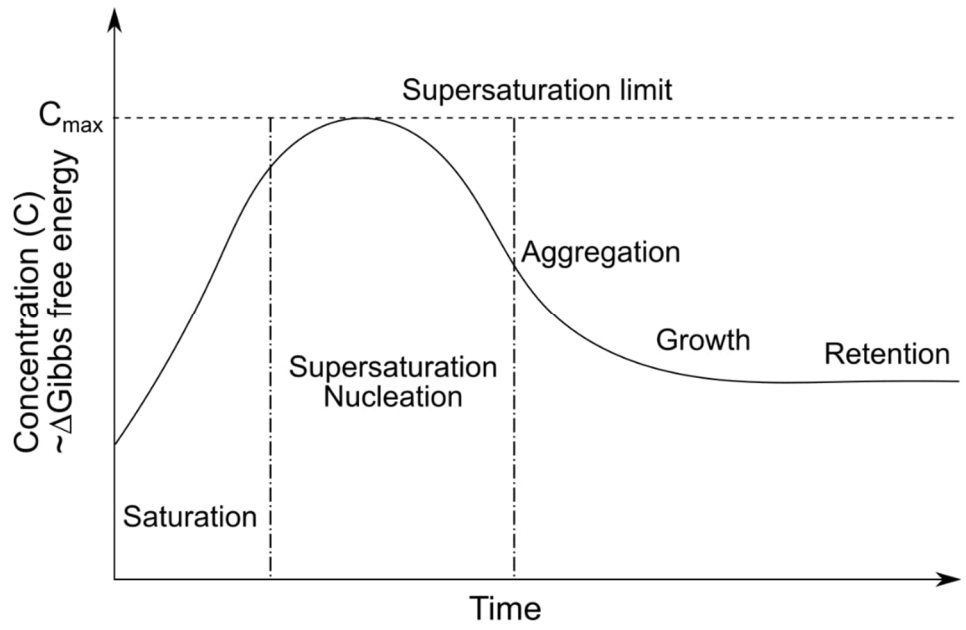


**Figure 2-1 Simplified stages of mineralisation. Change in Gibbs free energy, a measurement of reaction energy using concentration of ions, temperature, and pH**

Biomineralisation can be differentiated into two major mechanisms: biologically controlled mineralisation (BCM) and biologically induced mineralisation (BIM). However, multiple research projects and industries use a variety of terminology e.g., bioaccumulation and bioprecipitation is akin to BCM where organisms actively remove nutrients or metals[25–27]. Biosorption can be likened to BIM where precipitation can occur as result of changing chemical environments from metabolism, respiration and biological decay[28,29]. It is important

to consider the numerous studies into biomineralisation and its derivatives to improve the transfer of knowledge across disciplines i.e., from environmental science to bioengineering to medicinal science.

Organisms using BCM actively create conditions that favour precipitation of specific cations. Typically these produce unique crystal lattices which leads to structural benefit that are often difficult to replicate inorganically e.g., silicate precipitates of sponge spicules and diatom shells provide clear benefits to the organism such as protection or structure[15,30,31]. Biologically controlled mineralisation can be extracellular e.g. shell growth and the formation of diatoms (Figure 2-2) or intracellular e.g. the formation of magnetosomes in magnetotactic bacteria[32,33]. Intracellular biomineralisation occurs when mineral accumulation within the cell increases supersaturation and causes precipitation, either to improve cell function e.g., for cell mitosis or as a means of detoxifying its environment[26,34]. In magnetosomes, iron is concentrated within their cells and precipitated as magnetite, which causes the bacterium to follow the Earth's magnetic field, reducing the randomness of their feeding[32,33]. In some cases, microbes can accumulate nutrients or contaminants such as heavy metals to concentrations which are recoverable[12,35]. The unique properties of biominerals such as their structural benefits has led to breakthroughs in medicinal sciences, using biomineralisation-based virus shell-engineering to improve the deliverability of vaccines[36,37] and hydrogen fuel production, where iron-phosphorus nano-petals have been derived from biomineralisation processes to improve the reaction kinetics for hydrogen production[38].

Biologically induced mineralisation is the by-product of biochemical processes such as metabolism or respiration (Figure 2-2). This precipitation process is independent of the organism's function and has no direct benefit to the organism, it is extracellular, abiotic and

includes biomineralisation because of its death. For example, haematite can be a by-product from iron-reducing bacteria (Figure 2-2) or the biomineralisation of faeces (coprolites[39]).

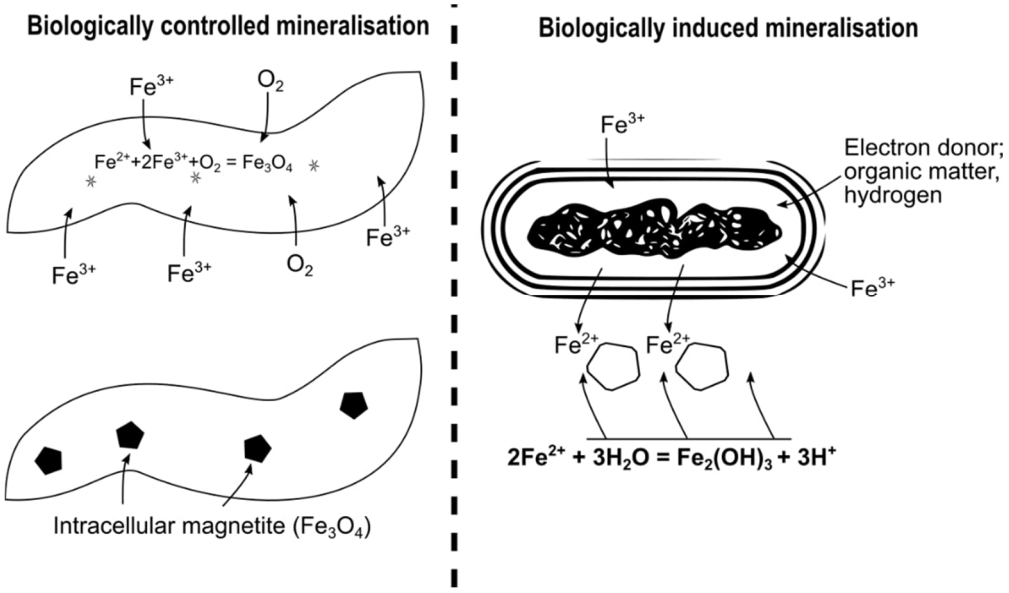


**Figure 2-2 Biologically controlled mineralisation compared to biologically induced mineralisation. Magnetosome using intracellular biologically controlled mineralisation (left) and iron reducing bacteria creating external precipitation of haematite via biologically induced mineralisation (right).**

Biomineralisation techniques could help address future challenges facing the water sector such as stricter effluent limits, recovery techniques becoming mandatory and to achieve greenhouse gas emission (GHG) targets by 2030 and 2050[40]. This review focusses on the removal and recovery of would-be contaminants from wastewater streams, including industry standard methods, gaps in knowledge and current research that can overcome today's environmental challenges.

### 2.2 Current phosphorus removal and recovery techniques

Removal of nutrients from wastewater to improve final effluent quality can be done in several ways e.g., culturing microbes and algae in facultative ponds is a relatively straightforward process which removes nutrients by locking them in biomass, which is removed as sludge,

and disposed of in landfill[41]. Additionally, chemicals can be added to sequester nutrients and other pollutants through coagulation or precipitation of minerals[42].

### 2.2.1 Coagulation

Dosing wastewater with inorganic coagulants such as metal salts of aluminium (alum) and iron (ferric) or calcium (lime), are able to reduce dissolved nutrients, organic loads and other contaminants in the WWTP[43,44] following Equation 2-1. The hydrolysation of metal salts or lime produces cations ($Al^{3+}$, $Fe^{3+/2+}$, $Ca^{2+}$) that can bind weakly charged ligands such as dissolved orthophosphate ($PO_4^{3-}$), which continue to bind until the density reaches a point where settling can occur, and sludge can be removed from settling tanks (Figure 2-3). Parts a) the hydrolysis of salts and b) the precipitation of phosphate of Equation 2-1 simplifies the coagulation of orthophosphate. Competing reactions, associated equilibrium constants, pH and alkalinity are some of the factors that will impact the completion of Equation 2-1a) and b). Equation 2-1 c) accounts for this slightly where the constant *r* is equal to 1.6 for ferric dosing and 0.8 for alum dosing[42]. However, bench tests and regular characterisation of the treated influent needs to be done to accurately dose the coagulants[42].

Equation 2-1 $a)\ Me - salt + H_2O \leftrightarrow Me^{n+}_{(aq)}$

$b)\ Me^{n+} + H_nPO_4^{3-} \leftrightarrow MePO_{4(s)} + nH^+$

c) $rMe^{n+} + H_nPO_4^{3-} + (3r-1)OH^- \leftrightarrow Me_rH_2PO_4(OH)_{3r-1(s)}$

Final effluent concentrations of <0.05 mg/L total P can be achieved using coagulation methods[45] and up to 82% and 94% total P can be removed with ferric and alum dosing respectively[46]. The benefits of using coagulation is the relatively short residence time required for sedimentation and removal of sludge through settling or filtration and its ability to be applied throughout WWTP[42] (Figure 2-3). Most coagulants are relatively inexpensive

costing £0.005 per $m^3$ wastewater treated[47] and until recently controls and costs of disposing the solids produced was moderate[48].

Sludge production for standard coagulants such as ferric sulphate is up to 600 mL sludge/L wastewater treated[49], as populations rise and with it the volume of wastewater required, the volume of sludge requiring disposal or treatment will increase. In 2000, the UK produced over 182 million tonnes of sludge from coagulant use, costing water utilities over £5.5 million to dispose[48]. However, global inflation and stricter environmental controls has led to research into recovering coagulants from sludge to bring the use of coagulants into sustainable 'green chemistry'[48]. Research into composite coagulants to reduce the volume of sludge to be treated can effectively reduce the volume of sludge produced by up to 41%[49]. Bulk reuse of sludge is the most utilised alternative to disposal in landfill, due to the properties of sludge it can make a suitable additive to building aggregates to reduce their permeability or sludge can be used to improve soil water retention and add nutrients for arable farming, however there is scrutiny over the efficacy of this and the threat of introducing heavy metals in the sludge into food chains[48]. Coagulant recovery techniques include acidification, ion exchange, filtration and electrodialysis, these have demonstrated coagulants can be recovered for reuse from batch scale up to pilot, however issues remain with the energy costs involved, fouling and regenerating exchange media[48] limiting the development of these processes.

Additional drawbacks of coagulation in conventional WWTP are the point of addition: in primary treatment additional polymers may be needed to enhance the flocculation and settling of solids and too much P removal can be detrimental to biological processes downstream. Dosing coagulants may also require additional treatment of the effluents to remove residual metals and changes to pH before biological treatment or other physico-chemical processes[42].

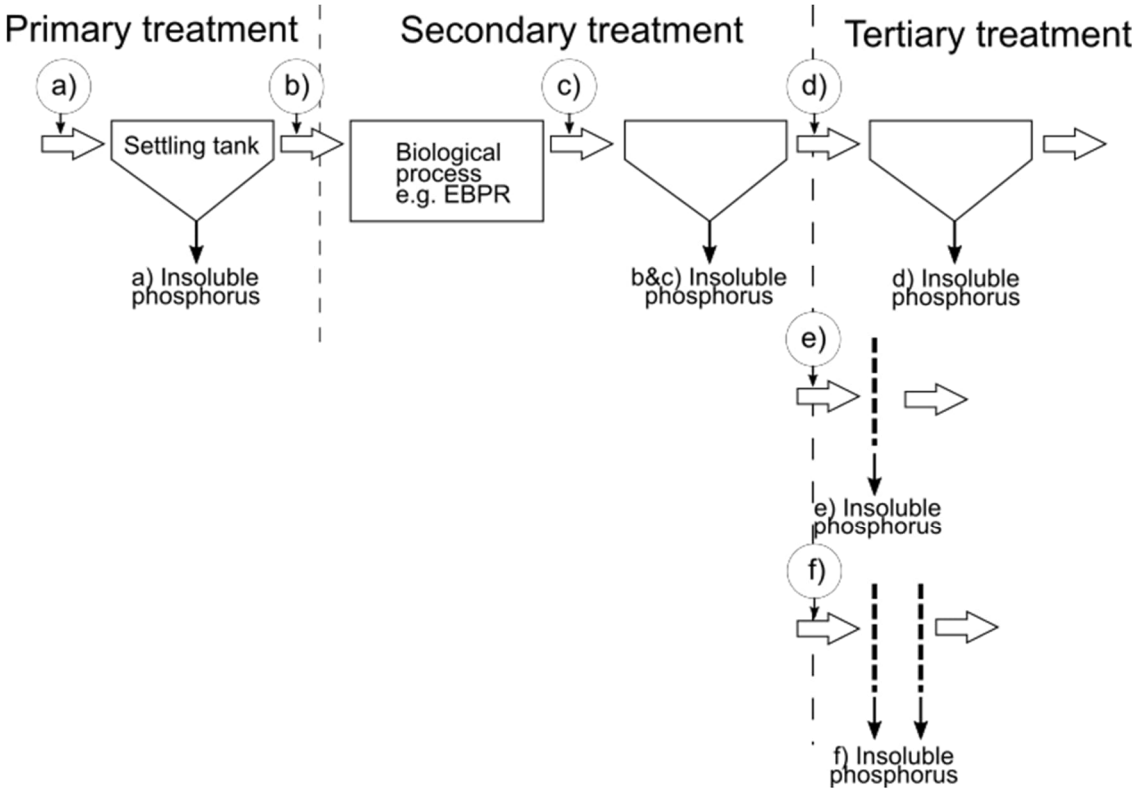


**Figure 2-3 An example of chemical dosing points of coagulants for P and sludge removal a) before primary sedimentation, b & c) before and/or after biological treatment process, d) post-secondary treatment, e) addition before single-stage filtration, f) addition before dual-stage filtration. Adapted from [42].**

The coupling of coagulation with biological nutrient removal in algal ponds has been proposed, where chemical coagulants achieved between up to 99.8% removal of algal biomass for bio-fuel production[50]. Research is growing into using sustainable polymers such as eggshells, for coagulation techniques, in particularly because of their affinity for heavy metals. For example, 80% of the contaminants were removed from synthetic wastewater using various hybrid states of ferromagnetite and eggshell[51]. Additionally, extracellular polymeric substances (EPS)[52] are an attractive coagulant for organic and heavy metal removal, that can be recovered using biopolymer extraction and could be applied to several industrial sectors such as, textile, construction and horticulture[52,53].

Increasing the nutrient load at WWTP will put pressure on current coagulation treatment processes, posing the risk for more environmental damage and costs due to stricter sludge handling and disposal of sludge and extraction of raw coagulants[54]. In the USA the cost of

coagulants are expected to increase from $1.37 billion in 2018 to $1.84 billion by 2023[54], to deal with increased loading at WWTP and increased pressure on raw materials (i.e., aluminium and iron).

For coagulation to remain a viable treatment method, sustainable alternatives are being researched to reduce or replace inorganic coagulants[55]. Natural coagulants include plant-derived gums such as: gaur gum and chitosan. Natural seed gum extracted from *Cassia obtusifolia* seeds has been shown to reduce quantity of alum needed to dose by 55%[56]. Ground *Moringa oleifera* seeds have been shown to aid the coagulation properties of alum and reduce the residue by 95%[55]. Furthermore, the use of natural coagulants has minimal impact on pH therefor reducing the need for pH adjustments downstream.

### 2.2.2 Enhanced biological phosphorus removal

Phosphate accumulating organisms (PAOs), such as *Candidatus Accumulibacter phosphatis* (*Accumulibacter*), are responsible for enhanced biological phosphorus removal (EBPR). To support POAs activated sludge processes create favourable conditions by cycling between anaerobic and aerobic conditions. Volatile fatty acids (VFAs) are broken down and converted to poly-β-hydroxyalkanoates (PHA) during the anaerobic phase. This produces the energy to accumulate phosphorus as intracellular polyphosphate during aerobic conditions, which is then removed in the accumulated sludge[57]. Enhanced biological phosphorus removal is argued to be the most economical and sustainable method of nutrient removal[58]. However, the availability of VFAs and biodegradable chemical oxygen demand (COD) to stimulate PAO growth and suppress competition from glycogen accumulating organisms (GAOs), is important to prevent EBPR failure[57,58]. In some cases, to overcome limited carbon sources and competition, dosing of specific carbon sources may be required depending on the species of PAOs and GAOs present in that reactor or influents. Research to improve EBPR has

shown that propionate increased the community of PAOs and decreased the GAO population[57,59]. Issues remain with the cost of adding external carbon sources due to insufficient urban carbon sources for POAs[60] and sustainable alternatives such as crude glycerol (a by-product from biodiesel production) have been shown to limit the long-term efficiency of EBPR due to the presence of inhibiting contaminants such as long chain fatty acids[60].

### 2.2.3 Chemical precipitation

Chemical precipitation of P is used in WWTP to prevent scaling of struvite ($MgPO_4NH_4.7H_2O$) in unwanted regions of the plant. The recovery of struvite as a resource is growing because of its potential as an alternative fertiliser[6,61]. Additionally, chemical dosing to recover vivianite ($Fe_3(PO_4)2 \cdot 8(H_2O)$) and hydroxyapatite ($Ca_5(PO_4)_3(OH)$) are being considered for their economic value in the agriculture and chemical industry[62–64]. Chemical recovery through mineralisation relies on abiotic measures, i.e., dosing reagents such as magnesium chloride (MgCl) or Fe(OH) to supersaturate Mg for struvite and Fe for vivianite precipitation, adjusting pH and controlling temperature so that the enthalpy of the chemical system supports precipitation[65].

Within WWTP chemical recovery can occur in primary or side-stream treatment, using sewage sludge (SS), anaerobic digestion (AD) liquors and sludge dewatering liquors from municipal, industrial, or agricultural sources (Table 2-1). The rationale for where to deploy P recovery technologies depends on the influent being treated e.g., agricultural wastes are rich in readily available P so can be recovered in a primary treatment step[66]. In contrast, in municipal wastewaters the required P-species are too low in primary effluents so require concentration before chemical recovery can occur; therefore the P-rich effluents in side-stream treatment are treated[67]. There are several patented technologies that are used

commercially across the globe (Table 2-1) that use a variety of external controls to encourage the precipitation of certain minerals with an economic benefit e.g., struvite and calcium phosphate for fertiliser substitution[22,68], sorbents[69] and cement additives[70,71]. The advancements in our understanding of chemical precipitation reactions have improved reactor efficiency. This has resulted in better quality products requiring less processing once recovered e.g., Crystal Green® from the Ostara Pearl© technology (Table 2-1) which produces 'market-ready' granules once dried[67]. Chemical recovery of minerals also provide savings to WWTP through the reduction in sludge produced, reducing final disposal costs[72].

Issues remain with the economic viability of chemical nutrient recovery technologies especially the cost of resources and energy for aeration and temperature control[69]. For example, the cost of sodium hydroxide (NaOH) in 2014 varied from \$1.05 to \$6.57 per $m^3$ effluent depending on the effluent treated[73–75]. Costs of magnesium chemicals varies depending on the quality of the source, such as low-cost magnesium oxide (MgO) at \$0.20 per $m^3$ effluent to magnesium chloride flakes (MgCl) at \$2.85 per $m^3$ effluent, in 2018[76]. Additionally, the price for phosphorus dosing varies from \$1.07 to \$5.65 per $m^3$ effluent based on its quality e.g., bone meal to phosphoric acid[75]. Whilst efforts are being made to find cheaper alternatives there are still additional costs or processes to include i.e., the acidification of bone meal[69]. When P content is greater than 100 mg $PO_4$-P/L chemical recovery remains viable[77,78], below this the process economics makes current chemical recovery technology unfeasible[69].

Table 2-1 Examples of commercially available or demonstration-scale phosphate recovery processes at WWTP (adapted from Kataki et al, 2016 and European Phosphate Platform Success Stories, 2019).

| Technology (mineral) | Influent | Reactor type | Treatment process | Recovery efficiency | First commercial plant | Plant size/capacity | Yields | Reference |
|---|---|---|---|---|---|---|---|---|
| **Pearl© Technology (struvite)** | Municipal wastewater | FBR | Side stream treatment of dewatering liquors via $MgCl_2$ and NaOH addition. | 80% $PO_4$-P, 10-15% N | 2009, WWTP Oregon, USA | 80 mg P/L, 600000 PE | 600-ton struvite/year *Crystal Green®* | 66,67,81 |
| **Crystalactor© (struvite or calcium phosphate)** | Municipal and industrial | FBR | Side stream treatment of dewatering liquors via $MgCl_2$, Aeration for stripping $CO_2$, or lime addition. Quartz sand for seeding | 70-80% $PO_4$-P | 1994, Edam Geestmerambacht, Netherlands WWTP | 230,000 PE | | 66,82,83 |
| **Phospaq™ and Anammox® (struvite)** | Food processing and municipal wastewater | CSTR | Aeration, stripping $CO_2$, increasing pH, and adding MgO | 80% $PO_4$-P, 90% $NH_4$-N | 2011, Waterstromen-Aviko | 260 kg P/L 160,000 PE | 400-ton struvite/year | 63,83,84 |
| **Struvia™ (struvite)** | Agricultural and municipal wastewater | CSTR Turbomix™ | Dosing of Mg-salts, NaOH buffers pH 8-9. Rapid mixing | 80-90% $PO_4$-P | 2016, Helsingør Denmark | 250 mg P/L 72,000 PE | 110 kg struvite/day | 85,86 |
| **AirPrex™ (struvite)** | SS | FBR air lift reactor | Aeration of digested sludge removes $CO_2$ increases pH. Dosing od Mg-salts | 90-95% P removal | 2009, MG-Neuwerk, Niersverband Germany | 995,000 | 1000 kg struvite/day | 87,88 |
| **Multiform (struvite)** | AD sludge dewatering liquors | FBR | Dosing of $MgCl_2$ and NaOH | 80% $PO_4$-P, 20% N | 2012, Idaho USA | | | 83,89 |
| **Polonite® and Sorbulite®** | Agricultural wastewater | Granulated beds | Filtration over $CaO.SiO_2$, $CaSiO_3$ (polonite®) | 100 mg P/g of | 4000+ farmsteads in | | Reclaimed granules are | 66,90 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **filter beds (struvite-granule mix)** | | | granules, P-precipitation nucleates around granules | Polonite® | Sweden since 2010 | | slow-release fertilisers | |
| **Seaborne (struvite)** | AD effluents/SS | CSTR | Acidification to separate heavy metals and P, struvite precipitation using $Mg(OH)_2$ and NaOH | | 2007, Gifhorn WWTP, Germany | 50,000 PE | 250 kg struvite/day and heavy metals | [91] |

PE – population equivalent
FBR – fluidised bed reactor
CSTR – continuous stirred tank reactor

## 2.3 Biomineral recovery techniques

### 2.3.1 Nutrient recovery and removal

Several microorganisms have been catalogued for their ability to remove ions from solutions through the precipitation of biominerals (Table 2-2). Wastewater has a rich variety of ions which, as the catalogue of biominerals grows, the opportunities for recovering these as biominerals as an alternative sustainable material increases[8,79,80]. Immobilised microbes are promising alternatives for seawater treatment for potable uses (industrial and domestic) by reducing the ion content, notable bacteria include *Bacillus subtilis* and *Halomonas* sp. (Table 2-2). These strains were shown to remove up to 100% calcium (Ca) and 60% Mg over a 14-day treatment period by inducing the biomineralisation of calcite, struvite, and anhydrite (*B. subtilis* only) (Table 2-2)[79].

*Lysinibacillus fusiformis* has been shown to produce both calcite and struvite from synthetic media (Table 2-2). Laboratory experiments tested the biomineralisation capabilities of *L. fusiformis* in varying Mg:Ca concentrations as 'free' and cells immobilised on activated carbon[80]. In the immobilised state, *L. fusiformis* removed ~90% Ca and ~80% Mg after 6 days, marking an improved removal rate compared to 'free' cell trials which achieved similar removal percentages over 30 days[80]. These trials showed that *L. fusiformis* could concentrate Ca intracellularly for improved calcite production, but Mg concentrations did not increase intracellularly. Indicating that the mechanisms for biomineralisation of struvite and calcite are different for *L. fusiformis*, calcite forming through BCM and struvite through BIM, *H. Yan et al.*[80] hypothesised that EPS produced by *L. fusiformis* could act as nucleation sites for struvite as weak negative charges attract positively charged Mg ions. The application of *L. fusiformis* to nutrient recovery from wastewater is attractive because of its ability to recover both Ca and Mg biominerals and has shown that immobilised bacteria can improve the rate of

nutrient removal and biomineral production. However, this remains to be tested in synthetic wastewater and in open culture conditions which could impact the productivity of *L. fusiformis* and the production of biominerals. Furthermore, evidence for the mechanisms of biomineral formation of both calcite and struvite needs to be clarified so that conditions can be optimised depending on which biomineral is to be preferentially recovered.

*Brevibacterium antiquum, Bacillus pumilus, Halobacterium salinarum, Idiomarina. loihiensis* and *Myxococcus xanthus* have been shown to repeatedly biomineralise struvite or 'bio-struvite' from sludge dewatering liquors[14,92]. Their biomineralisation mechanisms have been distinguished using cell dissolution and chemical analysis with transmission electron microscopy: *B. antiquum* and *H. salinarum* precipitated bio-struvite by intracellular BCM, *B. pumilus, I. loihiensis* and *M. xanthus* induced bio-struvite precipitation by BIM[92]. Importantly these microorganisms have been able to remove up to 92% of $PO_4$-P and recover between 99 and 198 mg bio-struvite per litre 'weak' sludge dewatering liquors where chemical struvite precipitation would be costly and inefficient[93,94]. The variety of biomineralisation mechanisms needs to be better understood so that processes can be tailored to give these bacteria the advantage in P-recovery from a variety of wastewater streams. Research also needs to be scaled up to see how these bacteria can function in open culture wastewater, where competition for nutrients may impact removal/recovery efficiencies and explore the options for biomass retention such as microbial immobilisation techniques and biocatalysts[95,96].

Diatoms have also been investigated for their use in nutrient removal and recovery. In studies of contaminated water such as those suffering from eutrophication or heavy metal pollution diatoms have been shown to be the most prevalent microorganism[97], suggesting they are better suited to deal with these hazardous environments. Also, it has been shown through

their photosynthesis and exoskeleton biomineralisation the productivity of symbiotic bacteria can be improved to break down organic pollutants in wastewater (Figure 2-4).

A proposed application for diatoms is direct input into high-rate algal ponds or similar wastewater treatment procedures. Diatom metabolism can improve the rate of organic pollutant breakdown by oxidising bacteria[101], and coupled with high rate algal ponds can remove up to 80% of N and P and up to 90% reduction in biological oxygen demand (BOD) over a few days[97]. The recovery of diatom biomass can be used for production of the nutraceuticals (animal feed supplements & aquaculture feed) such as carotenoids and polyunsaturated fatty acids (PUFAs) [102], biofuels (bio-diesel) and refinement into pharmaceuticals such as biosynthesised ovothiol for use in anti-inflamatories[103] (Table 2-3). However, recovery efficiencies need to be quantified and the feasibility assessed, furthermore the cost of bio-diesel production from diatom biomass exceeds that of fossil fuels currently[104].

Table 2-2 Summary of ongoing research into nutrient recovery using biomineralisation

| Microorganism | Biomineralisation mechanism | Recovered biomineral | Application | Removal/recovery | Reference (s) |
|---|---|---|---|---|---|
| *Brevibacterium antiquum* | BCM | Struvite | Sludge dewatering liquors and source-separated urine. | Up to 92% phosphorus recovery as bio-struvite. 99 and 198 mg struvite per L wastewater | [8,14,92,93] |
| *Bacillus pumilus* | BIM | | | | |
| *Halobacterium Salinarum* | BIM | | | | |
| *Idiomarina loihiensis* | BIM | | | | |
| *Myxococcus xanthus* | BCM | | | | |
| *Lysinibacillus fusiformis* | BIM | Struvite and calcium phosphate | Nutrient removal from wastewater | 95% and 80% recovery of calcium and magnesium, respectively | [80] |
| *Acinetobacter* *Aeromonas*<br>*Alcaligenes* *Arthrobacter*<br>*Corynebacterium* *Entero bacteriaceae*<br>*Kurhia* *Micrococcus*<br>*Murraya* *Plesiomonas*<br>*Psuedomonas* *Staphylococcus* | Mechanisms not specified | Struvite | Nutrient removal from freshwater, soils, and wastewater | - | [98–100] |
| *Bacillus subtilis*<br>*Halomonas* sp. | BIM through ureolysis | Calcite-struvite-anhydrite | Seawater treatment for industrial and domestic use | Up to 97% and 67% of calcium and magnesium recovery. Precipitates roughly 66:27:7 split calcite:struvite:anhydrite | [79] |

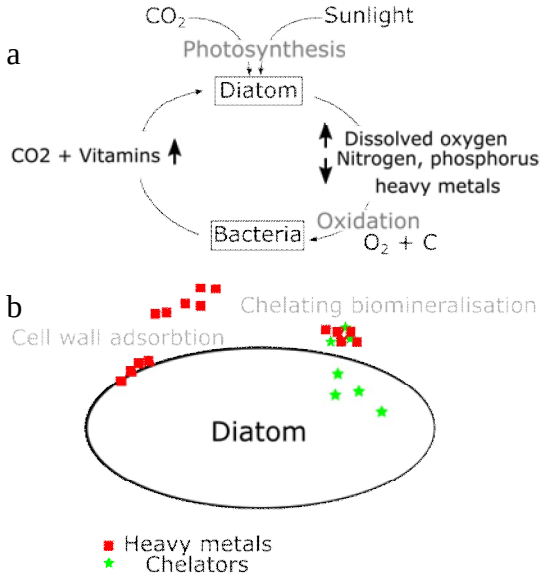


**Figure 2-4 Diatom application to wastewater refinement top improving bacteria oxidation to break down organics (C) (a) and heavy metal remediation (b). Adapted from Marella et al, 2020.**

**Table 2-3 Diatoms for wastewater treatment, adapted from Marella et al, 2020**

| Diatom strain | Recovered bioactive compound | Uses | Reference(s) |
|---|---|---|---|
| *Skeletonema marinoi* | Ovothiol | Pharmaceutical | [103] |
| *Navicula sp. Tabularia affinis* | PUFAs | Nutraceutical | [102] |
| *Chaetoceros calcitrans* | Carotenoids | Nutraceutical, pharmaceutical | [105] |
| *Phaeodactylum tricornutum* | Protocatechui/gallic acid | Nutraceutical, pharmaceutical | [106–108] |
| *Thalassiosira weissflogii* | Carotenoids | Pharmaceutical | [109] |
| *Thalassiosira pseudonana* | Nonyl 8-acetoxy-6-methyloctanoate Anticancer activity | Pharmaceutical, vaccine adjustments | [110] |

### 2.3.2 Remediation and metal recovery

The use of biomineral powders in absorption processes has been studied for several decades[111] but no industrial scale treatment techniques are being utilised. However, the opportunity biominerals have as a low-cost alternatives to filtration, chemical precipitation and electrodialysis techniques continue to be researched[111,112]. The abundance of shell materials and low processing costs make this heavy metal treatment method attractive[111]. Once sorbed to the biomineral, recovery of heavy metals remains to be researched and

perfected, so that these materials can recycled and reused. Promising techniques include electro-gravimetric methods after decantation and filtration of the material[111]. Sorption of copper, nickel, and zinc to shell ash has been demonstrated, removing 200 mg/L of each metal contaminant in synthetic wastewater[113]. However, issues remain with increased calcium loading as the biominerals deteriorate, increasing the risk of scaling and could lead to increased treatment costs further down the process[111].

Trials of using biomineralisation mechanisms to remove and recover contaminants from wastewater derived from mining or nuclear legacies are growing[29,30,114] (Table 2-4). These research projects have the ambition to show that bacteria can concentrate contaminants, producing cleaner effluents, trapping contaminants in insoluble precipitates. For example, research into the ability of microbes to concentrate and store uranium has grown as the global output of radioactive material increases, e.., *Saccharomyces cerevisiae* can reduce the solubility of uranium contaminants[28] (Table 2-4). The yeast successfully precipitated uranium-bearing phosphate crystals, chernikovite ($(H_3O)_2(UO_2)_2(PO_4)_2 \bullet 6(H_2O)$) and uramphite ($(NH_4)(UO_2)(PO_4) \bullet 3(H_2O)$) on its cell surface in acidic and alkaline initial conditions respectively, biomineralisation occurs due to pH bacteria buffering pH causing BIM[28] (Table 2-4). Additionally, research has shown with minor adjustments the function of microbial communities that have evolved at sites of contaminated land can be improved. For example, microbes naturally present in contaminated groundwater from nuclear facility legacies can be enhanced to remove radionuclides through the addition of new feedstocks (glycerol phosphate) improving the biomineralisation of phosphates which then binds the radionuclides[115].

Diatoms have also been shown to chelate heavy metals (BIM)[116]. A key characteristic of diatom metabolism is their complex urea cycle, it is believed that this allows diatoms to

Table 2-4 Metal concentrating microorganisms undergoing research for remediation and recovery

| Microorganism | Biomineralisation mechanism | Source | Recovered mineral/metals | Application | Reference(s) |
|---|---|---|---|---|---|
| *Saccharomyces cerevisiae* | BIM | Yeast | Chernikovite and uramphite depending on alkalinity | Biomineralisation of uranium and phosphorus from mine leachates. ~67% biomineralisation efficiency at equilibrium pH | [28] |
| *Bacillus megaterium* | BIM | A wide variety, inc. Antarctic Geothermal Lakes | Lead phosphate hydroxide, Chromium | Biologically induced mineralisation of lead and chromium from soils. | [27,114] |
| *Aspergillus alliaceous* *Trichoderma harzianum* *Clonostachys rosea* | BCM | Isolated from bare dump soil samples at Libola mine | Silver | Biomineralisation of silver, up to 50% recover from sulphide-rich wastewater and soils | [26] |
| *Viridibacillus arenosi* *Sporosarcina soli* | BIM | Isolated from abandoned mine soil | Lead and cadmium | Biologically induced mineralisation of lead and cadmium because of MICP | [13] |

survive for longer in nutrient depleted environments where other phytoplankton will struggle[117]. Diatoms can passively adsorb heavy metals to their biomineralised silica cell walls or can excrete chelators (organic ligands) and bind heavy metals which sorb to the diatom silicate exoskeleton[116] (Figure 2-4). It is likely this occurs to prevent intake of potentially toxic metals into the diatom[116].

## 2.4 Discussion

The broad range of applications of biominerals and biomineralisation is promising for the growth of sustainable development by reducing the raw resources and energy required without comprising on the quality of the product produced. In addition, research has shown microorganism performance can be improved using immobilisation techniques and biocatalysts, retaining biomass and providing an environmental niche for the microbe(s), to encourage the shift to these technologies.

To ensure that biomineralised products from wastewater are accepted, research needs to ensure that they meet regulatory requirements, demands, and address any negative perceptions. Such as them being 'inferior' compared to products from raw materials. Studies are underway to promote biomineralisation processes for resource recovery, which has significant potential in this area. The UK government's 'From Waste to Resource Productivity' report[118] identified the need for reassessment across all sectors what is defined as waste and how to recycle or recover resources to maximise the UK's resource resilience and compete with international changes. Furthermore, the NextGen water project supports demonstration sites of technologies that showcases circular water reuse, energy production and resource recovery across the European Union (EU)[119].

Within the UK it has been estimated that 14.8 kt of dissolved orthophosphate was lost in discharged effluents from domestic sources in 2015, ending up in fresh water catchments[120].

If nutrient recovery using biomineralisation were scaled up to commercial sizes and recovery efficiencies maintained (Table 2-3), then 13.6 kt P could be recovered as bio-struvite. A further 43 kt P could be recovered if this were applied to wastewater treatment plants, where removed P is currently disposed in landfill[120]. By incorporating P recovery at wastewater treatment plants, 31% of the inorganic phosphate applied to tillage crops in the UK could be replaced by sustainable bio-struvite recovered from domestic wastewater alone[121]. Furthermore, the opportunity to recover nutrients from high strength wastewater such as agricultural waste, using decentralised systems, would provide another source for biomineralisation technologies to provide more sustainable fertilisers. For the EU, analysis of P fluxes of 27 member states showed that 221 kt of P for 2005 was lost through municipal wastewater and mineral fertiliser imports reached 189 kt for 2005[122]. If biomineral recovery was implemented to this single loss of P all the imported mineral fertiliser could be replaced by recovered P removing the EU's reliance on imported P fertilisers. Assumptions have been made for these calculations and are likely 'best-case scenario', however, what studies have shown is that the current use of P has been, and continues to be, inefficient

## 2.5 Knowledge gaps and considerations

Previous studies of biomineral production and recovery from wastewater is possible in controlled settings using synthetic wastewater, pure cultures and nutrient supplementation. However, real-world wastewater environments are complex, with varying microbial communities and nutrients (Table 2-5). Key parameters such as the loading rates for intensified treatment and amount of biomass required to treat a litre of wastewater has not been quantified and accumulation rates of targeted ions such as P per mg of microbe (Table 2-5). Table 2-5 summarises the research gaps and other considerations for biomineral technologies to be developed into industry. For example, struvite recovery and phosphorus removal using biomineralisation requires research into the response each biomineralisation

mechanism has in open culture wastewater with varying nutrients conditions. Furthermore, it is not known how biomass can be retained or grown sufficiently for continuous treatment of wastewater and how the biomineralised struvite can be recovered at an industrial scale.

Table 2-5 Biomineral recovery knowledge gaps and considerations

| **Research gaps** | **Possible treatment trade-offs** | **Wider implications** | |
|---|---|---|---|
| | | Positive | Negative |
| Impact of nutrient competition by other microbes in open culture conditions on;<br>1. Selected microbes<br>2. Biomineralisation mechanisms | Relatively slower treatment process than chemical treatment | Local and cheap alternative to imported minerals | Social concerns for products derived from waste |
| Encapsulation techniques impacts on removal and recovery of biominerals | Recover biominerals are less pure that chemically recovered minerals | Resilience to global mineral market prices and fluctuations in supply | Biominerals may not provide the same benefit as chemically manufactured minerals |
| Consistent purity, quality, and possible presence of potential contaminants of concern | Influent conditions may be harmful selected microbes causing death and poor treatment and biomineral recovery efficiency | Development of circular economies across industries | Without regulatory controls, reuse could increase environmental risk from incorporated contaminants |
| Optimisation of nutrient loading rate and other parameters for efficient treatment and biomineral recovery | | | |

There is huge potential for biomineralisation technologies to revolutionise the movement towards ‘green chemistry’ utilising the productivity of microorganisms. Reducing the need for strict environmental controls which are required in chemical treatment, whist offering alternatives to extracted minerals with a biomineralised product. The considerations from Table 2-5 must be included in the development of these biomineral products to ensure they can meet requirements as well as demand without compromising the environment. Such as

the development of struvite recovery wastewater liquors using biomineralisation should be substituted into slow-release fertilisers, or methods be developed to improve its dissolution for faster nutrient release when required by farmers. Whilst ensuring that potential contaminants remain below regulatory limits for mineral fertilisers. This will benefit sustainable growth across developed and developing countries, providing local sustainable commodities such as fertilisers, reducing the risk of being driven out the market through fluctuating prices.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data Access Statement**

Data shown in this review paper can be found through the resources in the following References list.

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# 3. Nutrient recovery from urine using bio-mineral formation processes

Robert E. Colston[a], Ajay Nair[b], Peter Vale[c], Francis Hassard[a], Tom Stephenson[a] and Ana Soares*[a]
[a]- Cranfield Water Science Institute, Cranfield University, College Road, Cranfield, MK43 0AL, UK
[b]- Microvi Biotech, 26229 Eden Landing Rd, Hayward, CA 94545, United States
[c]- Severn Trent Plc. Severn Trent Centre, 2 St John's Street, Coventry, CV1 2LZ
*- Corresponding author: a.soares@cranfield.ac.uk

**Abstract**

Nutrient recovery from wastes is a promising step towards delivering a circular economy and address local shortages of mineral fertiliser around the world. The ability of selected microorganisms (*Brevibacterium antiquum, Bacillus pumilus, Halobacterium salinarum, Idiomarina loihiensis* and *Myxococus xanthus*) to remove and recover nutrients from fresh urine through bio-mineral formation struvite was investigated. The selected microorganisms outcompeted native microbes in open-culture urine batches and intact cell counts were 1.3 to 2.3 times larger than in non-inoculated controls. Orthophosphate removal reached 50% after 4 days of incubation and 96% when urine was supplemented with magnesium. Additionally, soluble chemical oxygen demand was reduced by 60% and urea hydrolysis was only <3% in controls however, increased up to 35% in inoculated urine after 10 days. The dominant morphology of recovered precipitates was euhedral and prismatic, identified using energy dispersive spectroscopy and X-ray diffraction as struvite (i.e., bio-struvite) but potassium was also present at 5%. Up to 1 g bio-struvite/L urine was recovered. These results demonstrate the ability of bio-mineral producing microorganisms to successfully grow in urine, enhance the rate of ureolysis and recover nutrients as bio-struvite, that could potentially be used as sustainable fertilisers or chemicals.

**Key words**

Nutrient recovery, urine treatment, bio-based economy, biomineralisation, bio-struvite

### 3.1 Introduction

Improving the sustainability of food production through nutrient recovery from urine[1,2] is a growing area of research. Urine only contributes to 1% of municipal wastewater yet it contributes to 80% of nitrogen (N), 50% of orthophosphate ($PO_4$-P) and 10% of chemical oxygen demand (COD)[3,4]. As such, treating urine separately has been inferred to increase the efficiency of nutrient recovery and reduce overall greenhouse gas emissions and costs to wastewater treatment plants (WWTP)[2,5]. Additionally, urine is rich in other key nutrients required for plant growth i.e., magnesium and potassium, but usually these are overlooked. There are many novel processes to treat urine (Table 3-1) many relying on a first step of volume reduction using renewable dehydration media (i.e. wood ash)[6] or alternatively, through reverse osmosis (Table 3-1), which can reach a flow 11.9 ± 1 L/day.$m^2$ with >70% N and 100% phosphorus (P) and potassium (K) recovered. The retentate of some technologies can be used as a liquid fertiliser such as volume reduction and ion exchange processes[6] (Table 3-1). In microbial fuel cells and electrodialysis technologies, electrons and protons released from the microbial reduction of organic matter within the urine are transferred between an anode and cathode to generate electricity, which can be used to power selective electrodialysis to remove nutrients such as $PO_4$-P[4,7] (Table 3-1). In developing countries where dry toilets have been installed as a decentralised option for safe sanitation, struvite recovery from urine could provide a source of renewable fertiliser for local use or resale[8]. Struvite has been proven to be a slow-release fertiliser, increasing research into recovering struvite as a sustainable fertiliser alternative[9]. Issues remain due to the value of chemical struvite not offsetting the cost of its recovery in traditional precipitation reactors to overcome the cost of mined mineral fertilisers. Further to this, urine can contain micropollutants and pharmaceuticals[10], further decreasing the value of recovered products. Life-cycle assessment has proven that source separating urine and treatment can reduce the global warming potential, eutrophication potential and cumulative

energy demand up to 63% by reducing freshwater use and nutrient-load to WWTP[1,2]. This reduces the overall energy required for wastewater treatment and recovered fertilisers can offset greenhouse gas emissions from the production and transport of synthetic/mined fertilisers[2].

The processes described in Table 3-1 have reached different stages of development and implementation, however the costs of materials and energy demand remains high for most[7,19,20]. Additionally, chemical recovery processes typically require two-stages of treatment to stabilise or completely hydrolyse the urea in urine, to limit ammonia volatilisation, increasing the pressure on urine storage at treatment sites[1,14,21]. Complete urea hydrolysis (ureolysis) or stabilisation is required to ensure consistent influent quality, transport urine and improve the ease of controlling pH as ureolysis causes pH to increase leading to ammonia volatilisation[22]. Stabilisation of urea requires acidification to bring the pH of the urine to below 4 to denature any free urease enzymes and prevent chemical hydrolysis of urea[6,14,19,23]. Without the presence of enzymes or chemical addition, complete ureolysis is a lengthy process, with an estimated half-life of 1.5 – 3.6 years at 25°C, depending on the concentration of urea[14,19,21,24]. To accelerate ureolysis, chemicals or enzymes are added, with temperature and/or pH control [19,21–24]. With the addition of urease enzymes and temperature increase to >50°C complete ureolysis can be achieved in hours[14,19]. After stabilisation or ureolysis, chemical recovery can take place which requires the addition of reagents to control pH, super saturation of desired ions, and crystal growth. These multiple steps, costs of materials and energy requirements of such technologies (Table 3-1) has meant commercial up take of them has been low. Alternative treatment methods should be investigated that can improve their viability to encourage wider uptake, by providing evidence for effective one-step nutrient recovery that has low energy and reagent requirements, whilst effectively recovering nutrients.

Table 3-1 Urine treatment processes producing fertiliser alternatives

| Process | Method | Advantages | Disadvantages | References |
|---|---|---|---|---|
| **Nutrient precipitation-Magnesium based** | Addition of Mg (i.e., MgO) to increase saturation of struvite, most efficient when Mg:P ratio raised to 2:1 and pH increased with NaOH to encourage precipitation | 99% $PO_4$-P recovery at room temperature and chemical struvite fertiliser alternative | 3-5% N-recovery<br>pH increase is still required | [1,11,12] |
| **Nutrient precipitation-Calcium based** | Ca(OH) was used to simultaneously increases pH and increase the saturation of Ca to encourage precipitation of hydroxyapatite (HAP) | Up to 95% $PO_4$-P recovery as $Ca_3(PO_4)2$ or HAP at 25°C, pH 11-13<br>More stable than struvite in temperatures >60°C | Hydrolysed urine promotes $CaCO_3$ over HAP due to abundance of $CO_3^-$ from complete urea hydrolysis | [1,13,14] |
| **Nitrogen recovery -Ammonia stripping** | Induced volatilisation of $NH_4^-$ through pH and temperature change<br>Bubble $NH_{3(g)}$ through weak acid (sulphuric or acetic acids), precipitating ammonium | Ammonium sulphate recovery up to 95% | Some processes have not accounted for ammonia loss during storage or transport of the urine.<br>Difficulty removing ammonia from absorption media | [1,15,16] |
| **Nitrogen recovery -Adsorption** | High electronegative materials are used to sorb $NH_4^-$<br>Materials used; zeolites, hybrid ion exchange resins (HIXR) (Fe-based) or activated carbon (coconut shell, sawdust, charcoal) | Up to 92% P recovered using HIXR<br>Removed 256 mg N/g coconut shell = 95% ammonia recovery | | |
| **Volume reduction-Evaporation** | Heat source (solar, burning biogas) evaporates the water fraction, increasing the concentration of nutrients in remaining liquid. The condensate can be used as water for re-use | Up to 95% water recovery and 95% ammonia recovery | $NH_4^-$ volatilisation increases, up to 93% loss.<br>High energy demand.<br>Risk of concentrating pharmaceuticals, parasites, pathogens, and heavy metals in liquid fertiliser products | [1,6,17,18] |
| **Volume reduction-Reverse osmosis** | 75% global desalination uses reverse osmosis.<br>Overpressure influent, increasing hydrostatic pressure drawing water from influent through membrane into buffer solution | Potable water (80% volume reduction), Nutrient-rich supernatant can be used as liquid fertiliser. ½ the cost of evaporation techniques. | Filter clogging<br>Risk of concentrating pharmaceuticals, parasites, pathogens, and heavy metals in liquid fertiliser products | |

| Process | Method | Advantages | Disadvantages | References |
|---|---|---|---|---|
| **Volume reduction-Freezer concentration** | Effluents chilled to -30°C to reach eutectic point of salts to cause precipitation. Suggested to use after pre-concentration effluents using reverse osmosis and before struvite recovery | 99% salts recovered and 95% water recovery | High energy demand to chill wastewater to -30-°C. Risk of concentrating pharmaceuticals, parasites, pathogens, and heavy metals in liquid fertiliser products | |

The growth of selected microorganisms in wastewater sludge dewatering liquors (SDL) has been shown to promote the recovery of struvite (henceforth referred to as bio-struvite) through bio-mineral formation without the addition of reagents[11,25–28]. Bio-mineral formation is the process in which minerals precipitate due to changes in solution chemistry controlled and induced by living organisms. The mechanisms can be split into biologically controlled mineralisation (BCM) e.g., magnetosome formation, providing clear benefits to the organism or biologically induced mineralisation (BIM) e.g., iron-reducing microorganism causing iron precipitation, which is a by-product due to chemical changes caused by their metabolic activity. Urine has been inferred to be a potential waste for P and other nutrients' recovery as bio-struvite[11,25,26,28], but not yet investigated in detail. Characterisation of five microbial strains known for producing bio-struvite and the mechanisms of precipitation involved was carried out by Simoes, et al,. and Leng, et al,. in wastewater[25,27–31]. Key characteristics of urine is an abundance of carbon sources including urea, which 4 of the 5 microorganisms can utilise as they produce urease[28], COD measures between 5000 and 12000 mg/L in fresh and hydrolysed urine[32,33] consisting of other carbon sources such as creatinine, hippuric acid and citric acid[28,34]. A public misconception is that urine is sterile, but microbial analysis has shown it is not the case[35,36]. Despite this, it is perceived that the selected microbes will be able to grow successfully in fresh (untreated), open culture urine[28], where open culture refers to native

microorganisms present. Due to the availability $PO_4$-P, $NH_4$-N and Mg being greater in urine compared to SDL, the likelihood of bio-struvite recovery taking place would be expected[30].

This study aims to provide evidence that bio-mineral formation can be applied for nutrient recovery in urine. The five microorganisms have never been studied in fresh urine and it is not known how native microbes will impact their growth rate, activity, nutrient removal and what type of bio-mineral would be forming. Each microorganism was inoculated into fresh, untreated urine and incubated to monitor their growth. Sacrificial bottles were analysed throughout the incubation period to analyse changes in urine chemistry and collect precipitates for analysis. The wider implications for this study are providing new data to understands if bio-mineral formation technologies can be applied to treat wastes and recovery nutrients as minerals for reuse as fertilisers.

## 3.2 Materials and methodology

### 3.2.1 Source of microorganisms and urine

Microorganisms previously tested and known for their ability to produce bio-struvite in wastewater[28,30] were selected for this study: *B. antiquum* and *H. salinarum* (DSM 21545 and DSM 671 respectively, German Resource Centre for Biological Material, Germany), *B. pumilus* (GB43, LGC Standards, Middlesex, UK), *I. loihiensis* and *M. xanthus* (CECT 5996/ MAH1 and CECT 422, Spanish Type Culture Collection, University of Valencia, Paterna, Spain).

Urine was collected from Cranfield University's toilets, adhering to established ethical research integrity protocols. Fresh (non-stabilised) urine batches were utilised to grow the selected microorganisms straight after collection or after storage at 4 $^{\circ}$C for a maximum of 2 days to limit urea hydrolysis.

### 3.2.2 Microorganisms' incubation in urine

Frozen starter cultures of each microbe were inoculated in 300 mL B4.1 synthetic media (4 g/L of yeast extract, 2 g/L of magnesium sulphate heptahydrate and 2 g/L of potassium phosphate), incubated in conical flasks at room temperature (20-22 °C) and agitated at 150 rpm (Stuart SSL, Fisher Scientific, Loughborough, UK) for 2-3 days to reach the stable growth phase[28]. After this period the growth media was filtered to remove precipitates greater than 10 µm (Whatman™, Grade 1 filter sheets).

Experiments were completed in batches of sacrificial bottles (i.e., the whole bottle content was used for each sampling point to ensure all precipitates were recovered and analysed) with 300 mL urine and inoculated with 50 mL of the filtered starter culture under sterile conditions to ensure only the inoculum introduced the targeted microbes to the raw urine, at room temperature (19-22°C) and agitated at 150 rpm (Stuart SSL, Fisher Scientific, Loughborough, UK) for 10 days, to ensure bio-minerals are at a recoverable particle size[25,28]. All experiments completed in duplicate, and control bottles contained only urine (non-inoculated).

Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) was added to two additional sacrificial bottles inoculated with *B. antiquum*'s to observe the impact on $PO_4$-P removal and precipitate recovery when Mg was not a limiting nutrient. Based on urine characterisation 15 mL of 0.8M $MgSO_4.7H_2O$ was added to reach a 1.2:1 Mg:P (based on initial $PO_4$-P concentration of the urine batch).

### 3.2.3 Analytical methods

Prior to each incubation the of urine was characterised, sampling occurred at time 0, 1, 2, 4, 7 and 10-days incubation through sacrificial bottles. pH was measured using a Fisherbrand hydrous 300 pH meter (Fisher Scientific, Loughborough, UK). Ammonium ($NH_4$-N), $PO_4$-P, total phosphorus (TP), total nitrogen (TN) and soluble chemical oxygen demand (sCOD), was

measured with Merck cell test kits according to the manufacturer's instructions. Magnesium, potassium, calcium and sodium were analysed using inductively coupled plasma-mass spectrometry (ICP-MS) (Beaconsfield, United Kingdom).

The urea content of each batch was estimated based on TN and $NH_4$-N measurements using Equation 3-1. In all urine batches nitrate and nitrite were not detectable, therefore it was assumed that, inorganic nitrogen (nitrates and nitrites) were not present[32,33].

Equation 3-1 $$\boldsymbol{Total\ nitrogen = Total\ kjeldhal\ nitrogen + inorganic\ nitrogen}$$

$$\boldsymbol{Total\ kjeldahl\ nitrogen = organic\ nitrogen(urea) + free\ ammonia}$$

$$\boldsymbol{Urea = Total\ nitrogen - free\ ammonia - inorganic\ nitrogen}$$

BD Accuri C6® flow cytometry with 488 nm solid-state laser (Becton Dickinson U.K. Ltd., Oxford, UK) was used to measure total cell counts (TCC), intact cell counts (ICC) and proportion of high nucleic acid (HNA) to low nucleic acid (LNA) in the urine at the same sampling intervals using SYBR Green I and propidium iodide staining[37]. Instrument noise was accounted for according to Gatza et al., (2013) using BD Accuri C6® software.

Stochiometric mass balances and the Geochemist's Workbench® modelling software was used to pinpoint abiotic minerals precipitates during urine incubation, using thermodynamic modelling (PHREEQC, US Geological Society (USGS)) based on the activities of measured ions and the environmental conditions (pH, temperature, and pressure = 1 bar). The measured ions ($Mg^{2+}$, $Ca^{2+}$ and $K^{+}$) provided estimates for struvite, calcium phosphate, potassium phosphate and the total precipitates. The mass balances considered changes in these measured ions at a stable pH, that would support abiotic nucleation in controls.

Precipitates were recovered from sacrificial bottles using vacuum filtration through a 10 µm filter paper (Whatman™, Grade 1 filter sheets), dried at room temperature (22 °C) and weighed. The quality of precipitates and their assemblage, morphological characteristics were analysed using optical microscopy (Olympus MX40) and scanning electron microscopy (SEM) coupled with energy dispersive x-ray spectroscopy (EDS) to distinguish the mineralogy using point ID analysis and element mapping (Tescan Vega 3, Oxford Instruments© AZtecCrystal™, Abingdon, United Kingdom). Powdered x-ray diffraction spectroscopy (XRD) was used to support SEM-EDS analysis by comparing precipitate spectra with pure struvite and calcium phosphate spectra's (Siemens D5005, Manchester, United Kingdom).

## 3.3 Results

### 3.3.1 Initial urine characterisation

The characterisation of each urine batch (UB) is presented in Table 3-2. On average, the pH was slightly acidic ranging from 5.76 to 6.42. There was high variability in the concentrations of sCOD, $NH_4$-N and TN among the batches collected (Table 3-2). Soluble chemical oxygen demand varied from 5740-15140 mg/L, $NH_4$-N varied from 152-595 mg/L and TN varied form 820-3250 mg/L (Table 3-2). $PO_4$-P and TP were very similar in all batches, $PO_4$-P varied from 228-466 mg/L and TP varied from 270-515 mg/L (Table 3-2). Magnesium, $Ca^{2+}$ and $K^+$ were relatively consistent across all urine batches recording values of 48-63 mg $Mg^{2+}$/L, 73-111 mg $Ca^{2+}$/L and 1394-1992 mg $K^+$/L (Table 3-2).

### 3.3.2 Microbial growth in urine

The microbial growth within inoculated and control urine bottles were observed by measuring intact cell counts (ICC) (Figure A1). Initial ICC in urine varied from 4.25 x $10^6$ to 3.64 x $10^{10}$ ICC/mL, which increased to 6.17 x $10^8$ to 3.16 x $10^{12}$ in controls and 7.36 x $10^8$ to 5.56 x $10^{12}$ ICC/mL in inoculated bottles after the 1 day of incubation (Figure A1). Tests showed an increase in ICC, in the inoculated bottles, 1.3 to 2.73-fold higher than in the controls (Figure

A1). After the 1 day of incubation the ICC in the inoculated tests and controls stabilised (Figure A1). The specific growth rates of the microorganisms in inoculated tests were between 0.09 - 0.18 l/h and 0.07-0.14 1/h in the controls, during the first day of incubation. From day 2 onwards the ICC remained constant and growth rate was close to zero.

High nucleic acid versus LNA percentages highlighted key differences between controls and inoculated tests (Figure 3-1). In the controls, percentages of HNA increased throughout the incubation period starting between 45% and 60% at day 1, increasing up to 85% by day 4. Inoculated tests showed a larger proportion of HNA compared to controls by day 1, measuring between 80% and 90% HNA (Figure 3-1). In the inoculated bottles HNA stayed between 80-90% throughout the incubation period.

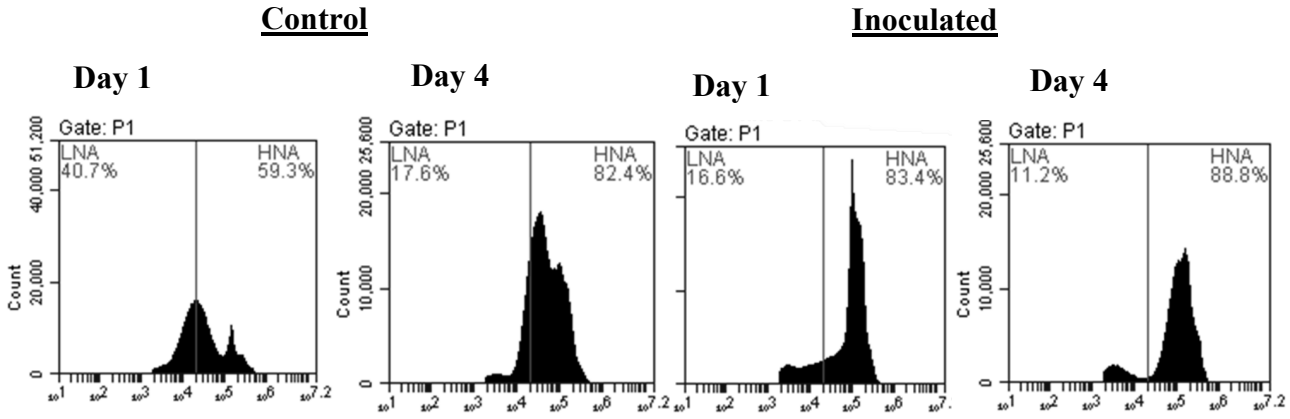


**Figure 3-1 Typical change in proportion of HNA to LNA between controls (left) and inoculated urine batches (right, data for *B. pumilus*) after 1- and 4-days incubation**

### 3.3.3 Solution chemistry

The pH in the inoculated tests and controls increased throughout the incubation period rising to between 7.1 and 9.5 (Table A1). In inoculated tests sCOD was reduced by 28-57% (1953 to 3820 mg/L) (Figure 3-2 a)), whilst in controls this was 13-46% (1060 to 3275 mg/L) by day 4 (Figure 3-2 a)). *H. salinarum* showed the poorest sCOD removal of all microorganisms tested, with 28% removal after 4 days incubation. The highest sCOD removal observed after 4 days

Table 3-2 Chemical characterisation of different UB collected with tested standard error for samples measured in duplicate and corresponding microbe inoculated

| | **pH** | **SCOD mg/L** | **$NH_4$-N mg/L** | **TN mg/L** | **$PO_4$-P mg/L** | **TP** mg/L | **$Mg^{2+}$** mg/L | **$Ca^{2+}$** mg/L | **$K^+$** mg/L | **Inoculated microorganism** |
|---|---|---|---|---|---|---|---|---|---|---|
| **UB1** | **6.42** ±0.00 | **5740** ±100 | **152** ±8 | **3250** ±50 | **228** ±1 | **270** ±14 | **32** ±2 | **66** ±1 | **1048** ±2 | ***B. antiquum*** |
| **UB2** | **6.09** ±0.00 | **7140*** | **316*** | **3500*** | **424*** | **384*** | **48** ±4 | **92** ±0 | **1772** ±2 | ***B. pumilus*** |
| **UB3** | **5.76** ±0.01 | **9290** ±30 | **595** ±11 | **4940** ±60 | **386** ±12 | **386** ±8 | **56** ±1 | **111** ±5 | **1778** ±21 | ***H. salinarum*** |
| **UB4** | **5.86** ±0.00 | **8200** ±320 | **404** ±12 | **7650** ±150 | **420** ±20 | **515** ±15 | **63** ±1 | **100** ±24 | **1394** ±20 | ***I. loihiensis*** |
| **UB5** | **6.11** ±0.00 | **15140** ±380 | **390** ±35 | **8250*** | **466** ±6 | **467** ±5 | **62** ±1 | **73** ±1 | **1992** ±30 | ***M. xanthus*** |

of incubation was in the *M. xanthus* bottles at 57% by day 4 (Figure 3-2 a)). By day 10, sCOD removal in the controls was 39-66%, whilst in inoculated tests removal reached up to 69%. Orthophosphate removal ranged between 15-52% (71 to 199 mg/L) for inoculated tests by day 4 (Figure 3-2 b)), narrowing to 28-52% by day 10. *Myxococus xanthus* measured the poorest $PO_4$-P removal of 15% by day 4, whilst *B. antiquum* achieved 52% removal in 4 days incubation (Figure 3-2 b)). In the controls the $PO_4$-P removal percentages were much lower, between 0-17% (0 to 66 mg/L) by day 4 (Figure 3-2 b)), increasing to between 11 and 34% by day 10.

When urine was inoculated with *B. antiquum* and supplemented with $Mg^{2+}$, a $PO_4$-P removal of 96% (219 mg/L) was measured, compared to 52% without $Mg^{2+}$ (Figure 3-2 b)). Soluble chemical oxygen demand removal increased to 49% compared to 39% in *B. antiquum* bottles without $Mg^{2+}$ added. Values of pH, TN, TP and $NH_4$-N had little to no difference compared inoculated tests without supplemented $Mg^{2+}$. The $Mg^{2+}$ and $Ca^{2+}$removals in inoculated tests were approaching 100%, by day 4 close to all $Mg^{2+}$ was removed with most also showing near complete $Ca^{2+}$ removal (Figure 3-2 c)). Potassium removal reached up to 5% in *B. antiquum, B. pumilus* and *H. salinarum* (Figure 3-2 c)). In controls cation removal was much more varied, in UB1 and UB2 $Ca^{2+}$ removal was close to 100% whereas UB3, UB4 and UB5 it was < 20%. In all controls $Ca^{2+}$ removal was higher than $Mg^{2+}$ and $K^+$, apart from UB2 whose $Mg^{2+}$ removal was similar to $Ca^{2+}$ (Figure 3-2 c)). Ammonia increased throughout the incubation period across all urine batches (Figure 3-3 a)). In *B. antiquum* $NH_4$-N increased by 6.5-fold (from 152 mg/L to 1870 mg/L, by day 4) and by 16-fold in day 10. In the other inoculated tests, $NH_4$-N increased by 3-fold whilst in the controls the it was up to 2-fold by day 4 (Figure 3-3 a)).

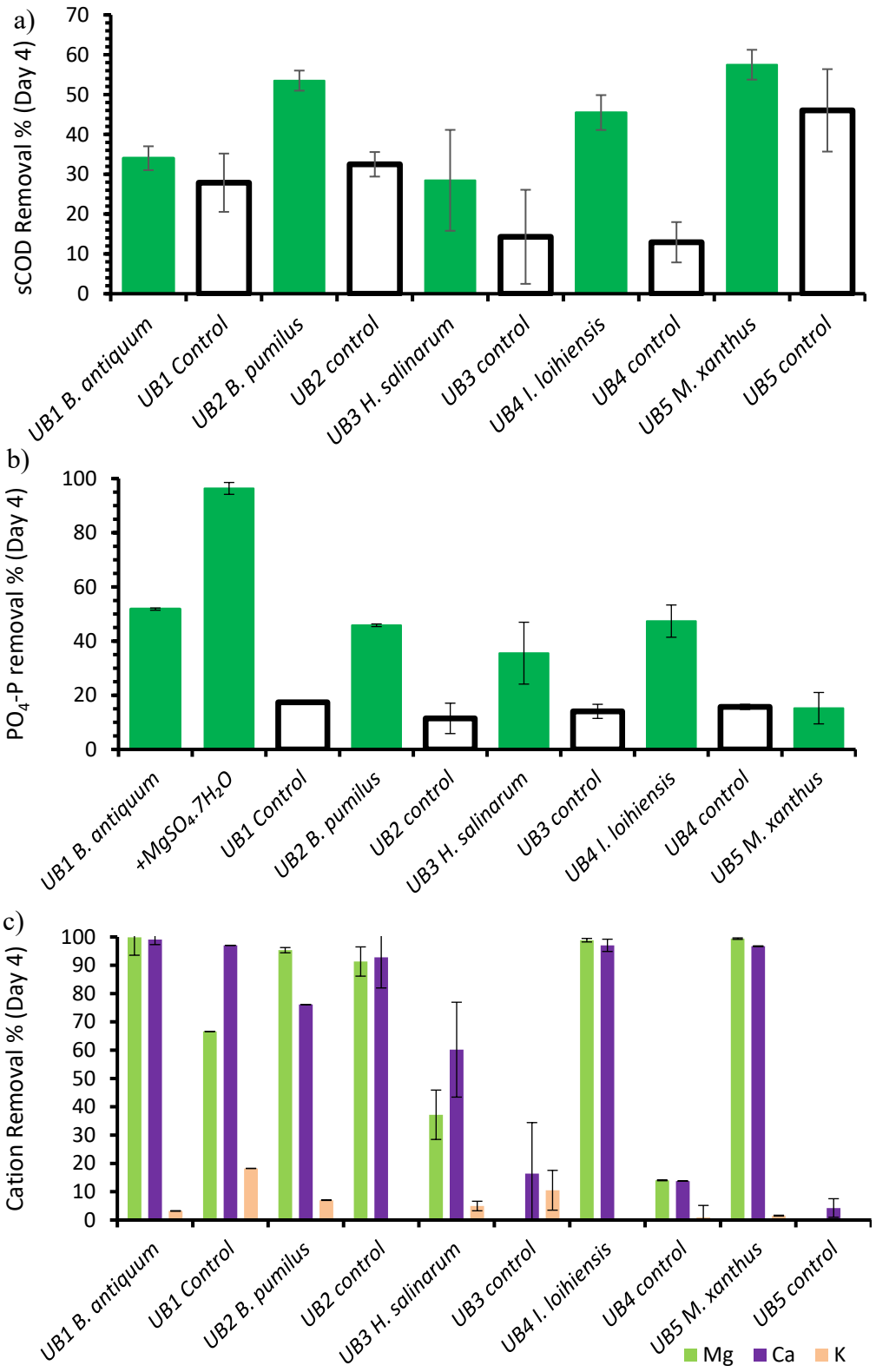


**Figure 3-2 Removal percentages from starting concentration (Table 3-2) by day 4. a) sCOD b) $PO_4$-P, including removal when dosed with magnesium sulphate to *B. antiquum* c) major cation (Mg, Ca, K) removal**

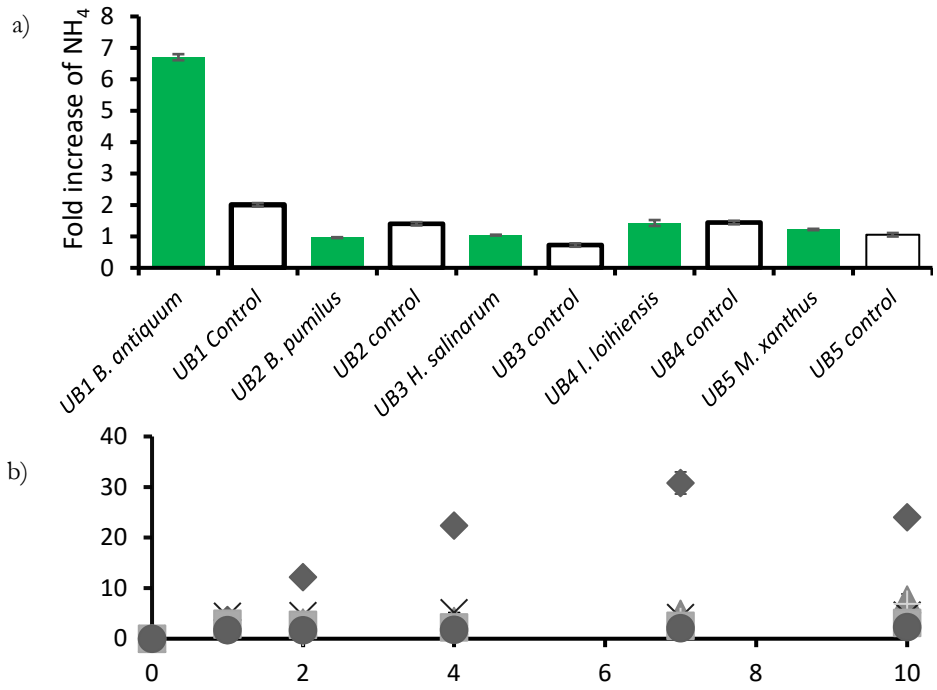


**Figure 3-3 a) Fold increase ammonia by day 4 from Table 3-2 b) hydrolysed urea during incubation. UB average for all controls (○), UB1 *B. antiquum* (♦), UB2 *B. pumilus* (▲), UB3 *H. salinarum* (×), UB4 *I. loihiensis* (+) and UB5 *M. xanthus* (■)**

The initial urea concentration in the various urine batches was estimated between 14.6-39.4 g/L urine. Based on these estimates the percentage of urea hydrolysed was calculated (Figure 3-3 b)). All microorganisms tested showed the ability to hydrolyse urea when compared to their respect controls, except for *I. loihiensis*. The bottles inoculated with *B. antiquum* recorded the highest urea hydrolysis, reaching 32% by day 7 (Figure 3-3 b)).

### 3.3.4 Mass balances and modelling

Stochiometric mass balancing of the measured ions throughout the incubation period in conjunction with geochemical modelling was used to identify abiotic mineral precipitates during urine incubation (Figure 3-4). The assemblages considered in this study included struvite ($Mg(NH_4)PO_4.6H_2O$), calcium phosphate ($Ca_3(PO_4)_2$), potassium phosphate ($K_3PO_4$), plus the total precipitate estimate and the actual mass of precipitates recovered. The estimated total precipitates mass in the controls was 706 mg/L urine by day 10, whilst actual mass recovered never exceeded 103 mg/L urine. Calcium phosphate accounted for 70% control

precipitate by day 10 (Figure 3-4 a)). In inoculated tests, the total precipitate calculated also exceed the actual precipitate recovered, except for *B. pumilus* 7 (Figure 3-4 c)). In inoculated tests struvite was calculated to account for 30-49% of all precipitates and exceeded the average control UB estimate of 15%. The predicted formation of $K_3PO_4$ in *B. antiquum, B. pumilus* and *H. salinarum* was between 12-20% and 15% in controls (Figure 3-4 b-d)).

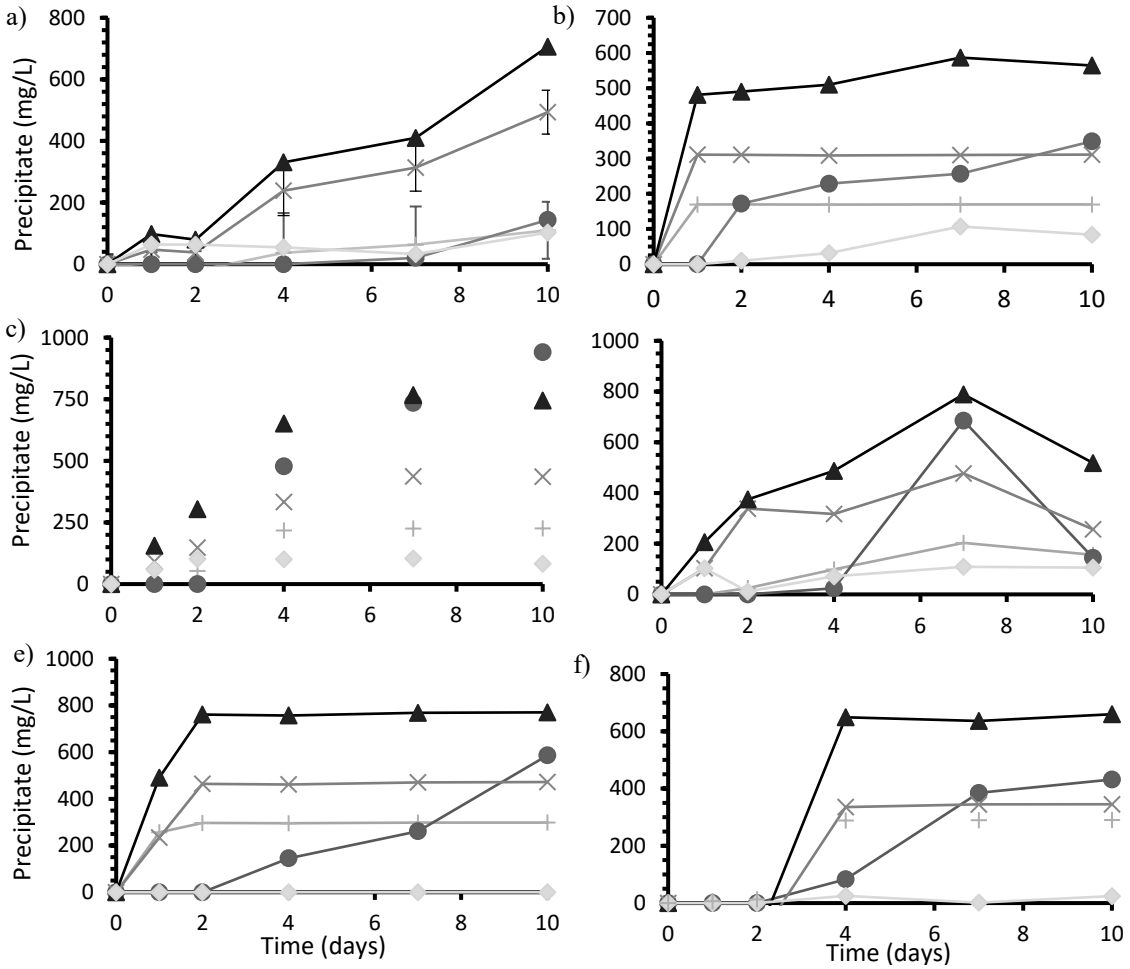


**Figure 3-4 Estimated mineral assemblages collected from UB. Struvite (+), calcium phosphate (×), potassium phosphate (♦), sum of precipitate estimates (▲) and actual precipitate measured (●) a) average UB control b) *B. antiquum* c) *B. pumilus* d) *H. salinarum* e) *I. loihiensis* f) *M. xanthus***

Using the pH, temperature and chemical data collected together with, thermodynamic modelling from the PHREEQC dataset (USGS) the saturation indices for precipitation of struvite, hydroxyapatite and calcium phosphate were calculated over the incubation period

(Figure 3-5). Saturation indices (SI) are calculated following the laws of thermodynamics[39], to predict what minerals can be precipitated from a system following the simple rules in Equation 3-2. The saturation index of a mineral is calculated from the ionic activity of the ions present and their solubility constants ($K_{sp}$) for the environmental conditions of the reaction (temperature, pH and pressure).

Ionic activity is dependent on the concentration of ions, temperature, pH and pressure following the Debye-Hückel equation, in environmental chemistry this is simplified to assume temperature is equal to 298 kelvin and pressure is constant at 1 bar[39]. Using the Debye-Hückel equation and Equation 3-2 the SI can be calculated and predict whether precipitation will occur if the mineral is supersaturated, SI > 0. Or if dissolution will occur as the mineral is undersaturated, SI < 0 (Equation 3-2).

**Equation 3-2**

$$SI = 0, \quad IAP = K_{sp}, \quad \textbf{saturated equilibrium}$$

$$SI < 0, \quad IAP < K_{sp}, \quad \textbf{undersaturated}$$

$$SI > 0, \quad IAP > K_{sp}, \quad \textbf{supersaturated}$$

Figure 3-5 shows that hydroxyapatite and other calcium phosphate minerals have positive saturation indices in all urine bottles (inoculated and controls) (Figure 3-5 b-c)). Indicating they are the most likely minerals to precipitate abiotically from solution based on thermodynamic principals. The saturation indices for struvite remains negative throughout the incubation period in all bottles (Figure 3-5 a)) indicating abiotic struvite is not expected to precipitate.

### 3.3.5 Precipitate recovery and characterisation

Across all inoculated tests, precipitates >10 µm were collected through filtration and exceeded the recovered precipitate weight recovered from the control bottles. Precipitates in inoculated tests were observed as early as day 2 and were recoverable by day 4. In *B. antiquum* tests, the

precipitates could be recovered from day 2 onwards. In controls no precipitates >10 µm were recovered before day 7. After 10 days of incubation, the weight of recovered precipitates varied between 330-1000 mg precipitate/L urine in the inoculated tests and between 54-168 mg precipitate/L urine in controls. *Bacillus pumilus* tests had the highest recovery of 1000 mg precipitate/L urine. In bottles with *H. salinarum*, 680 mg precipitate/L urine was recovered by day 7 but decreased to 142 mg /L urine at day 10. This re-solubilisation of the precipitates was only observed for this microbe and is supported by an increased $Mg^{2+}$ concentration measured in solution during that time (Figure 3-4 d)).

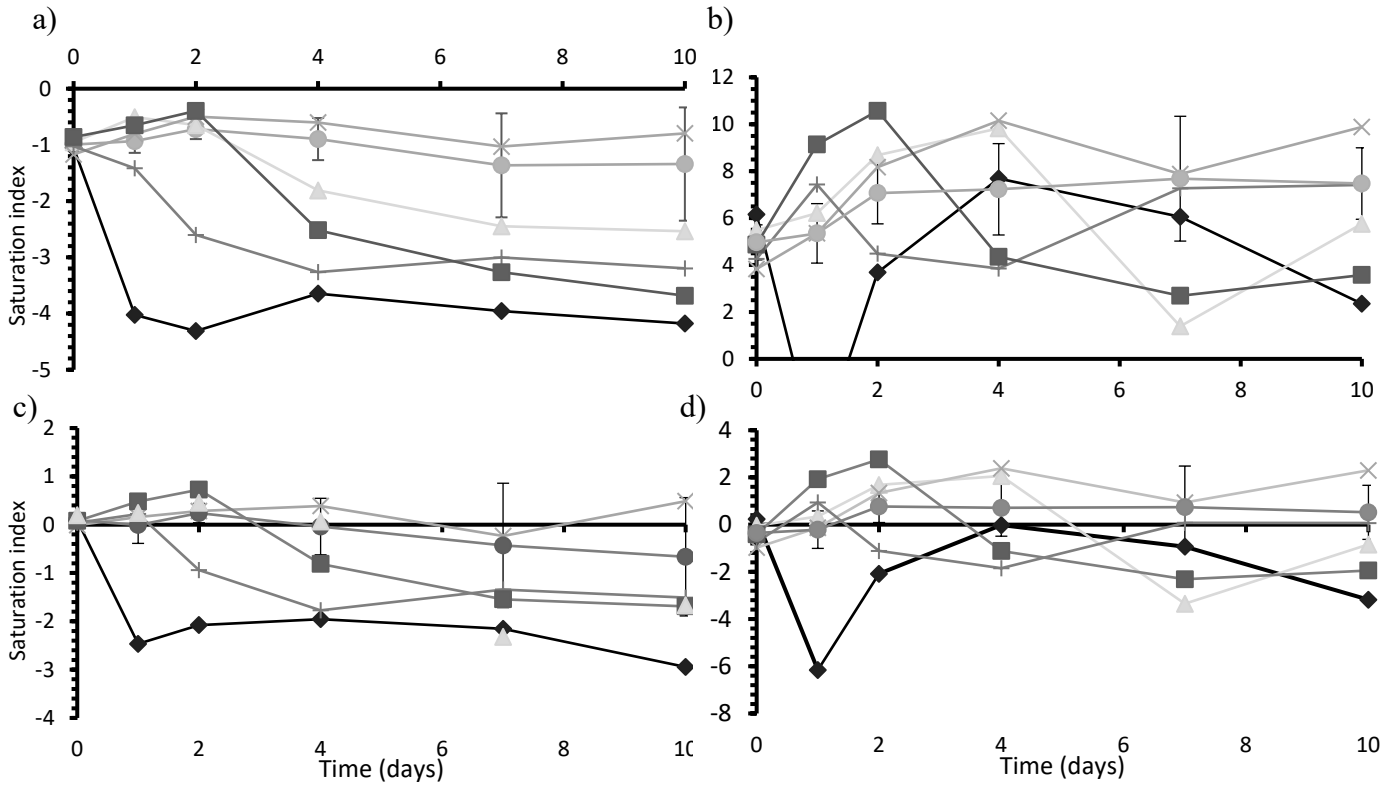


**Figure 3-5 Modelled saturation indices from Geochemist's Workbench®. UB average (●) *B. antiquum* (♦) *B. pumilus* (▲) *H. salinarum* (×) *I. loihiensis* (+) *M. xanthus* (■). a) Struvite b) Hydroxyapatite c-d) calcium phosphate**

Microscopy and optical microscopy revealed differences between the mineral assemblages recovered from controls and inoculated tests (Figure 3-6). Prismatic and tabular crystals, that were well formed (euhedral) and translucent were recovered from all microorganism tests (Figure 3-6 d-r)). However, the assemblages from *I. loihiensis* and *M. xanthus* had euhedral

crystals held within amorphous precipitates (Figure 3-6 o) and r)). Whereas *Brevibacterium antiquum*, *B. pumilus* and *H. salinarum* had less abundant and smaller amorphous crystals that typically coated the larger euhedral crystals. In contrast, the mineral assemblages from controls were dominated by amorphous, which were opaque under optical microscopy, with minor, translucent acicular crystals (Figure 3-6 a-c)).

Scanning electron microscopy with EDS of the precipitates recovered from inoculated tests measured a higher proportion of Mg than controls, reaching up to 40 wt.% and averaging at 36 wt.% (Figure 3-7 a-b). Phosphorus accounted for 50 wt.% of precipitates recovered from inoculated tests, except for *I. loihiensis* whose P wt. % was 40. The measured ratio of P:Mg is close 1:1 and indicated most precipitates from inoculated tests are bio-struvite ($MgNH_4PO_4.6H_2O$) (Figure 3-7 b). Element mapping of precipitates showed different element associations across the bi-modal distribution of morphologies (Figure 3-7 c-n)). Precipitates from inoculated tests showed clear distinction between amorphous crystals and prismatic crystals, with Ca (in yellow-pink) and Mg (in turquoise) aligned respectively (Figure 3-7 d-h)). The abundance of Ca-minerals within each precipitate assemblage varied, *B. antiquum, B. pumilus, H, salinarum* and *M. xanthus* presented some Ca-minerals coating bio-struvite precipitates, whilst *I. loihiensis* precipitates show struvite within amorphous masses of calcium phosphate (Figure 3-7 g)). Precipitates collected from controls had a higher proportion of Ca (up to 54 wt.%) compared to Mg, which fluctuated between 3 wt.% and 45 wt.%, averaging at 27 wt.% (Figure 3-7 a)). Measured P was between 32-50 wt.% in control precipitates. Element mapping showed no clear differentiation between Ca assemblages, Mg assemblages and P in controls (in yellow to pink) (Figure 7 c)).

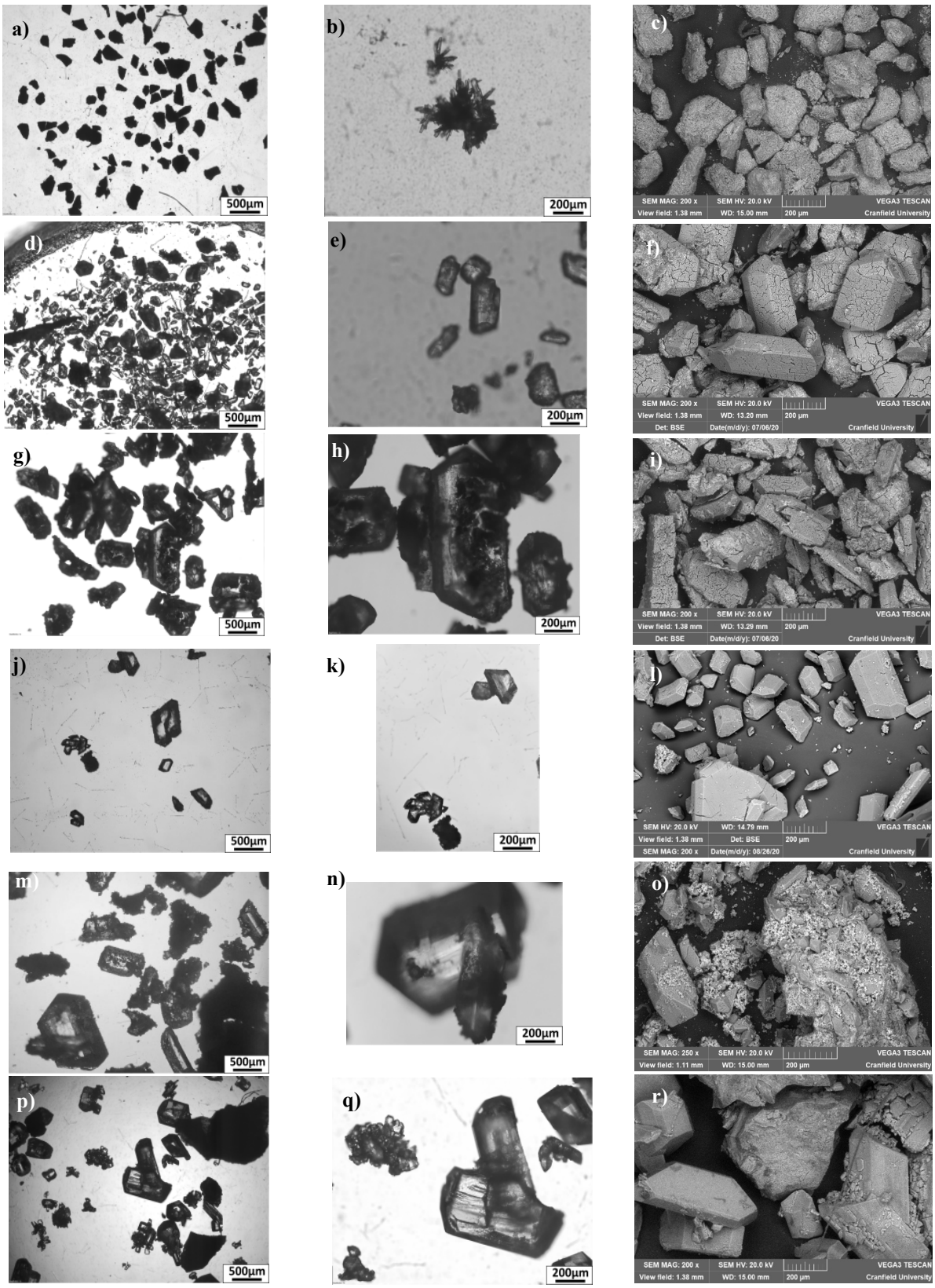


Figure 3-6 Optical microscopy and SEM images of precipitates collected after 4 days of urine incubation. Images from a) to c) are controls. d) to f) *B. antiquum*, g) to i) *B. pumilus*, j) to l) *H. salinarum*, m) to o) *I. loihiensis* and p) to r) *M. xanthus*

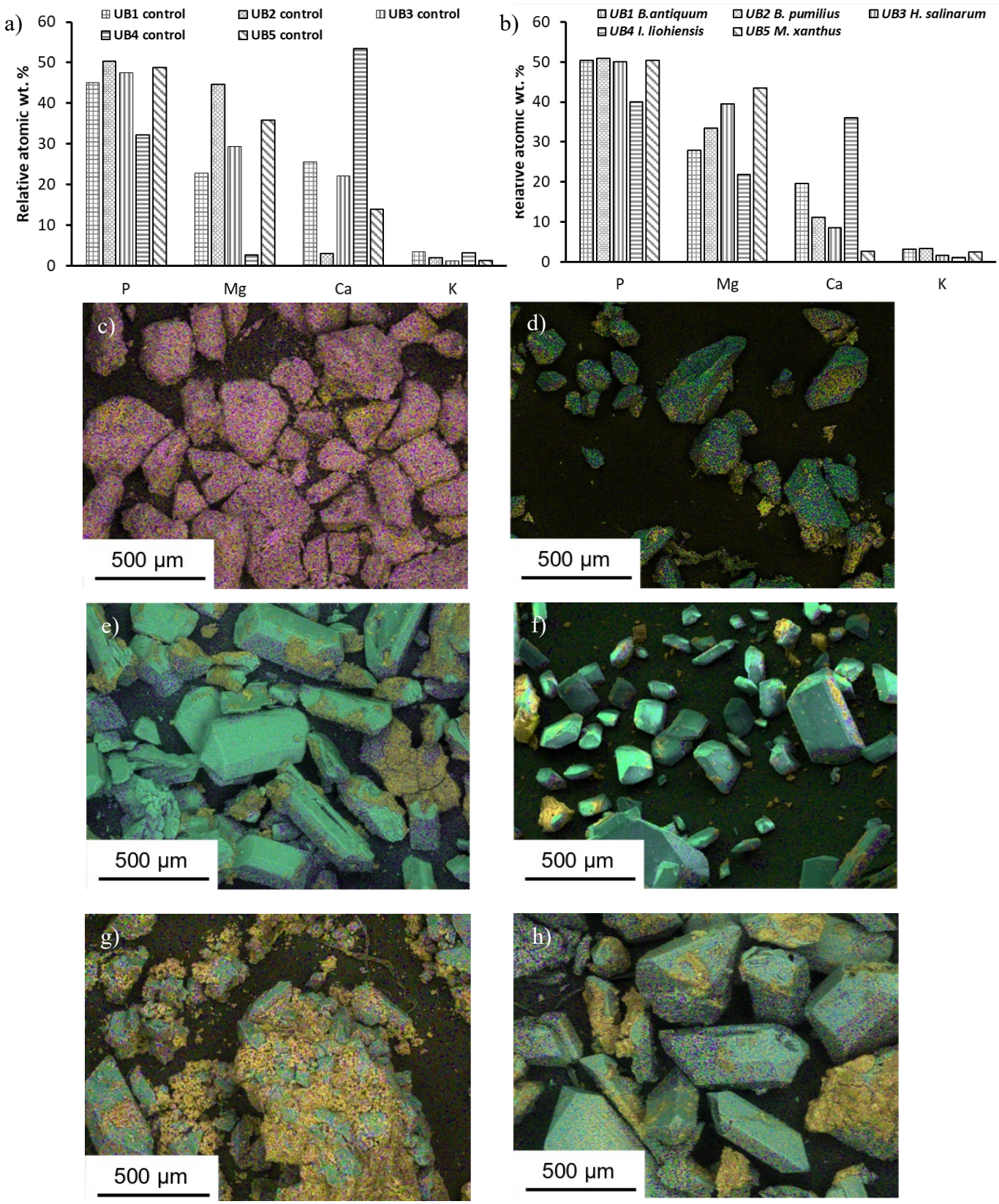


Figure 3-7 10-day precipitate assemblage. Relative atomic weight spectra of a) UB controls b) UB inoculated with microorganisms. Element mapping of precipitate assemblages where pink is P-rich, yellow is Ca-rich and green is Mg-rich, for c) control precipitates d) *B. antiquum* e) *B. pumilus* f) *H. salinarum* g) *I. loihiensis* h) *M. xanthus*

### 3.4 Discussion and conclusions

The fresh urine investigated in this study showed significant variability in pH, sCOD and key cation concentrations (Table 3-2). Compared to previous studies of fresh urine[3], the urine tested was weaker in strength in respect sCOD, $PO_4$-P and $Mg^{2+}$ to the study by Rose et al (2015). By the end of the 10 day incubation urea hydrolysis in controls was measurable, however final characterisations showed it was far from completely hydrolysed urine[32]. Which is to be expected considering the rate of urea hydrolysis measured in other studies at room temperature and no pH adjustment[14,24]

Intact cell counts throughout the incubation period showed that all the studied microorganisms were able to grow in fresh, open culture urine, in competition with native microbes by continually measuring higher ICC in inoculated tests. Growth rates of the inoculated microorganisms within urine were close to zero by day 2, indicating the stable phase of growth had been reached in inoculated tests and controls. Growth rates in sterile sludge dewatering liquors of the trialled microbes were between 0.02 and 0.14 1/h[25,28] and reached up to 0.18 l/h in urine inoculations, supporting the hypothesis that open culture urine is a good substrate for the growth of the selected microorganisms. This was further supported by HNA/LNA measurements which showed inoculated tests had a much higher proportion of HNA to LNA during the first 4 days of incubation (Figure 3-1). High nucleic acid is indicative of actively metabolising and replicating cells producing more DNA and RNA[40], which strongly suggests the inoculated microorganisms were responsible for the majority of measured growth, in tests during the first 4 days of incubation. These results support the hypothesis stipulated at the start of this experiment and past studies identifying *B. pumilus, M. xanthus, B. antiquum* and *H. salinarum* as urease producing microbes[28] that fresh urine is an effective growth media for the

studied microorganisms as it provides a suitable carbon source in the form of urea and is relatively sterile compared to municipal wastewater.

In the inoculated tests, there were clear differences in sCOD, $PO_4$-P, $Mg^{2+}$ and $NH_4$-N concentrations over the 10-day incubation period compared to controls. This was most obvious by day 4 of incubation (Figure 3-2 and Figure 3-3) where $PO_4$-P removal exceeded controls by up to 49% and $NH_4$-N production was 4.6 times greater in *B. antiquum* and at least 2 times greater in all other inoculated batches. The removal rates of *B. antiquum* and *B. pumilus* fall within the range of 63% to 76% $PO_4$-P removal measured within B4.1 growth media[27,28]. Additionally, $Mg^{2+}$ removal was similar to those seen after 5 days incubation in B4.1 synthetic media, where removal was up to 96%[28]. This supports the hypothesis that fresh urine provides the necessary nutrients and conditions for the studied microorganisms to achieve high nutrient removal rates and $PO_4$-P recovery.

The observed $NH_4$-N increase in inoculated tests is indicative of urease activity. *Idiomarina loihiensis* was the only microorganism not to produce urease[28] and measured the least ammonia production and ureolysis. All other microorganisms have been shown to produce urease[28] which accelerated the ureolysis compared to controls in this study. Most notably, *B. antiquum*, hydrolysed at least 34% of the urea present after 10 days. This rate of ureolysis is only matched or exceeded abiotically when reagents, additional enzymes and heating to >50°C which can bring complete ureolysis down to several hours[14,24]. A first step of urine ureolysis is needed for chemical struvite recovery and other urine treatment approaches (Table 3-1). The increased $NH_4$-N is beneficial for struvite reaction kinetics as it raises the molar ratio of N in M:N:P needed for struvite crystal growth[41] and the resulting increase in pH due to urea hydrolysis means that struvite precipitates would remain stable without the need to make pH adjustments[1,41].The ability of 4 of the microbes to produce urease means that this step can be

completed without chemical addition[28], and as a result cause biologically induced mineralisation of struvite for *B. pumilus, H. salinarum* and *M. xanthus*. *Brevibacterium antiquum* is able to induce struvite mineralisation (BIM) through urea hydrolysis by producing urease and exhibited the greatest ureolysis of all microbes measuring a 6.7 fold increase in 4 days, additionally it as able to concentrate ions intracellularly to control the biomineralisation of struvite (BCM)[27]. Additionally this $NH_4$-N-rich supernatant offers opportunities for secondary treatment and targeted ammonia recovery through means of stripping or ion exchange[42,43].

Magnesium addition to *B. antiquum* tests showed up to 97% $PO_4$-P can be removed, and in such tests it was also observed improved SCOD removal when the ratio of Mg:P was 1:1. To achieve maximum chemical struvite recovery the ideal Mg:P is 2:1[1,11,12]**,** this finding suggests that half the magnesium dosing would be required to completely remove $PO_4$-P using BCM struvite recovery with *B. antiquum*. Additionally, no negative impact on the growth of *B. antiquum* was observed. Another benefit is that the purity of magnesium, if added as supplement, may be of lower criticality, as the biological route for nutrient recovery through bio-mineral formation is likely to be less impacted by competing ions and lower saturation indices, when compared with traditional chemical recovery[1,11–14].

Modelling and mass balances allowed the calculation of saturation indices of minerals likely to precipitate in urine and it was demonstrated that struvite formation was not favourable in any of the urine batches. Whereas calcium phosphates (principally hydroxyapatite) were likely to precipitate based on the positive saturation indices. This indicates that without the biological mechanisms behind bio-mineral formation, struvite precipitation would not have been thermodynamically possible. The yields of the recovered precipitates >10 μm in inoculated tests were greater than controls by up to 37-fold (*B. pumilus*). Compared to recovery yields

from B4.1 growth media yields were 66% lower on average 1500 mg struvite per L B4.1 media[27,28] compared to an average yield of 456 mg struvite per L urine. *Bacillus pumilus* struvite yield was 33% lower than B4.1 media. The limiting magnesium cations and production of abiotic calcium phosphate is likely the cause for reduced yields in struvite. Recovery of precipitates greater than exceeded quantities recovered from SDL by up to 6 times[28,30]. This finding is promising for application to urine-only treatment and nutrient recovery as it shows recovery can become more efficient by source separating urine, as seen in life cycle assessments of other urine-only treatment techniques[5]. Precipitate analysis contradicts the calculated saturation indices for inoculated tests, which indicated that struvite precipitation was unfavourable throughout the incubation period. Mineralogical analysis showed that bio-struvite was clearly recovered from all inoculated tests and that the proportion of bio-struvite to Ca-minerals was greater than stoichiometric mass balances suggested. This finding provides evidence that the inoculated microorganisms were able overcome thermodynamic constraints to produce bio-struvite through their BCM and BIM mechanisms[28]. Furthermore, greater proportions of bio-struvite to Ca-minerals recovered from inoculated tests indicates that once nucleated due BCM/BIM crystal aggregation and growth of bio-struvite will continue. The greatest yield of bio-struvite were from bottles inoculated with *B. antiquum* and *B. pumilus*, interestingly these microorganisms use BCM and BIM mechanisms respectively to precipitate bio-struvite[27]. Suggesting the mechanism of bio-mineral formation did not influence the quantity of bio-struvite recovered from urine. Whether BIM or BCM is better for biological phosphorus removal and recovery as struvite remains to be investigated in more complex wastes such as sludge dewatering liquors where the interaction of suspended solids could inhibit the nucleation of BIM of struvite. When $Mg^{2+}$ was added to the bottles inoculated with *B. antiquum* precipitate yields increased 3-fold in the same incubation period which is

promising for improving the efficiency of $PO_4$-P recovery form urine and also for pilot and industrial scale studies.

These results show promise for the application of the biomineralisation technique to source-separated urine to recover bio-struvite, in a one-step process compared to multi-stage treatment methods such as complete urea hydrolysis or stabilisation before chemical or physical treatment can occur (Table 3-1). Developing these results to larger scale experiments can lead to sustainable, environmentally diligent nutrient recovery from wastewater and urine to secure recoverable fertilisers, improve food security and develop bio-based circular economy.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data Access Statement**

Data not represented in this paper is available in the accompanying appendices and available upon request from the 1st author data is not accessible in a public repository.

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# 4. Targeted phosphorus removal and recovery using encapsulated struvite bio-mineral producing microorganisms in sludge dewatering liquors in open culture systems

Robert Colston[a], Peter Vale[b], Ajay Nair[c], Tom Stephenson[a], Ana Soares[a1]
[a] Cranfield Water Science Institute, Cranfield University, Cranfield, MK43 0AL, UK
[b] Technology and Development, Severn Trent Water Ltd., Avon House, St. Martins Road, Coventry, CV3 6PR, UK
[c] Microvi UK Ltd., Sci-Tech Daresbury, Keswick Lane, Daresbury, Warrington WA44FS, UK
[1] Corresponding author: a.soares@cranfield.ac.uk

**Abstract**

Microbial encapsulation is a promising technique for improved efficiency and intensification of biological treatment processes to manage wastewater. This approach has the benefit of supplementing highly selective and significant biomass concentration into wastewater, which has a stable microbiome diversity that is difficult to manipulate at full-scale. Additionally, recent studies have shown the potential benefits of biomineral forming microorganisms to remove phosphate and recover struvite. However, these are not naturally found in wastewater, limiting enrichment options in conventional biological processes such as annamox. In this study encapsulated *Brevibacterium antiquum* and *Bacillus pumilus* cultures were tested in a synthetic media and sludge dewatering liquors from a full-scale treatment plant in pure and open culture conditions. These microbes have demonstrated their ability to utilise nutrients in open culture, source-separated urine and utilise their respective biomineralisation mechanisms. Results showed that encapsulation with microniche engineered (MNE) process enabled the selected microorganisms to thrive in wastewater, even in open cultures. Made clear by observed phosphorus removal up to 70% and a bio-struvite yield was 98 mg bio-struvite/L wastewater, by *B. antiquum* encapsulated in MNE tests. In comparison the removal of P in controls of suspended cells in open culture removed no more P than non-inoculated batches, and no bio-struvite was recovered. The results from this study clearly demonstrate that

encapsulation allowed the introduction of selected microorganisms to wastewater, outcompeting the pathways of native species leading to process intensification, and in this case, enabling P removal and recovery from sludge dewatering liquors in open culture systems.

**Key words:** *Bio-mineral, encapsulation, resource recovery, wastewater and biological struvite*

## 4.1 Introduction

Nutrient recovery from wastewater is important for closing the anthropogenic nutrient loop and can be achieved by building better circularity across the food and water sectors. Research into nutrient recovery is growing as the pressure on primary nutrient reserves grows i.e., limited reserves of mined phosphorus combined with rises in intense farming[1,2]. Nutrient recovery, in addition to better use of fertilisers, can alleviate the stress on rock P reserves and provide domestic fertiliser sources, key routes for nutrient recovery are in domestic, agricultural, and industrial wastewater[3–7]. Not removing nutrients from wastewater poses many environmental threats such as eutrophication[8]. To reduce this risk, regulation imposes limits on phosphorus (P) discharge of 2 mg/L for wastewater treatment plants (WWTP) with a population equivalent (PE) of 10,000 to 100,000 and <1 mg/L for those with >100,000 PE and at least 80% removal[9]. To manage P, technologies such as biological nutrient removal (BNR) and coagulation with iron dosing are applied. In BNR plants, struvite scaling in pipes is a re-occurring issue that reduces treatment efficiency and can cause blockages that are costly due to increased maintenance needs [10,11]. Struvite is a proven slow-release fertiliser[12–14], that could substitute for less environmentally friendly mined fertilisers. To improve the feasibility and uptake of nutrient recovery in WWTP, the costs of struvite recovery must be reduced[15].

Struvite bio-mineral (bio-struvite) formation using microorganisms has been proposed as a promising nutrient recovery method[16–18]. Biomineralisation occurs through biologically controlled mineralisation (BCM) where the microorganism actively concentrates the nutrients and facilitates the chemical change to nucleate the mineral. Biologically controlled mineralisation can occur intra-cellularly, i.e., magnetosomes, or extracellularly, i.e., coral polyps[19]. The other type mechanism is biologically induced mineralisation (BIM), where mineralisation occurs due to the change of the chemical composition of the micro-environment

around the microbial cells, as a by-product of the microbial biochemical cycles such as the metabolism of iron-reducing bacteria precipitating hematite on their cell membrane[20]. The application of a bio-mineral formation process to induce the precipitation of struvite in wastewater can reduce energy and reactant costs to WWTP[13,17,21,22]. Past experiments investigated struvite bio-mineral formation at lab-scale, reporting 82% orthophosphate ($PO_4$-P) removal and 54% P recovered as bio-struvite in various sterile sludge dewatering liquors (SDL)[17]. *Brevibacterium antiquum* (BCM) and *Bacillus pumilus* (BIM) have been proven to recover bio-struvite from synthetic media, sterile sludge dewatering liquors and source-separated urine under open culture conditions[13,17,23,24]. Both microorganisms have demonstrated that they can remove and recover bio-struvite, with yields between 1500-1700 mg/L B4.1 synthetic media and 125-130 mg/L sterile wastewater[17,25]. Removal and recovery of nutrient from urine was demonstrated in fresh urine as *B. antiquum* removed 70% of the initial $PO_4$-P and recovered 790 mg bio-struvite/L urine, and *B. pumilus* removed 55% of the initial $PO_4$-P and recovered 1000 mg bio-struvite/L urine[26].

To achieve efficient removal of P at WWTP using the bio mineral formation process, the target microorganisms need to out-compete native microbial cultures found in wastewater and ensure the key biomass is retained in the bioreactor. This has been a major bottleneck limiting the application of the technology, despite the significant removal and recovery of P in wastewater. To address this gap, microbial encapsulation was investigated.

Encapsulation and immobilisation are processes of containing microorganisms in an inert scaffold which still allows for mass transfer between the bulk solution and the microorganism. The difference between encapsulation and immobilisation has been eluded to the difference in type of scaffold utilised, i.e., typically in immobilisation the targeted microorganisms or enzymes are part of the matrix and incorporated in its structure, this includes naturally

occurring extracellular polymeric substances and basic alginate capsules[27]. Whereas encapsulation requires a semi-permeable membrane structure to carry the target microorganisms or enzymes, still allowing their movement inside the scafold[28,29]. However, in many studies the terms immobilisation and encapsulation have been interchangeable, as this technology grows and understanding deepens knowledge highlighting the differences in these techniques to become better defined[27,30]. The benefits of encapsulation/immobilisation are that the volume of biomass in contact with the wastewater is significantly increased, biomass retained, and overall the biological process is intensified[28,30,31]. There are several documented encapsulation and immobilisation techniques, such as cryogels and alginate polymers. Cryogels have been used to immobilise microorganisms such as, *Pseudomonas mendocina* and *Rhodococcus koreensis* through a one-step process, demonstrating that the cells remain viable and allow for improved degradation of phenols[31,32]. Alginate polymers have been used to intensify nitrification in activated sludge process as well as bio-sorption of heavy metals[33,34]. The use of specifically engineered microenvironments (microniche engineering) that enhance the productivity of an individual microorganism has been developed by Microvi biotech© to improve the efficiency of existing WWTP by accelerating the denitrification process, reducing biosolids production, and reducing biological oxygen demand in WWTP across the USA and Australia[35].

Whilst some encapsulation techniques have been made commercially viable, such as the microniche engineered (MNE) encapsulation process, most are in their infancies and mainly tested at lab-scale[30]. This is due to several factors and potential limitations of the various encapsulating or immobilising available that need to be investigated further. The toxicity of many cross-linking polymers to microorganisms has to be carefully monitored and balanced to ensure that they remain active whilst ensuring the framework remains stable[31,36]. Additionally

the interactions of the microorganisms and the encapsulation matrix is influenced by the characteristics of the cells and the media, such as adhesion determined by the interaction of charges between the cell wall and the polymer[37]. Furthermore, chelating agents and ion exchange has been documented to cause osmotic swelling and destabilisation of the immobilising alginate bead[38]. Finally, many trials have not taken encapsulation or immobilisation process beyond synthetic solutions or pure culture wastewater, the interaction of these scaffold structures with the variety of ions, organics and microorganisms in an open culture system remains largely unknown, along with the hypothesis that encapsulation materials can limit substrate and products mass transfer, which impacts process efficiencies and product recovery, such as bio-minerals[39].

The study investigated three different encapsulation or immobilisation processes, sodium alginate, cryogel and MNE biocatalysts obtained from a commercial supplier, to find the most suitable to achieve high P removal and recovery, when compared with suspended cultures. These scaffolds have been documented to maintain cell viability and have had promising functions removing pollution in different types of wastewater[28,31,37,40], and were chosen to compare the effectiveness differing encapsulating techniques from natural polymers to chemically derived polymers. Neither *B. antiquum* nor *B. pumilus* have been encapsulated before, so comparisons are made with suspended cells in synthetic media in this study. More importantly some tests were completed in open culture conditions to understand if bio-mineral production could be achieved.

## 4.2 Materials and methods

### 4.2.1 Microorganisms, synthetic media and sludge dewatering liquors

Pure cultures of *Brevibacterium antiquum* (DSM 21545, German Resource Centre for Biological Material, Germany) and *Bacillus pumilus* (GB43, LGC Standards, Middlesex, UK)

were inoculated into sterile B4.1 synthetic media (4 g/L of yeast extract, 2 g/L of magnesium sulphate heptahydrate and 2 g/L of di-potassium hydrogen phosphate – characterisation in Table 4-1 and incubated in conical flasks at room temperature (20-22°C) and agitated at 150 rpm (Heidolph Unimax-2010, Wolflabs, York, UK) for 48 to 72 hours to reach the stationary phase of growth of each microbe. B4.1 media was also used to inoculate cultures of suspended cells and those encapsulated or immobilised.

**Table 4-1 Characterisation of B4.1 synthetic media and sludge dewatering liquors (SDL) from 2 different WWTPs.**

| Solution | Source | | pH | sCOD mg/L | $PO_4$-P mg/L | $NH_4$-N mg/L | $Mg^{2+}$ mg/L | $Ca^{2+}$ mg/L | $K^+$ mg/L |
|---|---|---|---|---|---|---|---|---|---|
| **B4.1 synthetic media** | | | **7.65** ±0.01 | **10700** ±1273 | **535** ±49 | **-** | **195 *** | **-** | **900 *** |
| **SDL1** | Thermal hydrolysis process | **Open culture** | **8.13** ±0.05 | **1690** ±435 | **48** ±6 | **1350** ±55 | **9** ±6 | **50** ±15 | **100** ±2 |
| | | **Pure culture** | **8.30** ±0.12 | **1802** ±210 | **54** ±13 | **1350** ±55 | **5** ±3 | **21** ±6 | **57** ±2 |
| **SDL2** | Two-stage anaerobic digestion | | **8.51** ±0.26 | **603** ±18 | **120** ±16 | **998** ±218 | **22** ±8 | **36** ±10 | **228** ±62 |

sCOD = Soluble chemical oxygen demand
* Estimated from chemical composition of the synthetic media

Sludge dewatering liquors were collected from two full-scale WWTP. The first used primary treatment, biological nutrient removal (BNR) and combined sludge digestion with thermal-hydrolysis process. The digestate was then dewatered and collected after centrifugation and referred to as SDL1. The second WWTP used primary treatment followed by two-stage anaerobic digestion before centrifugation and collection of the dewatering liquors, referred to as SDL2 (this was sampled as SDL1 was not available due to WWTP refurbishment). Further processing was completed at the laboratory after collection, by removing excess solids using centrifugation at 3500 rpm (Sorvall Legend RT+, Thermo Scientific, Loughborough, UK)) and filtration through a 10 µm filter (Whatman™, Grade 1 filter sheets). The SDLs were characterised prior to inoculation and tests in batch mode (Table 4-1). Half of the collected

volume of SDL were filter sterilised (Thermo Scientific™ Nalgene™ Rapid-Flow™ vacuum filters) to inactivate native microbial species and create pure culture tests and also served as control for the experiments.

### 4.2.2 Microbial encapsulation and immobilisation

Immobilisation of the selected microorganisms in sodium alginate beads was completed following the method described by Zommere et al[41]. Separate solutions of 0.2 M calcium chloride ($CaCl_2$) (22.2 g/L) and 3% sodium alginate (30 g/L) were mixed and autoclaved at 121ºC for 15 minutes. The sodium alginate solution was mixed with steady state cultures of *B. antiquum* or *B. pumilus,* at 50:50 and stirred for 30 minutes, a second batch was mixed with sterile de-ionised water at 50:50 to make blank alginate beads to serve as control. The inoculated and blank sodium alginate solution was dropped using a 10 mL pipette into a beaker of 0.2 M $CaCl_2$ and left to solidify for at least 60 minutes. The beads were stored at 5ºC in the $CaCl_2$ solution until needed, where they were filtered and rinsed with de-ionised water.

Immobilisation of the selected microorganisms in cryogels was completed following the methodologies of Al-Jwaid, *et al.* (2018) and Berillo, *et al.* (2019)[32,31]. The cryogel were synthesised using 2% polyvinyl alcohol (PVA) and 2% polyethyleneimine (PEI) and mixed with 50% glutaraldehyde to achieve a final concentration of 1.5% glutaraldehyde. The cross-linking solution was made by mixing 60 g PVA with 1 L sterile deionised water, heated to 85°C and stirred until fully dissolved and left to cool. Then 30 mL of 50% glutaraldehyde solution was added and mixed for 1 hour. The cross-linking solution was then further processes using 1000 da dialyses for three days against 5 L deionised water, that was renovated twice per day. Pure cultures of *B. antiquum* or *B. pumilus* were centrifuged at 2400 relative centrifugal force, at 4°C for ten minutes. The supernatant was discarded, and the microbial pellets were rinsed with ice cold 0.9% sodium chloride (NaCl) to remove all traces of the growth media.

The pellets were then resuspended in 200 mL cross-linking solution to achieve a microorganism concentration of 10%. Inoculated cross-linked solutions were poured into ice-cube trays, covered, and stored at -12 °C for at least three days for cryogelation to occur. A hundred millilitres of un-inoculated cross-linked solution was also used to form a cryogel as a control.

Blank and encapsulated beads from the selected cultures were also obtained from a commercial company after production at their facilities with microniche engineering (MNE) technology (Microvi© MNE™, San Francisco, USA). The full protocol for obtaining the encapsulated cultures, also called biocatalyst, was not disclosed as this is protected intellectual property by Microvi© MNE™, San Francisco, USA. The biocatalysts beads were named *B. antiquum* (MNE-Ba) and *B. pumilus* (MNE-Bp).

### 4.2.3 Testing suspended and encapsulated cultures

Suspended, encapsulated or immobilised cultures of *B. pumilus* were inoculated at a filling ratio of 20% volume to volume (v:v) in 100 mL of B4.1 synthetic media. These were incubated in conical flasks, at room temperature (20-25$^{o}$C), and agitated at 150 rpm (Heidolph Unimax-2010, Wolflabs, York, UK). Experiments were run in duplicates, between 72-144 hours. *Brevibacterium antiquum* was not tested in synthetic media due to the close similarities in nutrient removal and recovery with past studies[17].

Both MNE-Bp and MNE-Ba as well as suspended cultures were inoculated in pure and open culture conditions in SDL1. These batches were run to the same filling ratios and physical conditions as in B4.1 media batches. Biocatalysts MNE-Bp and MNE-Ba, were filtered from the wastewater at the end of each trial, rinsed thoroughly in tap water and stored in fresh tap water at 4°C before being reused in subsequent wastewater batches. As a control, MNE blanks

were inoculated into 200 mL at the same filling ratio and physical conditions as all batches in SDL2 (Table 4-1).

To measure the absorption capacity of the MNE media, batches were incubated for 5 hours only, as earlier experiments of this study showed that the greatest nutrient removal differences between the encapsulated microbes and suspended cells was during this time. Samples were taken at 0.5, 1, 1.5, 3 and 5 hours for analysis.

The time when bio-mineral formation was observed to be taking place, i.e., white precipitated started to form, was noted for each flask. During the incubation period samples of the precipitates were taken for analysis, or sacrificial batch tests were filtered through 10µm sheets (Whatman™, Grade 1 filter sheets) and left to dry before analysis. At the end of the incubation period the remaining flasks were filtered through 10µm filter sheets and dried at room temperature for over 24 hours, before being removed and analysed.

### 4.2.4 Analytical methods

Spectroquant® Cell tests kits were used to measure the ammonia ($NH_4$-N), phosphate ($PO_4$-P) and soluble chemical oxygen demand (sCOD) according to the manufacturer's instructions (Merck Millipore, Gillingham, UK). Cations of concern, magnesium ($Mg^{2+}$), potassium ($K^+$), calcium ($Ca^{2+}$) and sodium ($Na^{2+}$) were analysed using inductively coupled plasma-mass spectrometry (ICP-MS) (PerkinElmer NexION 350D, UK)[42]. Ion chromatography was used for the analysis of $PO_4^{2+}$, $NH_4^+$, $Mg^{2+}$, $Ca^{2+}$ and $K^+$ in samples with the tests with SDL2 (Dionex Aquion,Thermo Fisher, UK) according to the operators manual[43]. The pH was measured using a Fisherbrand hydrous 300 pH meter (Fisher Scientific, Loughborough, UK). The weight of precipitates recovered was determined after drying for over 24 hours at20 to 25°C and expressed as weight recovered per litre of SDL. Morphological characteristics and mineralogy of the precipitates collected were documented after observation with optical microscopy

(Olympus MX40) and scanning electron microscopy (SEM). Further to this, coupled energy dispersive X-ray spectroscopy (EDS) to quantify the elemental composition and make potential links to crystal morphology (Tescan Vega 3, Oxford Instruments© Aztec™, Kohoutovice, Czech Republic).

### 4.3 Results and discussion

The cryogel with immobilised *B. antiquum* and *B. pumilus* were incubated in B4.1 media and the $PO_4$-P and sCOD removals were between 36-44% and 61-70%, respectively after 6 hours of incubation. These results indicate that the immobilisation through cryogel did not reduce the viability of *B. antiquum* and *B. pumilus* cells due to the potential toxicity of the polymers used, which was an initial concern. However, the cryogels were not stable as a self-supporting scaffold and there was a loss of structure of the polymer over the 72 hours incubation. Similar observations have been documented by others that have inferred that the structure of cross-linking polymers, PVA and PEI can be altered depending on whether the microorganisms are gram-positive or gram-negative[31,32] as well as the breakdown of cryogels can be due to the hydrolysis of poorly formed Schiff base groups[32]. Both *B. antiquum* and *B. pumilus* are gram-positive, suggesting that these microorganisms could have interfered with the stability of cross-linkers. Further to this it is important to balance the concentration of cross-linking polymers to produce a self-supporting structure in a way that toxicity of polymers remains low to the inoculated cells.

The alginate immobilised *B. antiquum* or *B. pumilus* were incubated in SDL and the $PO_4$-P and sCOD removals were between 80- 86% and 36-40%, respectively after 8 hours of incubation. Nevertheless, similar removals were observed in the control alginate beads, without microorganisms The results indicate that alginate beads acted as strong absorbent in SDL displayed a high affinity for $PO_4$-P hence the observed removal. Other studies have reported

that alginate can act as an inhibitor for struvite formation[44] , whilst having a high affinity for $PO_4$-P as removals of 87.5% were observed along with the co-precipitation of iron phosphates when wastewater was incubated with iron alginate polymers[45]. Although initial data showed $PO_4$-P removals in excess of 80%, it is likely due to the interaction of alginate with the $PO_4$ ions rather than the immobilised microorganisms. Furthermore, the alginate beads also changed in structure and shape during the incubation period, destabilising, becoming a flexible gel and later dissolving in the SDL1. The loss of consistency of the sodium alginate beads could be in part due to osmotic swelling related with the presence of chelating agents such as phosphate, which has been indicated to destabilise alginate[38]. Additionally the presence of sodium (Na) in concentrations greater than 180 mg/L causes ion exchange between the calcium (Ca) binding the alginate bead and $Na^+$, releasing up to 65% $Ca^{2+}$ within 3 hours of incubation, causing osmotic swelling and degradation[46]. The liquors in this study had sodium concentrations between 90-250 mg/L, therefore the higher strength SDL may have resulted in this process. Other studies also indicate that the microbial production of enzymes such as alginate-lyase can be due to biodegradation of the beads [47]. Nevertheless, further analysis of *B. antiquum* or *B. pumilus* is needed to establish if this enzyme is produced and whether native microbial communities in wastewater can produce the enzyme alginate-lyase.

On the other side, the encapsulation with MNE technology resulted in stable beads that were around 4 mm in diameter and no deterioration was observed throughout the test besides some staining observed when incubated in sludge dewatering liquors.

### 4.3.1 Inoculation of MNE in B4.1 synthetic media

The MNE-Bp beads were inoculated into B4.1 media at filling ratio of 20% v:v, alongside suspended cells inoculations of *B. pumilus* and non-inoculated batches acting as controls. During incubation, pH increased from 7.6 to 8.3-8.4 after 72 hours for both suspended cells of

*B. pumilus* and MNE-Bp. In comparison, the controls increased only slightly to 7.8. The pH increase observed in B4.1 media is consistent with reported pH changes in the same synthetic media, and can be attributed to the release of $NH_4$-N[25].

The in suspended cells and MNE-Bp removed $PO_4$-P and sCOD from the B4.1 media over 72 hours incubation (Figure 4-1). The sCOD removal was 78% after 6 hours of incubation in both suspended cells and MNE-Bp batches (Figure 4-1Figure 4-1). Orthophosphate removal was 38%, from initial concentrations of 535 mg $PO_4$-P/L, whilst suspended cells removed less $PO_4$-P in the first 6 hours (15%) (Figure 4-1). The same removal percentage was reached after 72 hours incubation of suspended cells and MNE-Bp, achieving between 72% and 75% $PO_4$-P removal (Figure 4-1). These results suggest that the encapsulation by MNE had no negative impact on nutrient removal due to potential toxicity from the encapsulating media when compared to suspended cells of *B. pumilus* (Figure 4-1). Additionally, the similarity in pH increase between MNE-Bp and suspended cells is a simple indication that the encapsulated microorganisms were performing like their suspended counterparts and as expected in B4.1 synthetic media[25]. This is important for the continued development of bio-struvite recovery using MNE encapsulated microorganisms, as a pH greater than 8 is needed for struvite nucleation and growth[48,49]. Furthermore, sCOD removal was equal between suspended cells and MNE-Bp reaching 89% after 24 hours incubation (Figure 4-1), suggesting that the activity of the microbes was not reduced by the encapsulating media as carbon (measured as sCOD) was still metabolised at the same rate.

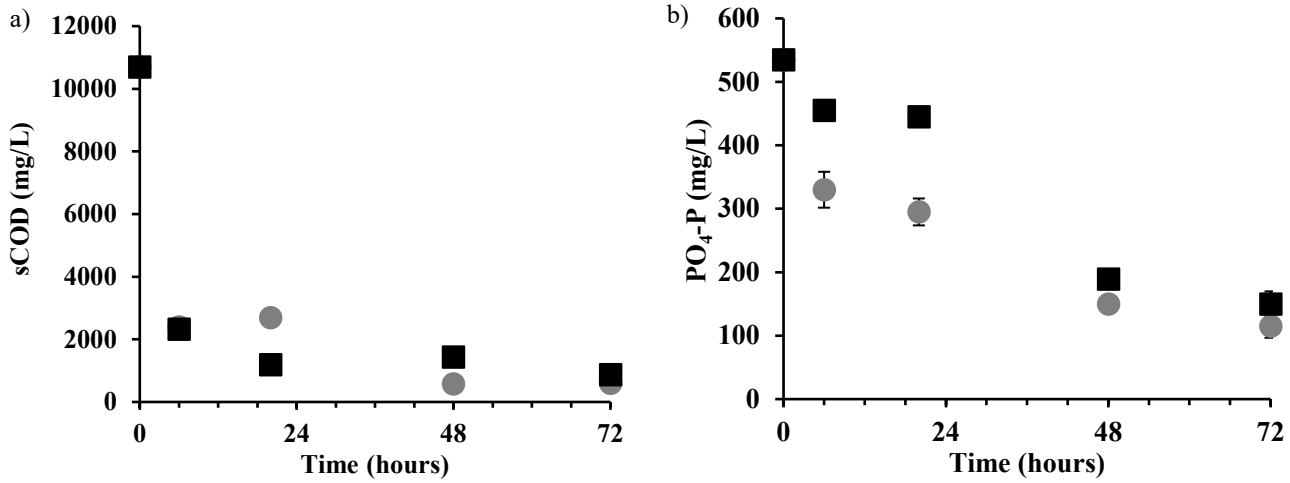


**Figure 4-1 Variation in SCOD and $PO_4$-P concentrations in B4.1 media when inoculated with *B. pumilus* as; suspended cells (■) and encapsulated *B. pumilus*. MNE-Bp (●) of sCOD a) $PO_4$-P b) over 72 hours incubation.**

The rate of removal of $PO_4$-P increased from 13 mg/L.h in controls to 34 mg/L.h by MNE-Bp over the first 6 hours of incubation. This increase in removal rate highlights one of the key benefits of encapsulation, which is process intensification, relative to suspended cell incubations. In comparison to past studies, MNE-Bp removed the $PO_4$ twenty times quicker than suspended cells of *B. pumilus* in B4.1 meida[17]. Based on these results MNE-Ba was not trialled in B4.1 media these batches showed that MNE encapsulation has no negative impact on *B. pumilus* in comparison to suspended cultures and past studies in B4.1 media. Furthermore, *B. antiquum* exhibited similar removal efficiencies as *B. pumilus* in suspended cell inoculations in B4.1 media[17].

### 4.3.2 Inoculation in sludge dewatering liquors

Only suspended cells and the encapsulated MNE cultures were inoculated into SDL1. Over a 24-hour period, both MNE-Ba and MNE-Bp in open and pure culture conditions measured similar increases in pH, from 7.8 to 9.2. Suspended cell controls in pure and open culture SDL1 also measured an increase pH to 9.2 and 8.9, respectively. Similar to the B4.1 incubation, sCOD removal of 22% was equal in pure culture conditions for MNE-Bp and MNE-Bp and suspended

cell controls, over the initial 6 hours of incubation. The sCOD removal for MNE-Ba and suspended cells of *B. antiquum* in pure culture SDL was 47% and 45%, respectively. After 24 hours, $PO_4$-P removal in controls and suspended cells in open culture conditions was between 10-20% $PO_4$-P (Figure 4-2). Suspended cells in pure culture removed up to 60% $PO_4$-P over 24 hours. MNE-Ba showed the same rate of $PO_4$-P removal in both open and pure culture conditions over 3 and 6 hours, with 48% and 61%, respectively (Figure 4-2). However, $PO_4$-P removal by MNE-Bp was slower in open culture conditions compared to pure culture in between 3 and 6 hours of incubation, on average 20% less $PO_4$-P was removed (Figure 4-2). Overall, suspended cells in open cultures showed significantly lower $PO_4$-P removal when compared with immobilised *B. antiquum* or *B pumilus* (Figure 4-2).

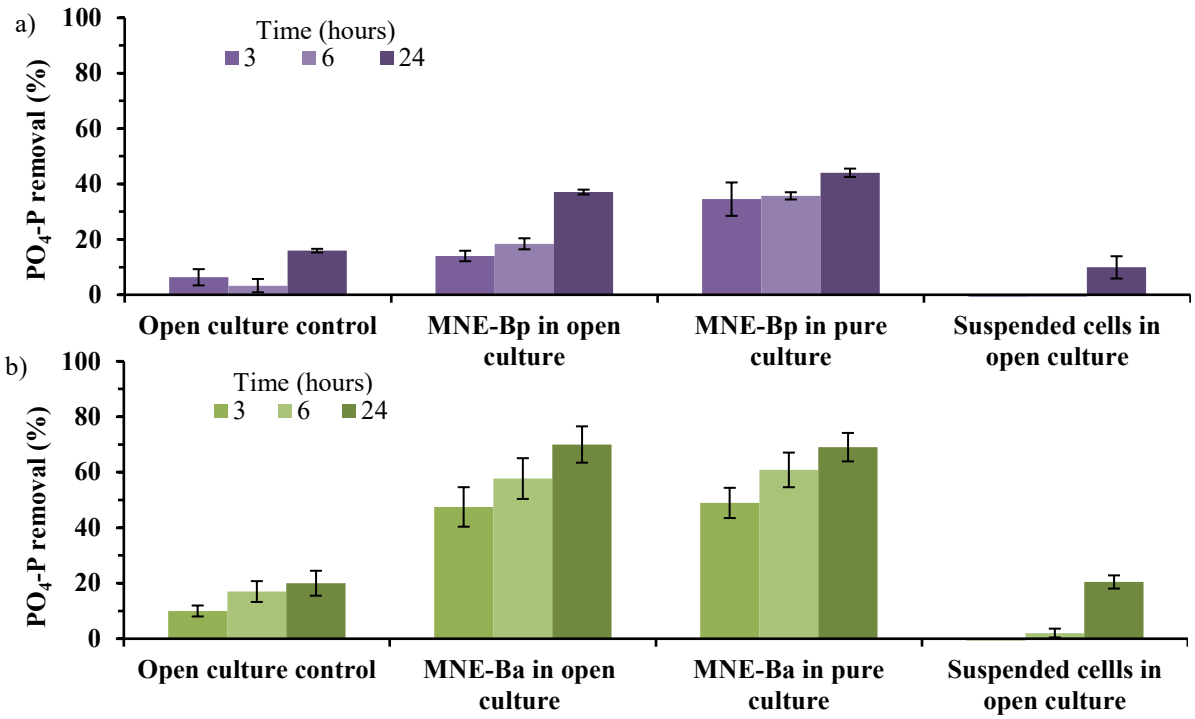


**Figure 4-2 $PO_4$-P removals over 24 hours incubation in SDL1 comparing open culture and pure culture system a) MNE-Bp b) MNE-Ba**

The initial magnesium concentration in SDL1 was between 6-15 mg/L. In the tests with MNE, 45-57% was removed after 24 hours. This accounted for a removal of 7 mg Mg/L SDL over the incubation period, on average. Other ions measured ($Ca^{2+}$, $Na^{+}$, $K^{+}$) had minimal removal

across the whole incubation period in all batches. Using stochiometric mass balances, the estimated production of bio-struvite would be 71 mg/L SDL as magnesium remains the most limiting nutrient. The initial ratio of P:Mg was 1:0.19, this is far from the minimal ratio needed for abiotic mineralisation of struvite[49] and below magnesium concentrations in previous batch experiments of suspended cells in supplemented wastewater[50]. Previous experiments incubating suspended cells of *B. antiquum* and *B. pumilus* have reported magnesium removals of between 92% and 98% in synthetic solutions and source-separated urine[17,24]. The reduction in Mg removal could be attributed to the lower starting concentration, as treated effluent concentrations of Mg in urine batches were similar which implies there may be a fraction of Mg in wastewater that is not biologically available i.e., sorbing to organics. However, this was not investigated in this study and could be studied further. Based on the stoichiometry for struvite this accounts for 28 mg $PO_4$/L removal on average.

From the data collected, the impact of open culture conditions versus pure culture conditions when the selected microorganisms were encapsulated in MNE was negligible after 24 hours incubation (Figure 4-2), The batches with MNE-Bp measured lower a $PO_4$-P removal over the first 6 hours of incubation in open culture SDL 1 before reaching 37% removal by 24 hours, like MNE-Bp in pure culture conditions. Whereas MNE-Ba removed the same $PO_4$-P in open culture conditions as pure culture conditions. This difference may be due to the mechanism of biomineralisation being different between *B. antiquum* and *B. pumilus*. Biologically induced mineralisation requires the attraction of ions to the cell wall of *B. pumilus*[25], the MNE can potential add a barrier for this mechanism resulting in the observed decrease in removal of $PO_4$-P relative to MNE-Ba and also lower than in previous experiments[17]. However, the similarities between the results when MNE was used in pure and open culture conditions in addition to the lack of any $PO_4$-P removal by suspended cell batches in open culture conditions strongly

supports the ability of encapsulation to overcome competition from native microbial communities found in SDL. The results from this study compare well against other encapsulation techniques in other forms of resource recovery, a 25% improvement was observed in the first 56 days of start up the production of methane by encapsulated methanogens compared to suspended cultures in an anaerobic membrane bioreactor[28].

The MNE media was retained after each initial batch and after rinsing in tap water and incubated into fresh batches of SDL1. After four cycles in SDL1 in open culture conditions, $PO_4$-P removal in MNE-Bp tests was between 15- 33% after three hours increasing to between 24-56% after 24 hours incubation (Figure 4-3). Over three cycles $PO_4$-P removal by MNE-Ba removal was between 38- 49% the first 3 hours, increasing to between 63-70% after 24 hours incubation. The results obtained over multiple cycles clearly indicate the potential bio-mineral forming microorganisms and the dominance of their metabolism when added to wastewater in an encapsulated form to open cultures. This corroborates with previous studies into using encapsulated microbes in open culture wastewater, such as brewery wastewater[28] and high strength ammonia wastewater[29]. The results have shown an improved rate of removal relative to suspended cells in synthetic media and wastewater by the same encapsulated microbes[25]. Furthermore, studies into engineered encapsulating polymers have shown improved removal ammonia of 97% in encapsulated algae in membrane bioreactors relative to sodium alginate beads and suspended cells[29].

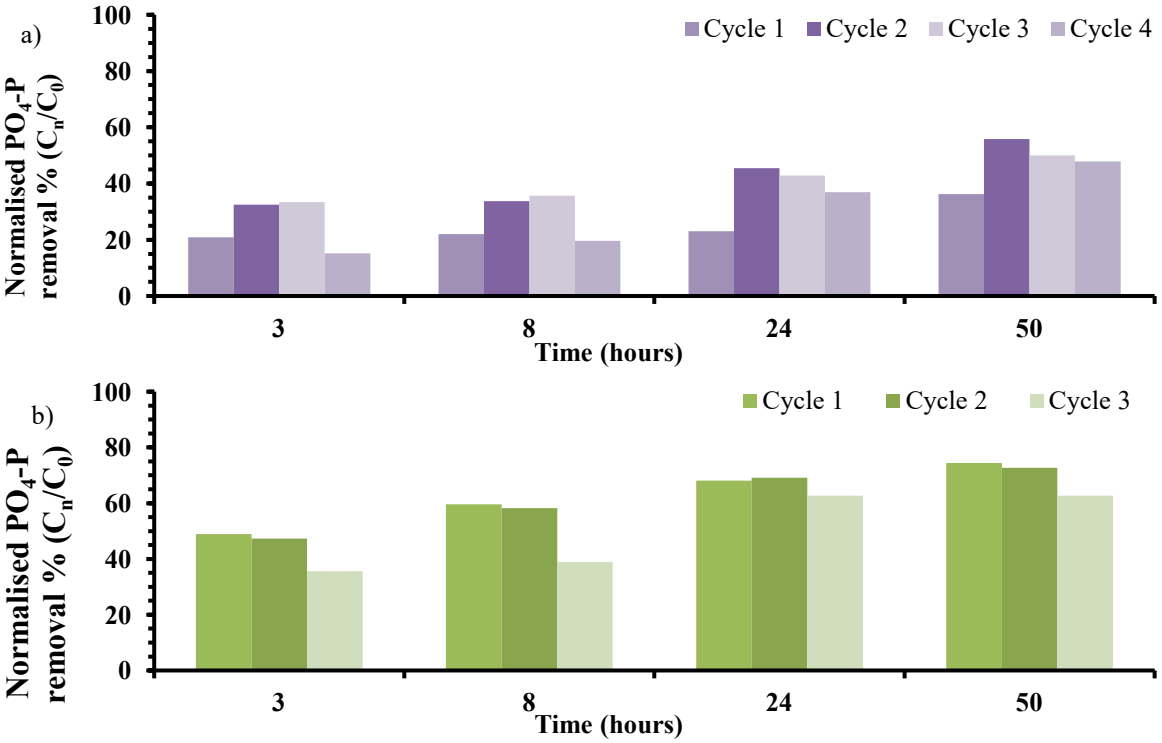


**Figure 4-3 $PO_4$-P removal over multiple cycles with immobilised cultures with MNE technology. Cycles are denoted by the first number in each key with microbial conditions labelled as open or sterile and wastewater source for a) MNE-Bp and b) MNE-Ba**

### 4.3.3 Phosphate absorption capacity of MNE media

The $PO_4$-P absorption of the blank and encapsulated cultures in MNE was monitored (Figure 4-4). During the incubation time, the $PO_4$-P did not change from an initial concentration of 120.1 mg $PO_4$-P/L in the controls, except at the anomalous result at 0.5 hours (Figure 4-4). Within the first hours of incubation, the tests the MNE blank noticed a 19% $PO_4$-P removal and remained stable for the rest of the incubation period, likely due to the initial diffusion inside the porous material, that reached quick saturation. For MNE-Ba and MNE-Bp $PO_4$-P removal reached 37- 34%, respectively (Figure 4-4). These results indicate that 20% of initial $PO_4$-P was sorbed/adsorbed by the blank MNE beads equating to an absorption capacity of 1.28 mg $PO_4$-P/g MNE media, increasing the $PO_4$-P availability to the encapsulated microbes after contacting the solution.

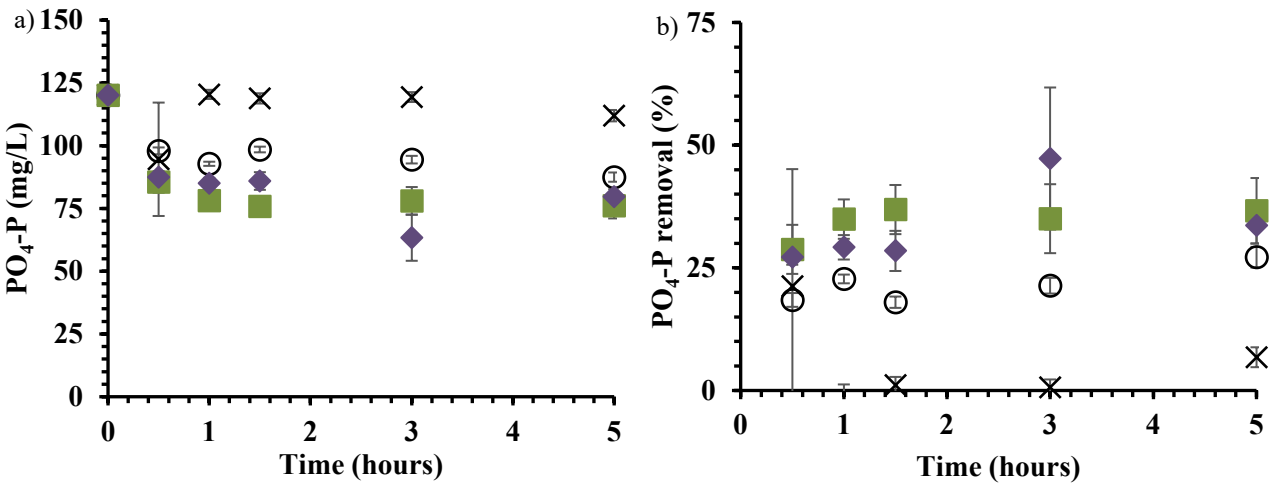


**Figure 4-4 a) Orthophosphate concentration and b) percent removal over 5-hour incubation period. Control (×), MNE blank (○), MNE-Ba (■), MNE-Bp (♦)**

### 4.3.4 Mineral recovery, morphology, and mineralogy

#### *4.3.4.1 Minerals from B4.1 media*

Minerals precipitates were recovered from all inoculated forms of *B. pumilus,* with 550 mg precipitate/L B4.1 media recovered from suspended cells and 400 mg precipitate/ L B4.1 for the MNE-Bp. The morphology of precipitates recovered from all inoculated solutions (Figure 4-5) show well-formed tabular and prismatic crystals, >50 µm and up to 200 µm in length. The precipitates from suspended cells of *B. pumilus* presented a bimodal distribution of morphologies, needle-like precipitates less than 50 µm and tabular prismatic precipitates (Figure 4-5 a and c). The precipitates from MNE-Bp inoculations were much more homogenous in morphology, under optical microscope and SEM precipitates were between 50 µm and 200 µm and were consistently prismatic and tabular. There was no precipitation in controls of B4.1 media. This morphology is akin to euhedral (regular crystal shape) abiotic struvite grown in in super saturated conditions[48]. In comparison to other studies of biomineralisation in B4.1 media the yield was reduced 50% in MNE-Bp batches, however the morphology was the same[25]. The reduction in yield in MNE-Bp batches could be due to hypothesised mass transfer limitations of encapsulating media[39], this is not unexpected as the passage of solid material through the MNE media will be slower relative to cells expelling their

minerals from their cell walls (BIM) or from within their cells (BCM). This could be beneficial for continuous systems as the seeding of bio-struvite from the MNE media will be more resilient to fluctuations in the influent chemistry.

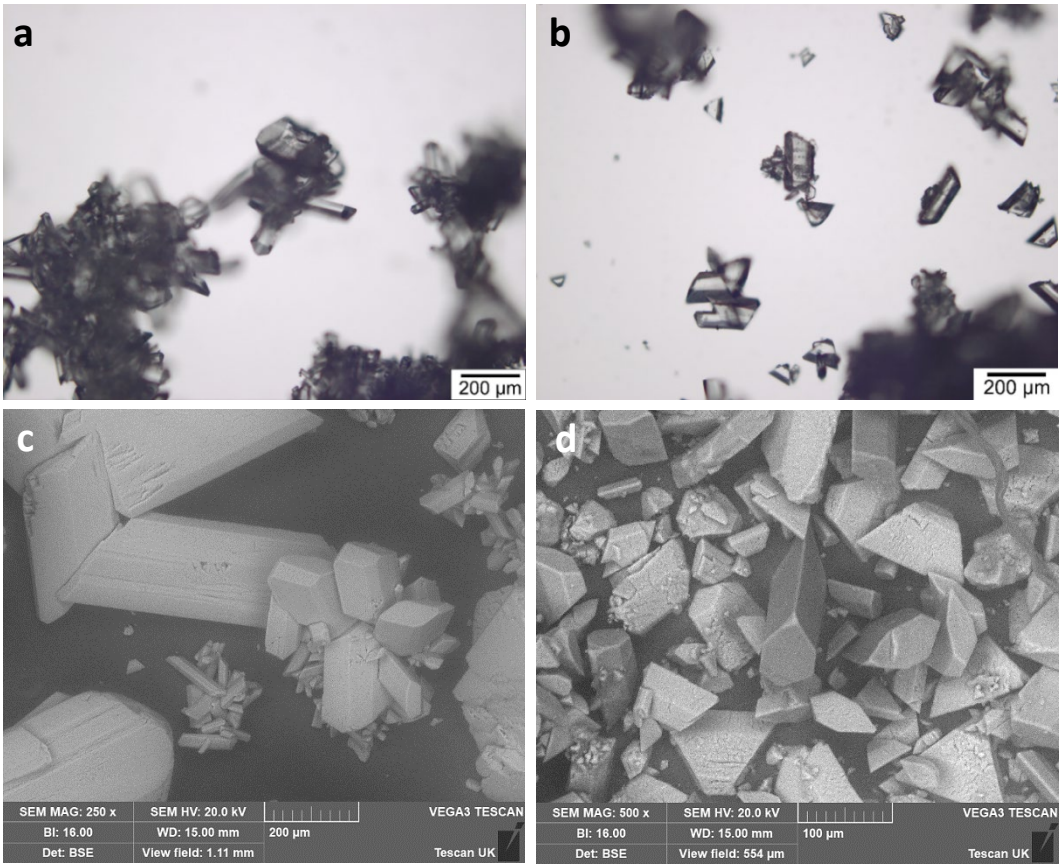


**Figure 4-5 Precipitates recovered from B4.1 media inoculated with *B. pumilus* as; a) suspended cells, b) MNE-Bp, under optical microscope. And under SEM, suspended cells of c) *B. pumilus* and d) MNE-Bp**

#### *4.3.4.2 Minerals from sludge dewatering liquors*

Mineral precipitates were observed as early as 1 day of incubation in suspended cells in pure culture SDL1 and all SDL1 conditions inoculated with the active MNE beads. After 5 days of incubation precipitates greater than 10 µm in diameter were recovered, dried, weighed and, analysed under optical microscope and SEM. No precipitates greater than 10 µm were recovered from controls or open culture conditions inoculated with suspended cells. The yield was up to 140 mg of precipitate/L SDL in pure culture conditions inoculated with suspended cells. The inoculations of MNE-Ba and MNE-Bp precipitate yields ranged from 38 mg/L to 98 mg/L in both pure and open culture conditions, averaging at 63 mg/L. There was little to no

difference in yield between the two encapsulated microorganisms in MNE despite *B. antiquum* using biologically controlled mineralisation and *B. pumilus* using biologically induced mineralisation. This decrease in precipitate yield is consistent with what was observed in B4.1 growth media and is inferred to be due to a lag in precipitate growth as the precipitates are extruded from the MNE. Also, to consider are the limiting concentrators of magnesium, hindering the precipitate formation, as described above.

The precipitates recovered from all inoculated batches of pure and open culture SDL exhibited a bi-modal distribution of morphologies, tabular and prismatic precipitates ranging from 50 µm to 100 µm, as seen in B4.1 media batches (Figure 4-5) and, equigranular (the same diameter), but amorphous precipitates (Figure 4-6). There is a coupling between Mg and P, evident by SEM-EDS, mapping with the tabular and prismatic precipitates and, Ca with the equigranular precipitates (Figure 4-6). The culmination of morphology and element mapping indicates that the tabular precipitates are struvite biomineralised by the microbes encapsulated in the MNE media and, that the equigranular precipitates are calcium phosphates. Furthermore, weight percentage based on spectral analysis shows the ratio P:Mg and Ca is 1:1, with calcium phosphates making up less than 20% of the assemblage of the precipitates recovered.

The precipitate observations and data collected show that struvite was successfully recovered through biomineralisation based on this studies' data and corroborates with observations made in past research in synthetic media and wastewater[17,22,26]. The formation of precipitates in open culture conditions has likely occurred in two stages, firstly, biomineralisation of struvite due to larger precipitate sizes and euhedral (well-formed) morphology indicating little competition for ions or environmental changes during its growth phase. Secondly, calcium phosphates precipitated abiotically, more quickly than the first biomineralisation stage, hence the smaller size and poorer quality crystal shape[51]. This evidence further proves that *B. antiquum* and *B.*

*pumilus* can overcome kinetic restrictions to biomineralise struvite before abiotic calcium phosphates, as seen in previous studies[26]. To produce bio-struvite in conditions not favourable for abiotic mineralisation of struvite. Furthermore, the SDL characterisation showed the P concentration was below 100 mg/L and the ratio of P:Mg was well below 1:2, making chemical struvite recovery unfeasible[49,52].

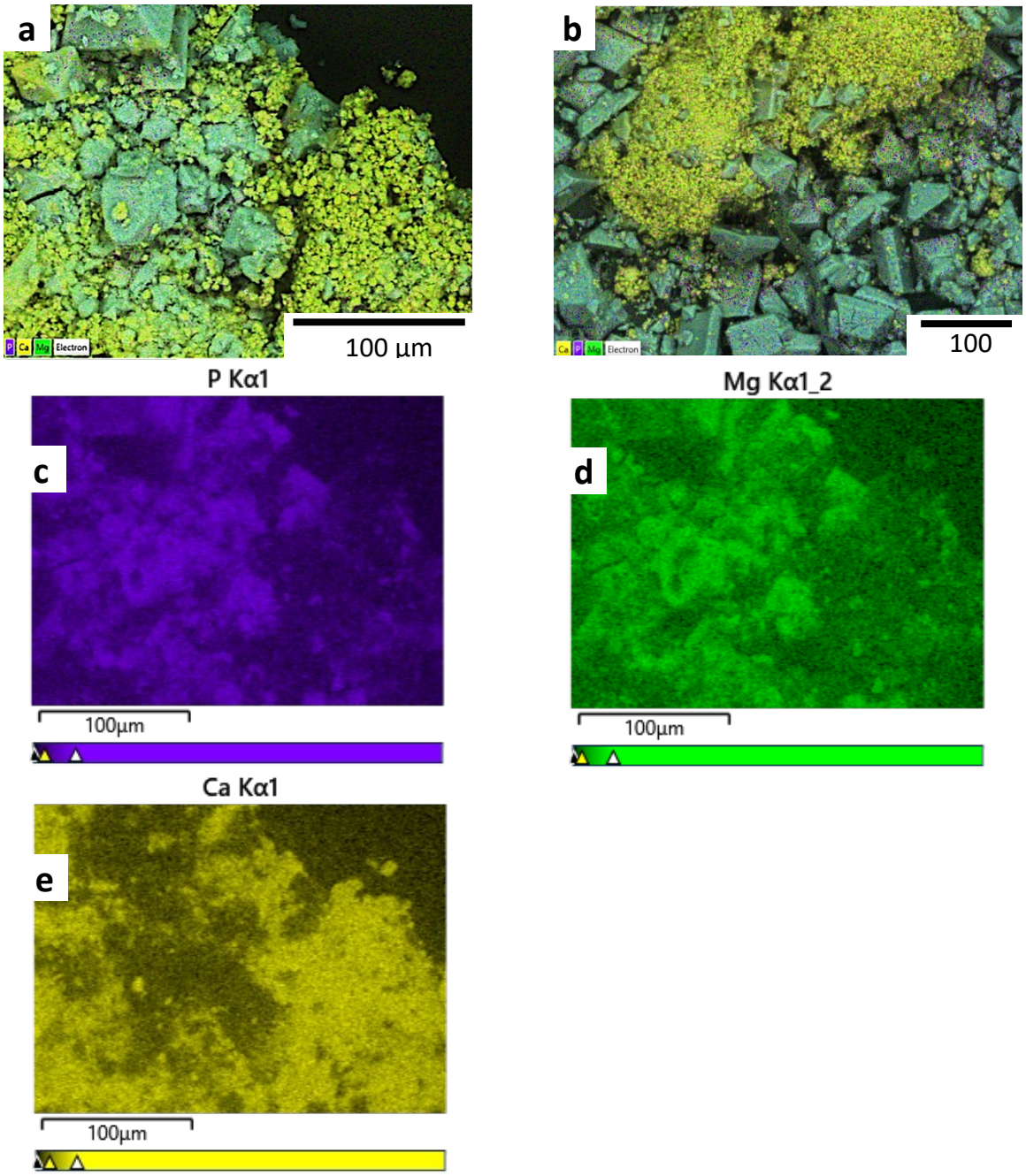


**Figure 4-6 Images of SEM-EDS elemental mapping of precipitates recovered. Turquoise represents P- and Mg-rich precipitates, yellow represents Ca-rich precipitates. Recovery of precipitates from MNE-Ba inoculated in a) pure culture SDL, b) open culture SDL, c) P element mapping, d) Mg element mapping, e) Ca element mapping, of a).**

However, in a continuous treatment system this may prove beneficial to ensure that the reactor is constantly seeded by bio-struvite to encourage abiotic precipitate growth in the reactor, studies have shown that seeding at a constant rate (up to 25 g/L) improves the recoverability and phosphorus removal in abiotic struvite reactors, by reducing the time between nucleation and mineral growth[53]. The controlled release of bio-struvite from the MNE media can potential help maintain a constant seeding of struvite fines into solution to improve the growth rate. This hypothesis should be tested through mineralisation kinetic experiments similar to those that have been done in chemical struvite reactors[53,54].

### 4.4 Conclusions

To conclude, this study demonstrated that the encapsulation of *Brevibacterium antiquum* and *Bacillus pumilus* in MNE successfully facilitated their activity in open culture conditions, where suspended cells could not. Initial comparison in batches of B4.1 media, showed that MNE encapsulation improved the removal of P by 20% compared to suspended cell inoculations during the first 6 hours of incubation. Further to this, MNE allowed for the cultivation and retention of selected microorganisms in sludge dewatering liquors from a full-scale wastewater treatment plant in an open culture system. The MNE media improved the resilience of encapsulated microbes to open culture conditions, $PO_4$-P removal was unaffected at the end of 24 hours incubation in both MNE-Ba and MNE-Bp in pure and open culture SDL, reaching 70% and 44% $PO_4$-P removal respectively. In comparison, suspended cells of each microbe were not able to remove more $PO_4$-P relative to the controls under open culture conditions. The refreshment of SDL with the same MNE batches showed limited reduction in $PO_4$-P removal and recovery of bio-struvite, indicating that the MNE structure has not been degraded during incubation which has been observed in other encapsulation/immobilisation processes.

The $PO_4$-P absorption capacity of the blank MNE was around 20% and reached equilibrium within an hour, this equates to an absorption capacity of 1.28 mg $PO_4$-P/g MNE. This indicated that $PO_4$-P removal was due to the microbial activity when active MNE media were utilised. Further to this, the quick adsorption of $PO_4$-P within the MNE can improve the availability nutrients to the encapsulated microorganisms, reducing the start-up time for the bio-mineral production enabling the recovery $PO_4$-P as struvite.

The yield of bio-struvite from MNE encapsulated batches were reduced up to 50% relative to when suspended cells could facilitate biomineralisation (B4.1 media or pure culture SDL). This has been alluded to be due limitations in the MNE media pathways. However, this may provide consistent loading of bio-struvite seed material for improved $PO_4$-P removal and recovery in continuous reactors.

The findings of this study provide clear evidence supporting the progression of MNE media in continuous reactor pilots to investigate the impact loading rate has on $PO_4$-P removal and bio-struvite recovery in open culture conditions, to develop the biomineralisation technology into full-scale treatment for $PO_4$-P removal and recovery as a viable fertiliser alternative.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

**Data Access Statement**

Data not represented in this paper is available in the accompanying appendices or available upon request from the 1st author, data is not accessible in a public repository.

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# 5. Struvite bio-mineral production in continuous pilot scale with encapsulated microorganisms in sludge dewatering liquors

Robert Colston[a], Peter Vale[b], Ajay Nair[c], Francisco Simoes[a], Tom Stephenson, Ana Soares[a1]

[a] Cranfield Water Science Institute, Cranfield University, Cranfield, MK43 0AL, UK
[b] Technology and Development, Severn Trent Water Ltd., Avon House, St. Martins Road, Coventry, CV3 6PR, UK
[c] Microvi UK Ltd., Sci-Tech Daresbury, Keswick Lane, Daresbury, Warrington WA44FS, UK

[1] Corresponding author: a.soares@cranfield.ac.uk

**Abstract**

The biomineralisation of struvite (bio-struvite), as a process to remove and recover nutrients from wastewater, is emerging as promising technology and has been demonstrated at lab-scale. Retaining the desired biomass and intensification of the biomineralisation process remains to be demonstrated due to wash-out of the biomineral producing species and competition from native microorganisms in sludge dewatering liquors fed reactors. This pilot-scale study establishes the operations of continuous reactor using *Brevibacterium antiquum* and *Bacillus pumilus* encapsulated in Microniche Engineering™ media fed with digestate dewatering liquors with orthophosphate ($PO_4$-P) concentrations 48-65 mg/L. The initial removal of $PO_4$-P and bio-struvite recovery was limited by the poor carbon source availability (biochemical oxygen demand, BOD < 36 mg/L) and concentration of magnesium (16-22 mg/L). With the addition of 100 mg/L of albumin as BOD and magnesium (50 mg/L, to match equimolar concentration of $PO_4$-P), the $PO_4$-P removal reached an average of 80 and 94% for *B. pumilus* and respectively, in open culture conditions. The effluent $PO_4$-P was <3 mg/L with *B. antiquum* corresponding to 96% removal. The precipitates recovered from the reactor were identified as struvite with high purity. The results of this pilot-plant study provide an evidence-based proof

of concept with the opportunity to scale up encapsulated microbes for nutrient removal and recovery of bio-struvite to full scale once the technology is fully optimised. Further to this, the encapsulation technique utilised could be extended to other desirable microorganism enabling full control of biological pathways in wastewater open systems, leading to turn-on/turn off plug and play intensified synthetic biology driven treatment processes by design.

**Key words:** *Microbial encapsulation, bio-mineral recovery, continuous pilot, sludge dewatering liquors*

## 5.1 Introduction

Global fertiliser use remains largely linear, as global population increases, farming also needs to intensify and relies evermore on mineral fertilisers. The pressure this puts on finite resources, such as phosphorus[1] is leading to large fluctuations in market price and circular economy needs to be applied. This will sustain food security in the future which can be implemented at the end of use i.e., composting and recovery of nutrients at wastewater treatment plants (WWTP)[2–6]. Additionally, not removing nutrients from wastewater poses environmental threats such as eutrophication[7]. To reduce risks, regulation enforces discharge limits. In the UK (at the time of writing) the annual mean for phosphorus discharge is 2 mg/L for WWTP with a population equivalent (PE) of 10,000 to 100,000 and < 1 mg/L for >100,000 PE and at least 80% of the annual mean load of phosphorus must be removed to reduce the impact on receiving catchments[8]. As population numbers and density increase, the nutrient pressure on WWTP will too, threatening wastewater treatment infrastructure and the environment. High pH and high aqueous phosphate concentrations lead to mineral-scaling of pipes in WWTP and dramatically reduces treatment efficiency and can cause blockages. Therefore, WWTP employ phosphorus removal technologies such as enhanced biological nutrient removal and coagulation. Struvite precipitation reactors have been implemented in some WWTP to recover phosphorus as the mineral struvite for reuse, as struvite is a proven slow-release fertiliser[9–11]. The market price of struvite has fluctuated between 7.50-9.50 €/kg over the past two years[12]. Chemical recovery of nutrients is largely uneconomical due to the reagents required. For example magnesium hydroxide is a common reagent used for chemical struvite precipitation in WWTP, that costs between 5.95-6.3 €/kg (2019 and 2021)[12]. Further to this, strict reaction parameters and concentration of influent orthophosphate ($PO_4$-P) needing to be greater the 100 mg P/L to be feasible[13]. To improve the profitability and uptake of phosphorus recovery techniques by

WWTP, the costs of struvite recovery must be reduced to unlock the potential of substituting struvite for less environmentally friendly mined fertilisers[13].

One technique being investigated for its nutrient removal and recovery of nutrients ad metals is biomineralisation. The biomineralisation of struvite ('bio-struvite') using microorganisms has been proposed as a promising nutrient recovery method. The precipitation reaction is induced (biologically induced mineralisation (BIM)) or controlled (biologically controlled mineralisation (BCM)) by the microorganism, energy and reactant costs could be reduced[10,14,15]. Laboratory scale experiments have distinguished the carbon sources, the species of phosphorus recovered, and the mechanisms of biomineralisation of five known bio-struvite producing microorganisms including *Brevibacterium antiquum* and *Bacillus pumilus*[14,15]. Past experiments have reported 82% orthophosphate removed and 54% recovered as bio-struvite in various sterile or sludge dewatering liquors (SDL)[14].

To scale up the biomineralisation process to full scale WWTP, development needs to show that it is possible to enrich the biomass of the selected biomineralising microorganisms and retain it in the reactor. So that the selected microorganisms out-compete native cultures within wastewater to ensure the process is effective. The use of sequencing batch reactors (SBRs) is commonly used to enrich specific microorganisms, for example enriching anammox bacteria and implementing the process[16]. Additionally, novel techniques for the accumulation of polyhydroxyalkanoates (PHAs) to produce bio-plastics have been trialled at pilot-scale using SBRs to achieve a relative abundance of >70% *Plasticicumulans acidovorans* in fermented wastewater[17]. Sequencing batch reactors are relatively simple to operate and offer the flexibility of being able to independently control parameters such as the oxygen content (aerobic, anoxic, and anaerobic), residence time and solid retention time. Furthermore, SBRs can be combined

with physical processes such as sedimentation and membrane technology which could be useful for the harvesting of bio-struvite.

Encapsulation is a process of containing microorganisms within a substrate which still allows mass transfer of nutrients between it and the solution it is suspended in. Compared to immobilising microorganisms in a biofilm, encapsulation improves the contact time of the biomass with the solution. Encapsulation techniques include cryogels and have been shown effective for *Pseudomonas mendocina* and *Rhodococcus koreensis* that remained viable, and were responsible for improved degradation of phenols[18,19]. Additionally, nitrification in activated sludge and the bio-sorption of heavy metals using biocatalysts has also been investigated through alginate polymers[20,21]. Alginate encapsulation has been studied at pilot-scale for the treatment of brewery effluents in a two-stage anaerobic process to recover hydrogen ($H_2$) and methane ($CH_4$) gasses, utilising encapsulated hydrogen-producing microbes in the first stage, the second stage was split between encapsulated methanogenic microbes and an anaerobic membrane bioreactor[22]. Despite some success, there are issues surrounding the stability of the encapsulation substrate such as breakdown over time leading to biomass leakage and washout.

The use of specifically engineered microenvironments that enhance the productivity of an individual microorganism has been developed using MicroNiche Engineering™ biocatalyst technology (MNE) to improve the efficiency of existing wastewater treatment plants by; accelerating the denitrification process, reducing biosolids production, and reducing biological oxygen demand in full-scale WWTP across the USA and Australia[23].

Previous batch trials of *B. antiquum* and *B. pumilus* encapsulated by MNE media, MNE-Ba and MNE-Bp respectively, were able to achieve the same $PO_4$-P removal from B4.1 synthetic

growth media compared to suspended cells inoculations. Furthermore, 70% and 44% $PO_4$-P removal was achieved respectively by MNE-Ba and MNE-Bp incubated in sludge dewatering liquors (SDL) in open culture conditions over a 24-hour period, equal to batches in pure culture conditions[24]. The removal of $PO_4$-P from SDL in open culture conditions has not been observed in suspended cell inoculations of either *B. antiquum* or *B. pumilus*, due to the competition from native microbes preventing the biomineral producing microorganisms from growing. The biomineralisation process continued when microbes were encapsulated in the MNE media to recover $PO_4$-P as bio-struvite precipitates during the 24-hour incubation period. Past batch studies of MNE encapsulation treatment of wastewater eluded to delayed crystal growth phase due to the time taken for biominerals to be transferred through the MNE media[24]. However, staggering the extrusion of bio-struvite from the biocatalysts may benefit a continuous system by continually crystal seeding the reactor regardless of fluctuations in nutrient concentrations. This pilot-plant study investigates the *B. antiquum* (BCM) and *B. pumilus* (BIM)[10,14,25] encapsulated in the MNE biocatalysts for $PO_4$-P removal and bio-struvite recovery in a continuous reactor, to assess the impact nutrient loading rate has on the encapsulated microbes in terms of $PO_4$-P removal and recovery or bio-struvite. Additionally, this study was key to understand the longevity of the MNE biocatalyst when continually treating SDL with intermittent stop starting. The research here presented underpins the ability to proliferate selected microorganisms in open culture in wastewater sludge dewatering liquors using for recovery of bio-struvite using a biomineralisation. This was done through two differing techniques, attempting to enrich *Brevibacterium antiquum* in SBRs and continuous reactors inoculated with MNE-Ba and MNE-Bp.

## 5.2 Materials and methodology

### 5.2.1 Microbial encapsulation and sludge dewatering liquors

Starter cultures of *Brevibacterium antiquum* and *Bacillus pumilus* strain were inoculated at a ratio of 10% (v/v) in B4.1 synthetic media (4 g/L of yeast extract, 2 g/L of magnesium sulphate heptahydrate and 2 g/L of di-potassium hydrogen phosphate), incubated in conical flasks at room temperature (20-22$^{o}$C) and agitated at 150 rpm (Stuart SSL, Fisher Scientific, Loughborough, UK) for two to three days to reach the stable growth phase.

Encapsulated beads from the selected cultures were also obtained from a commercial company after production at their facilities with MicroNiche Engineering technology (MNE) (Microvi© MNE™, San Francisco, USA). The full protocol for obtaining the encapsulated cultures, also called biocatalyst, was not disclosed as this is protected intellectual property (Microvi© MNE™, San Francisco, USA). The biocatalysts beads were named *B. antiquum* (MNE-Ba) and *B. pumilus* (MNE-Bp).

Sludge dewatering liquors (SDL) were collected from full scale wastewater treatment plants (WWTP) in the East Midlands of England, United Kingdom. Samples were taken of centrifuged centrate, post two-stage anaerobic digestion (Figure B1) and post-biological nutrient removal. To aid the centrifugation process influent sludge was dosed with cationic polymer flocculant, anti-scale, and anti-foam agents. Sludge dewatering liquors were transported to Cranfield University, characterised, and stored for a up 7 days before passing through the pilot-scale reactor.

### 5.2.2 Operation and set-up of SBR enrichment of *B. antiquum*

Each SBR had a 500 mL volume, kept at a constant temperature of 25℃ using a water bath, to ensure optimum growth conditions for *B. antiquum*. Magnetic stirrers kept each SBR contents suspended and ensured aeration. Three hundred and sixty millilitres of SDL post-BNR were added to each SBR. Two SBRs were inoculated with *B. antiquum* (replicates Ba and Bb) and two control SBRs were not inoculated (replicates Sa and Sb). Pure cultures of *B. antiquum* were centrifuged and resuspended in 0.9% sterile NaCl solution and inoculated into two SBRs with a one-time addition at 10% v/v. 0.9% sterile NaCl solution was inoculated into the two controls at the same ratio.

All SBRs were ran for a total of 25 days, consisting of 6 cycles. Each cycle was split into 3 stages according to the finding from Simoes et al[15]; stage 1: 1 day supplement with 3% w:v NaCl and 562 mg COD/L as acetate. Stage 2: 3 days supplement with 562 mg COD/L only. Stage 3; settling (30 minutes) before decanting 90%, leaving 40 mL of spent SDL in the SBR to seed the next cycle where the SBRs were refreshed by 360 mL SDL. ON-off pH controllers were used to ensure pH remained below 8, each controller activated a peristaltic pump (Watson Marlow 120s, Falmouth, UK) that supplied 5M HCl solution to the SBR to lower pH as necessary. pH controllers were washed using 2% Decon 90 alkaline solution and recalibrated using pH 7 and pH 10 standard solutions (Fischer Scientific, Loughborough, UK) during stage 3. Dissolved oxygen was measured at the end of stage 1 for each cycle and average concentrations of 6.2 mg $O_2$/L were measured.

### 5.2.3 Microbial community profiling

DNA extraction was achieved by mixing SDL samples with an equal volume of 100% ethanol and kept frozen at -80°C until analysed. Once thawed, samples were placed in a lysing matrix

tube and DNA extraction was carried out using the MPBio FastDNA Spin Kit for Soil (Santa Ana, USA). The V4 and V5 regions of the 16S ribosomal RNA gene were targeted with the universal primers 515F and 926R[26]. Golay barcodes were used to correct errors to achieve sample multiplexing[27]. Polymerase chain reaction (PCR) amplified products (amplicons) were purified using HighPrep magnetic beads (Magbio, Gaithersburg, USA) and quantified using QuantiFluor ONE (Promega, Madison, USA). Illumina MiSeq with 2x300 v2 chemistry (Illumina, San Diego, USA) was used to sequence an equimolar pool of amplicons, with reads per samples measuring between $3.4 \times 10^5$ and $1.6 \times 10^6$. The sequences were analysed in QIME 1.9 software and the SILCA 16S rRNA gene database v123.1[28,29].

### 5.2.4 Pilot-plant description and operational conditions

Two continuous reactors were set up in parallel for each MNE encapsulated microorganisms, *B. antiquum* (MNE-Ba) and *B. pumilus* (MNE-Bp). These consisted of an aerated bioreactor (4 L) and settling tank (2 L) (Figure 5-1). Sludge dewatering liquors were pumped into each biocatalyst reactor (Tank A) using a peristaltic pump (Watson Marlow 120R, United Kingdom) at varying flow rates to control the nutrient load (Table 5-1). MNE beads filled Tank A at 20% volume to volume filling ratio and each tank was run continuously for 21 to 30 days at each selected feeding low rate (Figure 5-1). Each pilot rig was operated at ambient temperatures from June 2022 to September 2022, temperatures ranged from 12°C to 25°C during this period and was not controlled to mimic real world WWTP. Each biocatalyst reactor (Tank A, Figure 5-1) was aerated enough to provide uplift and mixing of the MNE media and ensure no limiting dissolved oxygen concentrations were provided[14] that were measured with an average 7 mg $O_2$/L were measured.

The $PO_4$-P loading rates tested were achieved by varying the influent flow that in turn impacted the hydraulic retention time (HRT), first at 14 hours and second at 10 hours (Table 5-1).

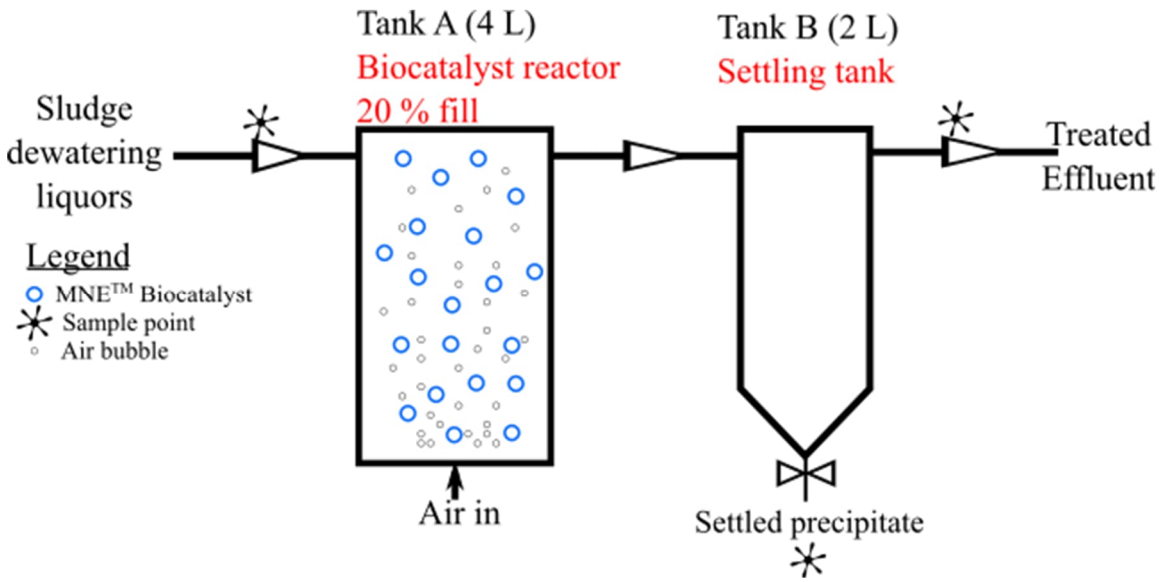



**Figure 5-1 Pilot-plant flow scheme**

**Table 5-1 Operation conditions for pilot plants**

| ***Flow rate*** | ***HRT*** | ***sCOD*** | ***$PO_4$-P*** | ***BOD*** | ***$NH_4$-N*** | ***Mg*** | ***Ca*** |
|---|---|---|---|---|---|---|---|
| L/d | hours | kg/$m^3$.d | kg/$m^3$.d | kg/$m^3$.d | kg/$m^3$.d | kg/$m^3$.d | kg/$m^3$.d |
| **7** | **14** | **4.22** ± 0.12 | **0.43** ± 0.13 | - | **6.89** ± 1.42 | **0.16** ± 0.06 | **0.45** ± 0.06 |
| **10** | **10** | **6.18** ± 0.33 | **0.64** ± 0.15 | **0.37** ± 0.01 | **12.75** ± 1.72 | **0.19** ± 0.08 | **0.62** ± 0.04 |
| ***10** | **10** | **7.96** ± 0.12 | **0.48**[1] ± 0.18 | - | **13.01** ± 2.00 | **0.71** ± 0.03 | **0.33** ± 0.03 |

*HRT =Hydraulic retention time; sCOD = Soluble chemical oxygen demand; * = Dosed with 100 mg/L BOD and 50 mg/L magnesium;* [1] = ***influent concentrations of $PO_4$-P was lower*** *in the SDL collected from site* ***during this period hence*** *the* ***lower loading rate of 0.48 kg P/$m^3$.d****, even if the HRT was kept constant.*

### 5.2.5 Dosing of reagents

Serum albumin in the form of dehydrated egg white protein was dosed via an in-line to dose each reactor with a suitable carbon source for each microbial biocatalyst after initial operation conditions (Table 5-1), in addition to magnesium sulphate heptahydrate ($MgSO_4.7H_2O$). To ensure magnesium and carbon were no longer limiting to investigate the maximum phosphorus removal rate possible using the biomineralisation process.

The dosing solution was made of Serum albumin (1 g/L) and $MgSO_4.7H_2O$ (70 g/L) at a flow rate of 0.5 to 1 L/d using a Watson Marlow 120s/DM3 peristaltic pump and in-line mixer. These concentrations were defined by past characterisations, so that BOD increase was between 150 mg/L and 200 mg/L, and Mg was increased by 50 mg/L to reach a concentration where the ratio of P to Mg was less than 1:2.

### 5.2.6 Precipitate production

The precipitates collected in Tank B were regularly monitored by visual observation and sampling of the settled precipitates. The samples of suspended precipitates were taken for analysis and left to settle by gravity and filtered through 10µm sheets (Whatman™, Grade 1 filter sheets). The precipitates were then left to dry at room temperature (20-22°C) before analysis and weighed once all precipitates were collected from each pilot.

### 5.2.7 Analytical Methods

The wastewater chemistry was measured using ion chromatograph. Dionex ICS-900 with a Dionex IonPac column (CS12A) (Thermo Fisher, USA) for cation analysis; ammonium ($NH_4$), magnesium ($Mg^{2+}$), potassium ($K^+$), calcium $Ca^{2+}$ and sodium ($Na^{2+}$). Dionex Aquio (Thermo Fisher, USA) for anion analysis of $PO_4^{3-}$. The temperature, pH and dissolved oxygen levels were measured using a Hach HQ40d multi-metre probe (Hach UK, Manchester). Soluble chemical oxygen demand was measured using Merck cell test kits following manufacturer's instructions and biological oxygen demand was measured using the 5-day standard method[30].

The weight of dried precipitates recovered was noted to estimate the recovery of minerals per litre SDL. Morphological characteristics and mineralogy of the precipitates collected were analysed using optical microscopy (Olympus MX40) and scanning electron microscopy (SEM) to observe their crystallography and distinguish direct and indirect bio-mineralisation (if

possible), with the addition of energy dispersive x-ray spectroscopy (EDS) to quantify impurities and understand their relationship to crystal morphology (Tescan Vega 3, Oxford Instruments© Aztec™). The recovered precipitate yield, relative weight percentage and stoichiometric mass balancing will be used to estimate the proportion of the precipitate as bio-struvite and the proportion of $PO_4$-P recovered in precipitates compared to the total $PO_4$-P removed using Equation 5-1.

Heavy metals and other micro-pollutants were analysed through the dissolution of collected minerals by 2M HCl. Heavy metals, pathogens and coliforms were measured at an external laboratory (ALS Environmental, Coventry, Manchester) using UK accredited services. 99 Micropollutants were analysed using a 1290 series liquid chromatograph (Agilent, Santa Ana, USA) equipped with a Waters Acquity BEH (2.1×100 mm, 1.7 µm). A full method can be found in Appendix B. In short, the liquid chromatograph was connected to a 6540 accurate-mass quadrupole time-of-flight mass spectrometer (QTOF) (Agilent, Santa Ana, USA) which ionised the sample to analyse the spectra returned of each compound present, this can be compared with the known molecular weights of potentially hazardous contaminants measured in wastewater[31]. All analyses were run in duplicate for quality assurance of data collected. A full list of the micropollutants, their compounds and molecular weight analysed can be found listed in Appendix B.

**Equation 5-1 Calculations for recovery of bio-struvite and percentage of $PO_4$-P removed that is recovered in precipitate. Where the constants 0.226 and 0.436 are the proportion of $PO_4$ in struvite and in calcium phosphate, respectively.**

$$R_{Bio-S} \sim \frac{W_{Mg}}{W_{Mg}+W_{Ca}}$$

$$R_{PO_4-P} = \frac{\Sigma(Y \times R_{Bio-S} \times 0.226):(Y \times (1-R_{Bio-S}) \times 0.436)}{X_{PO_4-P}} \times 100$$

| Notation | Meaning |
|---|---|
| $Y$ | *Yield (mg/L)* |
| $X_{PO_4-P}$ | *Average $PO_4$-P removed (mg/L)* |
| $W_{Mg}$ | *Weight percentage as Mg* |
| $W_{Ca}$ | *Weight percentage as Ca* |
| $R_{Bio-S}$ | *Percentage or precipitate recovered as Bio-struvite* |
| $R_{PO_4-P}$ | *Percentage of removed $PO_4$-P recovered* |

## 5.3 Results and discussion

### 5.3.1 Sludge dewatering liquor characterisation

Throughout the pilot-scale study the characterisation of the dewatering liquors fluctuated for the major ions $PO_4$-P, $NH_4$-Nand $Mg^+$ with average concentrations at 59 mg $PO_4$-P /L, 1165 mg N/L and 19 mg Mg/L, respectively (Table 5-2). The initial characterisation of SDL collected from full scale BNR WWTP had an initial concentration of 40 mg/L for $PO_4$-P (Table 5-2), this falls within reported values for $PO_4$-P which range between 37 mg/L and 167 mg/L[32,33]. Soluble chemical oxygen demand and Mg measured at 418 mg/L and 73 mg/L respectively (Table 5-2), above typical ranges for BNR sites, 70 mg COD/L to 306 mg COD/L and 11 mg Mg/L to 51 mg Mg/L[32,33].

Overall, yielding an average ratio of Mg to $PO_4$-P of less than 0.5:1 and the $PO_4$-P concentration was also much lower than that required for chemical struvite. The concentration of $Ca^{2+}$ and $K^+$ ions was also higher than $Mg^+$ (Table 5-2). Soluble chemical oxygen demand between 603 mg/L and 618 mg/L (without dosing) (Table 5-2). Biological oxygen demand was between 30 mg/L and 40 mg/L, this was considered a potential limit for the biomineralisation of struvite so dosing was applied in one of the tests.

The ratio of key struvite ions for optimum bio-struvite recovery has been reported at 1:2:12 [$PO_4$-P]:[$Mg^{2+}$]:[$NH_4$-N][36]. In this study, the dewatering liquors presented a ratio of 1:0.3:15 and 1:0.3:20 when the reactor was operated at loading rate of 0.4 and 0.6 kg P/$m^3$.d, respectively. The ratio of [$PO_4$-P]:[$NH_4$-N] lies within the optimum range for $PO_4$-P removal by *B. antiquum* and *B. pumilus* however, the ratio of [$PO_4$-P]:[$Mg^{2+}$] was below ratios tested in previous batch studies[15,24,36]. Nevertheless, the low BOD of the SDL raised concerns as it may be limiting, as it has been shown that *B. antiquum* and *B. pumilis* required readily available carbon to grow[14,15].

The reactors with the MNE microorganism were operated at loading rates between 0.43 and 0.64 kg P/m3.day (Table 5-2). In comparison with other pilot-scale studies and enhance biological phosphorus removal (EBPR) in WWTPs, the loading rate of $PO_4$-P was up to an order of magnitude higher than those measured in EBPR[37].

**Table 5-2 Sludge dewatering liquor characterisation at each operational condition**

| ***Loading rate*** | ***n*** | ***pH*** | ***Temperature*** | ***sCOD*** | ***$PO_4$-P*** | ***$NH_4$-N*** | ***Mg*** | ***Ca*** | ***K*** |
|---|---|---|---|---|---|---|---|---|---|
| kg P/$m^3$.d | | | *°C* | mg/L | mg/L | mg/L | mg/L | g/L | mg/L |
| **0.43** | **18** | **8.7** ± 0.1 | **16.3** ± 2.0 | **603** ± 18 | **65** ± 19 | **985** ± 202 | **22** ± 8 | **65** ± 3 | **218** ± 49 |
| **0.64** | **30** | **8.4** ± 0.3 | **19.8** ± 2.2 | **618** ± 33 | **64** ± 15 | **1275** ± 172 | **16** ± 7 | **64** ± 5 | **261** ± 55 |
| ***0.48** | **20** | **8.6** ± 0.1 | **18.7** ± 1.6 | **796** ± 57 | **48** ± 18 | **1235** ± 270 | **74** ± 5 | **33** ± 3 | **-** **-** |
| BNR | **12** | **7.8** - | **25.0** - | **418** ± 4 | **40** ± 1 | **-** **-** | **73** ± 1 | **-** **-** | **-** **-** |

***n*** = *number of samples* - = *No data* collected
* = *Dosed* BNR = biological nutrient removal, sample collected from full-scale WWTP after 10 days storage post-BNR

### 5.3.2 Enrichment of *B. antiquum* in SBRs

The enrichment of *B. antiquum* was attempted in a SBR with acetate and salt dosing, following the characterisation in batch experiments[15]. Bio-struvite precipitates were monitored through visual observations during stage 3 of each cycle and morphology was investigated under optical microscope. Throughout the SBR cycles very little mineralisation was observed, samples that were collected had weak morphological resemblance to struvite. The comparison between

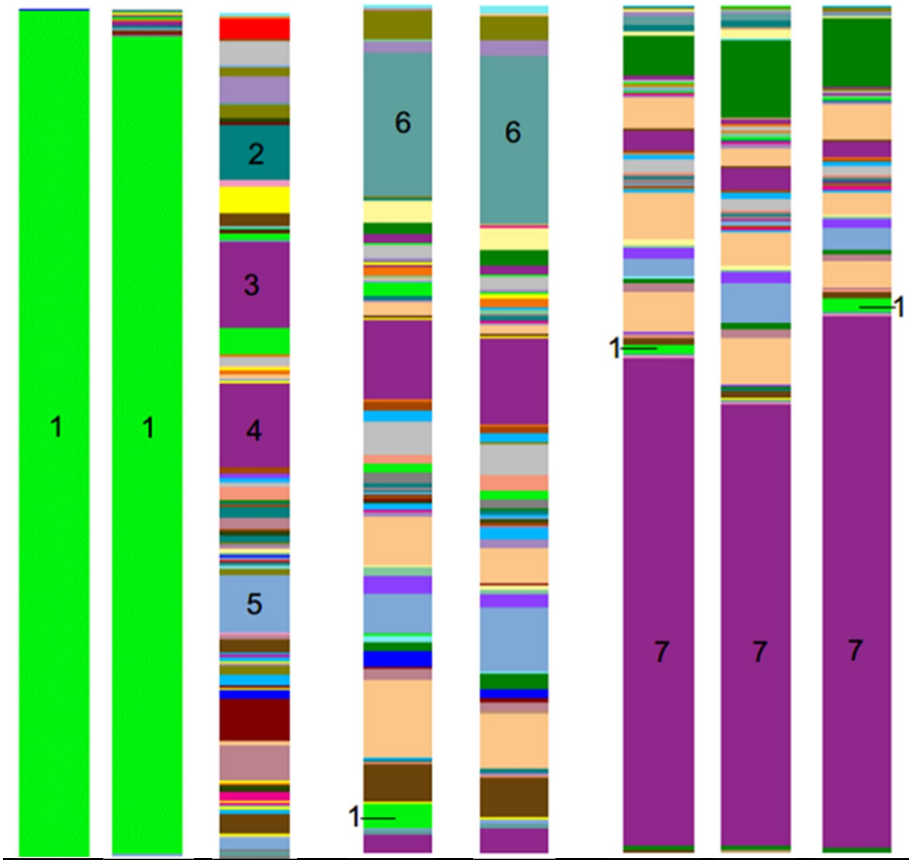
inoculated SBRs and non-inoculated SBRs did show a qualitative difference in minerals observed, with inoculated *B. antiquum* SBRs having more struvite-like morphologies in the assemblage of precipitates collected. Whilst this study focussed on enriching *B. antiquum* in SBRs the observation of struvite was reassuring, as precipitation was occurring in conditions where abiotic struvite was thermodynamically unfavourable[38].

Microbial community profiling of SDLs prior to and during the SBRs operation showed that the abundance of *B. antiquum* was 96.4% at the time of inoculation into the SBR (Figure 5-2). The abundance of *B. antiquum* then decreased to 2.8% at the end of cycle 3 and 0.9% at the end of cycle 6, whilst non-inoculated SBRs measured a relative abundance <0.01% (Figure 5-2). These results show a steep decrease in the relative abundance of *B. antiquum* compared to other microbial genera such as *Smithella, Simplicispira, Rhizobiales, Proteiniclasticum, Acinetobacter* and *Corynebacterium. Corynebacterium* genera were enriched to 56.1%, 61.9% and 50.5% in relative abundance in the SBRs Ba, Bb and Sa, respectively by the sixth cycle (Figure 5-2). The evidence from the SBR study shows that the enrichment of *B. antiquum* was not successful and therefore further optimisation is needed to either improve the retention of *B. antiquum* or increase the selective pressures to limit the growth of native microbes i.e., selecting a carbon source which *B. antiquum* has a higher affinity for.

The enrichment of specific microbial communities has been successful in a variety of other wastewater treatment technologies. Ammonium oxidising bacteria (AOB) has been enriched relative to nitrite oxidising bacteria (NOB) in the CANON process by lowering the concentration of $O_2$ in wastewater as the affinity for oxygen is lower in AOB at 0.99 g $O_2/m^3$ compared to NOB at 1.4 $O_2/m^3$ [39,40].

| SBR | Inoc. | Ba | Sa | Ba | Sa | Ba | Sa | Bb |
|---|---|---|---|---|---|---|---|---|
| Cycle No. | - | 0 | | 3 | | 6 | | |
| Relative abundance of *Brevibacterium antiquum* (%) | 99.8 | 96.4 | 0.0 | 2.8 | 0.0 | 0.9 | 0.0 | 1.5 |

Relative abundance of the bacterial *Genera* detected




1=*Brevibacterium*
2=*Smithella*
3=*Simplicispira*
4=uncultured bacterium of the Order *Rhizobiales*
5=*Proteiniclasticum*
6=*Acinetobacter*
7=*Corynebacterium*

**Figure 5-2 Microbial community profile and relative abundance of *Brevibacterium antiquum* relative to other taxonomic genera in samples collected from 3 of the 4 SBRs (Ba, Bb and Sa) and the starter culture (Inoc.)**

The AOBs were enriched from activated sludge through full biomass retention, controlling ammonium (>30 mg $NH_4$/L), oxygen (0.1 mg $O_2$/L), nitrite (< 15 mg $NO_3$/L), pH at 7.8 and 30 °C[41]. The enrichment of microbes naturally occurring in sewage sludge or activated sludge is well documented, phosphate accumulating organisms (PAOs) and microbes able to accumulate polyhhydroxyalkanoates (PHA) have been enriched through similar controls as the CANON process, utilising feast-famine stages to encourage the most accumulation of phosphate or

PHAs[42–44]. These processes target microbial communities with a common function such as, phosphate accumulation and denitrification, but not a specific organism like *B. antiquum* in this study.

The enrichment of single cell cultures in wastewater in open culture conditions has been successful only when the selected strain had been initially isolated from wastewater source and bioaugmentation, where the culture is repeatedly regenerated with pure culture inoculum[45]. There are issues and limitations with bioaugmentation, if wash out of microbes is greater than the growth more regeneration is needed, inefficient inoculum size, predation and competition from native communities, presence of inhibiting substances are amongst the causes for poor bioaugmentation success[46].

### 5.3.3 Continuous pilot-scale reactor operation with encapsulated microorganisms

#### 5.3.3.1 MNE-Ba

Throughout the pilot-plant operation, an increase in pH was observed from an initial range of 8.3 to 8.7, to between 9.1 and 9.5 (Table 5-3), conforming to previous batch studies[24]. In addition to biological increases of pH from the presence of MNE-Ba, degassing of $CO_2$ would also contribute to some of the pH rise (Figure 5-1). A pH above 9 that remained stable is

beneficial for bio-struvite harvesting as this allows a large build up of struvite to occur before collecting[9,47], reducing the need for a continuous collection system.

Throughout the 60 days of incubation where no dosing of Mg or carbon took place, the average $NH_4$-N removal was between 5% and 9% (Table 5-3). At a loading rate of 0.4 kg P/$m^3$.d, the removal of $PO_4$-P and Mg was on average 32% and 36%, respectevely. The removals were 23% ($PO_4$-P)and 34% (Mg) when the loading rate was increased to 0.6 kg P/$m^3$.d. Little if any

changes were observed in Ca and K concentrations throughout the incubation period when no dosing occured.

The $R^2$ value decreases form 0.7629 to 0.297 with the increased loading of $PO_4$-P, this suggests that there were limitng factors independent of the availability of $PO_4$ which casued the lower removal of $PO_4$-P and Mg by MNE-Ba. Additonally, Mg removal was much lower compared to bach trials indicating that despite the avialbiity of key ions for bio-struvite mineralisation there was a limitng factor, which has been eluded to the availability of an apporpriate carbon source earlier in this discussion.

**Table 5-3 pH change and nutrient removal at different operational conditions**

| ***Loading rate*** | **Minimum/ Maximum/ Average** | ***pH*** | | | ***$PO_4$-P*** | | ***$NH_4$-N*** | | ***Mg*** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **kg P/$m^3$.d** | | **Influent** | **Effluent** | | MNE-Ba | MNE-Bp | MNE-Ba | MNE-Bp | MNE-Ba | MNE-Bp |
| | | | MNE-Ba | MNE-Bp | | | | | | |
| **0.43** | Min. | 8.4 | 9.0 | 8.7 | 17% | 3% | -10% | -13% | 8% | 17% |
| | Max. | 9.0 | 9.6 | 9.6 | 55% | 46% | 16% | 30% | 43% | 68% |
| | **Ave.** | **8.7** | **9.5** | **9.5** | **32%** | **22%** | **5%** | **9%** | **31%** | **36%** |
| | | ±0.1 | ±0.1 | ±0.2 | ±14% | ±14% | ±7% | ±12% | ±12% | ±19% |
| **0.64** | Min. | 8.1 | 8.8 | 8.9 | 12% | 1% | -4% | -20% | 0% | 0% |
| | Max. | 9.1 | 9.5 | 9.5 | 55% | 58% | 43% | 18% | 68% | 62% |
| | **Ave.** | **8.4** | **9.1** | **9.2** | **23%** | **24%** | **8%** | **3%** | **34%** | **30%** |
| | | ±0.3 | ±0.3 | ±0.3 | ±11% | ±13% | ±13% | ±10% | ±21% | ±23% |
| ***0.48** | Min. | 8.5 | 9.0 | 8.6 | 80% | 30% | -37% | -43% | 75% | 23% |
| | Max. | 8.9 | 9.5 | 9.5 | 98% | 91% | 43% | 10% | 96% | 96% |
| | **Ave.** | **8.6** | **9.5** | **9.3** | **94%** | **61%** | **4%** | **-6%** | **85%** | **80%** |
| | | ±0.1 | ±0.1 | ±0.3 | ±6% | ±16% | ±34% | ±16% | ±11% | ±29% |

* = Dosed with 100 mg/L BOD and 50 mg/L magnesium

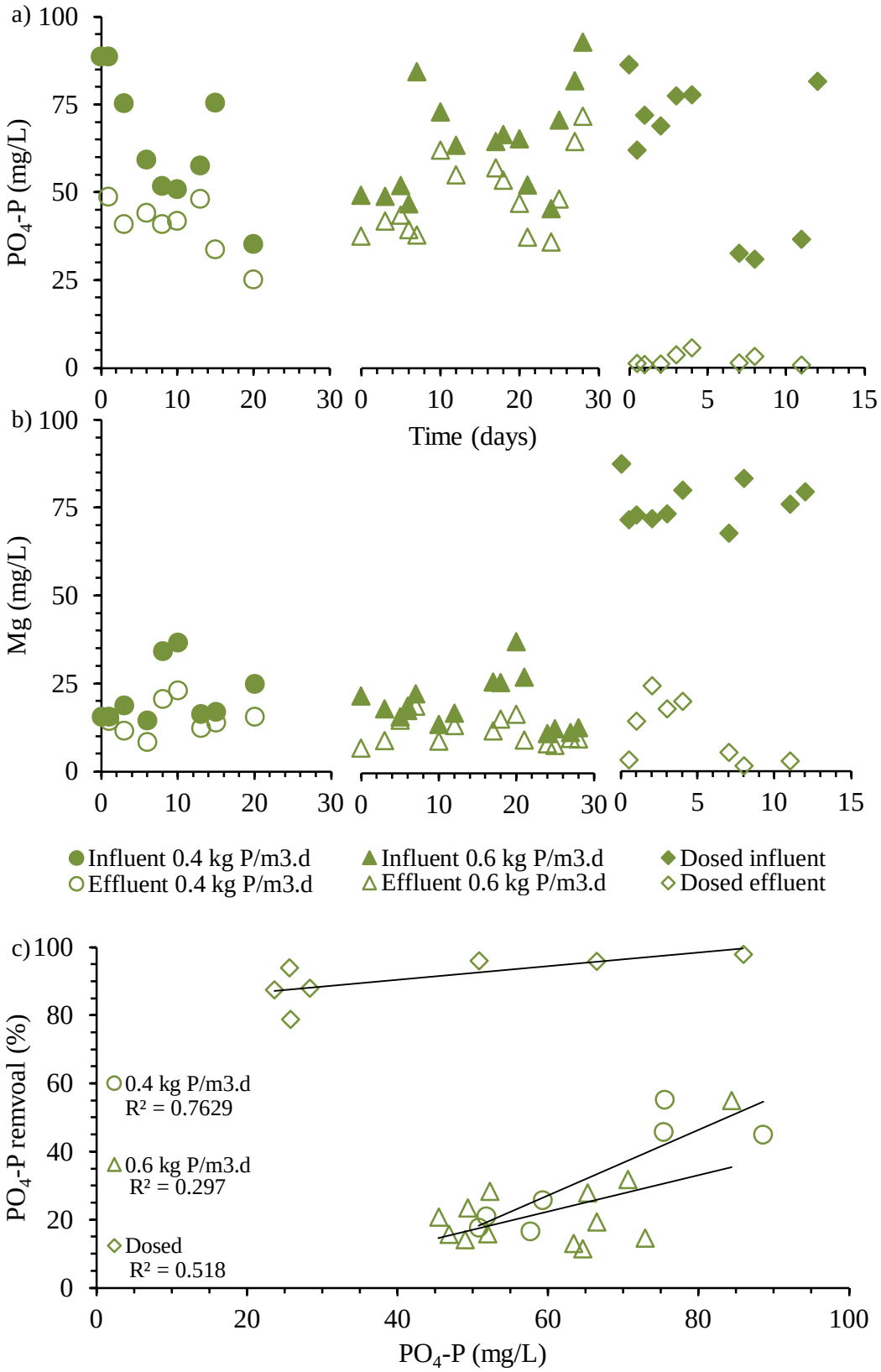



**Figure 5-3 Influent and effluent characterisation during pilot study at loading rates 0.4 (open and closed circles), 0.6 (open and closed triangles) and 0.5 (Dosed, open and close diamonds) kg P/m$^3$.d for MNE-Ba. $PO_4$-P a), Mg b) and $PO_4$-P removal versus influent $PO_4$-P and $R^2$ statistics on trendline c)**

The removal of $PO_4$-P after dosing the SDL with albumin and Mg increased $PO_4$-P removal to between 80% and 98%, achieving a treated effluent $PO_4$-P quality < 3 mg/L (Table 5-3 and Figure 5-3). When correlation initial PO4-P concentration and removal, the $R^2$ value increased to 0.518 (Figure 5-3). This suggests that the addition of carbon and Mg has relieved the limitations observed at 0.6 kg P/$m^3$.d.

#### 5.3.3.2 MNE-Bp

The pilot-plant containing MNE-Bp exhibited similar chemical changes to MNE-Ba. The pH increased and stabilised between 8.7 and 9.5 in final effluent (Table 5-3). These results suggest the MNE-Bp facilitates the same pH changes as MNE-Ba. The $NH_4$-N removal average was between a 3- 9% decrease in $NH_4$-N (Table 5-3).

When the reactor was operated with a loading rate of 0.4 kg P/$m^3$.d $PO_4$-P removal by MNE-Bp was 22% on average, 10% lower than MNE-Ba. However, increasing the loading rate to 0.6 kg P/$m^3$.d had no impact on the $PO_4$-P removal, which was 22% on average (Table 5-3). The $R^2$ values of when correlating P removal and initial $PO_4$-P concentration without dosing decreased from 0.6198 to 0.3406 as the loading rate increased (Figure 5-4), also suggesting that other variables were influencing the removal of $PO_4$-P. Magnesium removal measured between 30- 35% during both loading rates without dosing, this was also below levels observed in batch trials like the MNE-Ba pilot (Table 5-3).

There appears to be very little difference between MNE-Ba and MNE-Bp, until dosing occurs when carbon and Mg were no longer limiting. Once dosing occurred the $PO_4$-P removal increased to between 30% and 91% (Table 5-3 and Figure 5-4), averaging at 61% removal and a treated effluent $PO_4$-P was <15 mg/L. This large fluctuation could be attributed to the biomineralisation mechanism of *B. pumilus* known as BIM. A limitation of this mechanism is

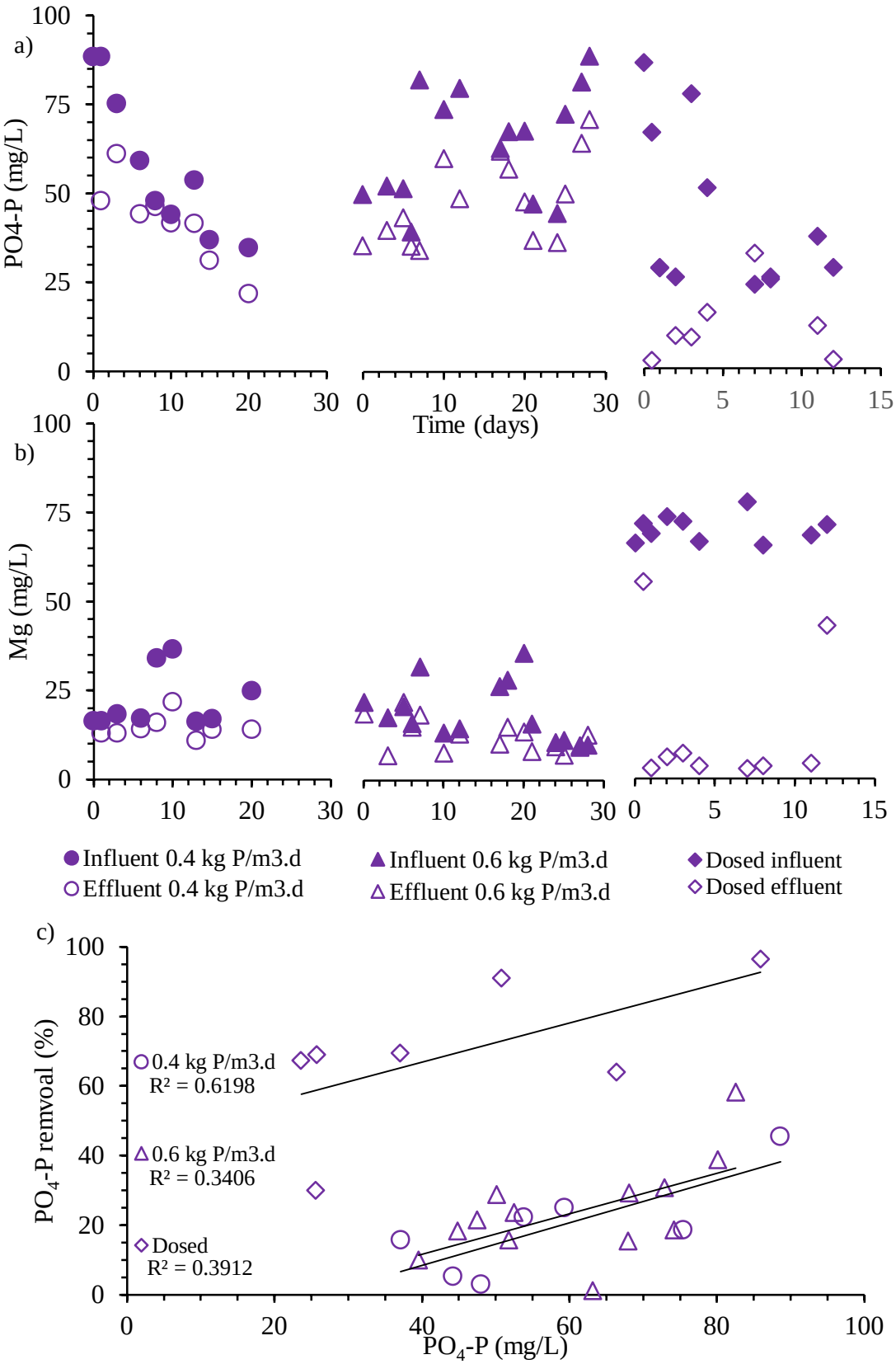



**Figure 5-4 Influent and effluent characterisation during pilot study at loading rates 0.4, 0.6 and 0.5 (plus dosing) kg P/m$^3$.d for MNE-Bp. $PO_4$-P a), Mg b) and $PO_4$-P removal versus influent $PO_4$-P and $R^2$ statistics on trendline c)**

the reliance on the mass transfer of ions through the MNE media to the cell wall of *B. antiquum* where biomineralisation occurs[50]. If the concentration of ions at the cell wall are not high enough biomineralisation via induction will not occur.

#### 5.3.3.3 Overall pilot-plant performance with MNE

The results of the MNE-Ba and MNE-Bp pilot-plant studies, have demonstrated that there was a limiting factor or factors due to the influent for the biological processes of *B. antiquum* and *B. pumilus*, i.e., $PO_4$-P removal was reduced from the 44-70% observed in batch trials[24] to between 20- 30% removal in the pilot-plant studies without external dosing. Because of the low BOD and Mg in the SDL (Table 5-2), these compounds were hypothesised as limiting factors. This was then verified with the reactors were operated with dosing to achieve a BOD increase between 100 mg/L and 200 mg/L and P:Mg of at least 1:1. This dosing improved the P removal of both MNE-Ba and MNE-Bp to 98% and 61% on average, respectively. The addition of an external carbon source is not uncommon in WWTPs, and it is current practice in biological nutrient removal (BNR) treating weak wastewater. The use of fermented surplus activated sludge (AS) as an additional carbon source that can be utilised by WWTP without the incurred costs of chemicals and transport[51]. Furthermore, studies in BNR have shown that the addition of surplus activated sludge improved the release of P in BNR processes by 89% compared to those dosed by acetate and by 2.5 fold, compared to no carbon addition[51]. In laboratory experiments carbon sources in the form of bovine serum albumin and acetate have been required for the growth of *B. antiquum* and *B. pumilus* in pure and open culture SDL[36,47]. The use of internal carbon sources like surplus activated sludge, in WWTP should be tested with MNE-Ba and MNE-Bp to test its effectiveness as a carbon source, saving expenses in external sources and transportation. Furthermore, in WWTP, incorporating smart monitoring systems and modelling will provide real-time data to adjust flow rates to ensure nutrient loading

is optimised for the biomineralisation process which will improve efficiency of treatment techniques when nutrient loading fluctuates[34,35].

The chemical recovery of struvite was not feasible under the initial conditions of the SDL, the $PO_4$-P was significantly <100 mg/L (Table 5-2) and the P:Mg was 1:0.34, also much lower than the required ratio of 1:2 for required in chemical recovery of struvite and a P-removal >80%[38,52]. Commonly used chemical struvite recovery technologies have removal and recovery efficiencies between 80% and 90%[53], this typically leaves a treated effluent that requires secondary treatment as final concentrations of $PO_4$-P can be as high as 30 mg/L[53,54]. The treated effluent from MNE-Ba had a concentration <3 mg $PO_4$-P/L when BOD was 100-200 mg/L and Mg was also present, this is a permissible for UK discharge limits, meaning no secondary treatment for P-removal would be required. The treated effluent still needs to be treated for ammonia, this could be achieved via a range of processes from anammox, to standard nitrification/denitrification, ammonia stripping and ion exchange process to recover $NH_4$-N[55,56].

### 5.3.4 Mineral recovery, morphology, and mineralogy

Precipitates were successfully removed using gravity settling from Tank B, draining and passive filtration. Significant amounts of precipitates were observed scaling the walls of both Tank A and B, the connection and around the sinter. The precipitate recovered were there for a low estimate of the true recovery yield and could be improved with optimisation of the reactor design/materials[57].

#### 5.3.4.1 MNE-Ba, biologically controlled mineralisation

Settled precipitates were observed within five days of the pilot-plant start-up for each loading rate without dosing. With a loading rate of 0.4 kg P/$m^3$.d the precipitate recovered was 23 mg/L

of SDL treated. By increasing the loading rate to 0.6 kg/$m^3$.d, increased the yield to 44 mg/L, twice the mass of precipitate was recovered by intensifying the loading rate by 1.5 times. The recovered precipitate yield, when there was limited BOD and $Mg^+$, was below yields reported in batch experiments in autoclaved SDL, which have recovered between 44 mg/L and 98 mg/L[15,24].

The morphology of precipitates recovered was bimodal, the primary morphology was tabular and prismatic crystals, between 50 µm and 200 µm in length, for all loading rates tested. The second, less abundant morphology was amorphous, <50 µm in length and was typically observed as crystal masses incorporating or coating the primary morphology (Figure 5-5). The analysis under electron dispersive spectroscopy revealed a clear relationship between $Mg^+$ and P with the primary morphology and Ca and P with the secondary morphology described (Figure 5-5). This finding was consistent with analyses from previous studies into the biomineralisation of struvite which indicates well-formed (euhedral) minerals from *B. antiquum*[14,15,24,49] and is conclusive evidence in support of biomineral recovery remaining viable when encapsulated in the MNE media.

The spectroscopy results provide an estimate for the relative weight percentage of the measured elements (Figure 5-5). Using Equation 5-1 the proportion of bio-struvite within precipitate recovered was 61%, at a loading rate of 0.4 kg P/$m^3$.d, increasing to 71% bio-struvite at a loading rate of 0.6 kg P/$m^3$.d. This suggests intensifying the process further would improve the purity of bio-struvite. It is possible this was occurring because BCM in this open system is facilitating faster precipitation of bio-struvite against the abiotic precipitation of calcium phosphate, as the active removal of ions by *B. antiquum* reduces the availability of ions for calcium phosphate precipitation. The proportion of $PO_4$-P recovered in minerals form the total $PO_4$-P removal was 33% at a loading rate of 0.4 kg P/$m^3$.d and increased to 84% at the loading

rate 0.6 kg P/$m^3$.d. This would suggest the intensified loading rate has reduced the availability of $PO_4$-P for native microbes and to allow more removal directly by MNE-Ba. Pearson's statistical testing of the loading rates and ratio of Mg:Ca in precipitates had a $r^2$-value of 0.06, at a 10% confidence level this would strongly suggest that the loading rate of P has a significant impact on the proportion of magnesium recovered compared to calcium. This can be related to proportion of bio-struvite recovered compared to abiotic calcium phosphate in the precipitate assemblage for BCM.

The mineral assemblage when the pilot was dosed by carbon and magnesium showed no significant change in the morphology of minerals recovered, SEM-EDS revealed clear tabular and prismatic minerals rich in P and Mg (Figure 5-5). This strongly suggests that the minerals recovered from the MNE-Ba pilot during dosing were bio-struvite. The proportion of the recovered assemblage was 60%, a reduction to the proportion of bio-struvite recovered at a loading rate of 0.6 kg P/$m^3$.d.

#### 5.3.4.2 MNE-Bp, biologically induced mineralisation

*Bacillus pumilus* encapsulated in the MNE media had a precipitate yield of 19 mg/L at a loading rate of 0.4 kg P/$m^3$.d, this increased to 30 mg/L when the loading rate was 0.6 kg P/$m^3$.d. This increase was directly proportional to the magnitude of the increase in loading rate. This was a reduced yield compared to MNE-Ba.

The precipitates recovered from un-dosed influents to MNE-Bp had the same morphological and elemental assemblage as MNE-Bp (Figure 5-5) typically bio-struvite minerals were less euhedral there was a lack of clear crystal faces and orthorhombic indices. This suggests pressure during the growth phase resulting in a weaker packing structure.

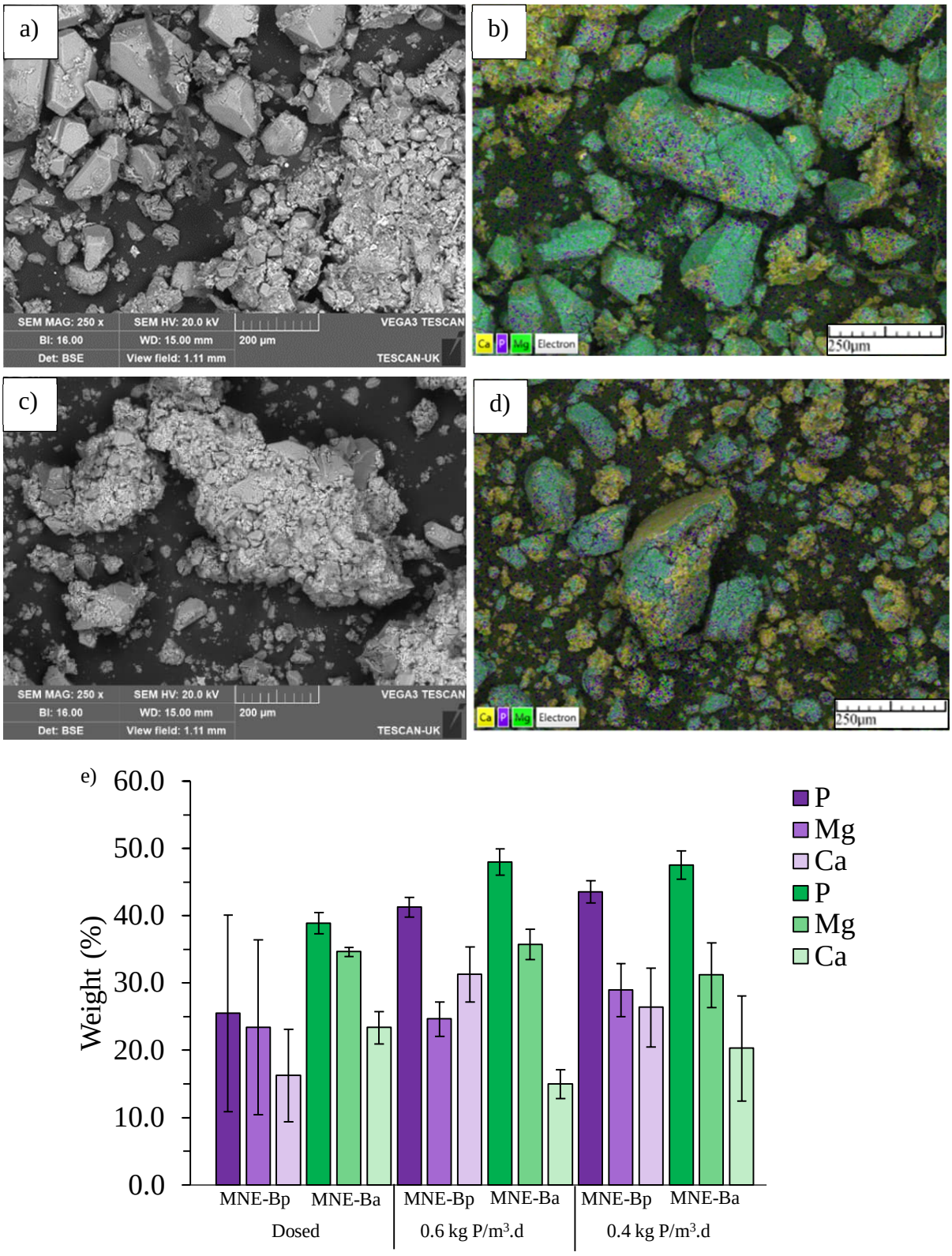



**Figure 5-5 Recovered precipitate morphology imaging and element mapping, MNE-Ba precipitates electron image a), MNE-Ba elemental mapping b), MNE-Bp precipitates electron image c), MNE-Ba elemental mapping d), Relative weight % of major ions present in precipitate assemblage e)**

Using the spectroscopy results of MNE-Bp assemblages the proportion of bio-struvite was less at both loading rates compared to MNE-Ba, 53% at 0.4 kg P/$m^3$.d and 45% at 0.6 kg P/$m^3$.d. Intensifying the treatment process appears to have reduced the proportion of bio-struvite in the mineral assemblage with MNE-Bp. This suggests that biologically induced mechanisms was influenced more by external factors compared to biologically controlled mineralisation, despite increasing the input of ions and irrespective of the encapsulation by the MNE media. The proportion of removed $PO_4$-P recovered as minerals from MNE-Bp 46% and 68% at loading rates 0.4 kg P/$m^3$.d and 0.6 kg P/$m^3$.d, at the lower loading rate a greater proportion of the removed $PO_4$-P was recovered compared to MNE-Ba. Using Pearson's statistical testing of loading rate compared to the ratio of Mg:Ca returned a $r^2$-value of 0.28, this rejects the hypothesis that the loading rate of P was significant for biologically induced mineralisation of struvite. This indicates that the induced mechanism was more susceptible to external factors outside the saturation of the desired ions for bio-struvite mineralisation.

Like MNE-Ba, MNE-Bp mineral assemblages when dosed by additional carbon and Mg showed clear prismatic and tabular minerals with a high affinity for P and Mg as seen in Figure 5-5. However, variance across the mineral assemblage was observed in SEM-EDS analysis with mineral assemblages having as little as 10% P up to 40% P, this was significantly more than MNE-Ba and loading rates not receiving dosing in the MNE-Bp pilot (Figure 5-5).

The mineralogy and morphology of the mineral assemblage was consistent with previous studies of the biomineralisation process to recover $PO_4$-P in synthetic media and open culture wastewater[14,49]. The mineral assemblage was also consistent with those recovered in batch trials using the MNE media[24].

#### 5.3.4.3 Bio-struvite purity and contaminants

The results from chemical analysis of recovered minerals are summarised in Table 5-4. There was minimal difference in the purity in terms of $P_2O_5$, MgO and Ca between minerals recovered from MNE-Ba and MNE-Bp. There was up to a 5% reduction in the purity of P from the recovered minerals when influent dosing has occurred and the proportion of calcium in recovered biominerals increased by as much as 15% when dosing occurs (Table 5-4). It appears dosing has led to an increase in abiotic mineralisation of calcium as well as the biomineralisation of struvite, calcium is a secondary nutrient needed for the formation of plant tissues and is vital for maintaining soil structure through the solidification of finer particles and adsorption of heavy metals[58,59].

The presence of heavy metals in recovered bio-struvite and scaling from WWTP was minimal, 50% of the samples were below the limit of detection when 1.5 g of sample was analysed. Zinc and arsenic were measured in all minerals recovered from MNE pilot trials, measuring between 0.15 and 0.6 mg/kg minerals and 5.86 and 61 mg/kg minerals (Table 5-4). When compared to published standards for heavy metal limits in fertilisers, all recovered minerals and scaling measured well below the range of limits across several EU and non-EU nations (Table 5-4). There was no detection of *E. coli, Legionella,* and faecal coliforms in any of the mineral assemblages recovered from MNE-Ba and MNE-Bp pilots, nor scaling recovered from WWTP. The lack of microbes of pathogenic material in the recovered minerals is consistent with other studies into their presence in recovered minerals[60].

QTOF analysis returned the mass-to-charge ratio of 50 compounds within bio-struvite samples collected throughout the pilot study. None of these compounds had the same mass-to-charge of the compounds f concern listed in Appendix B, Table B2.

It has been shown that when influents were spiked with pathogenic microbes and eggs they did remain active in struvite recovered from human urine, inactivity was exponentially increased through removal of moisture and air temperature[60]. However, based on the methods of recovering the minerals from this pilot-plant study the presence of the pathogenic material has not been detected.

**Table 5-4 Purity and heavy metal concentrations of recovered minerals from pilots and WWTP scaling**

| | **P loading rate ($kg/m^3.d$)** | | **0.4** | | **0.6** | | **0.5*** | | **Regs. ▲** |
|---|---|---|---|---|---|---|---|---|---|
| | **Analyte** | **Scaling** | **MNE-Bp** | **MNE-Ba** | **MNE-Bp** | **MNE-Ba** | **MNE-Bp** | **MNE-Ba** | |
| Purity (%) | Calcium | 39 | 17 | 11 | 18 | 13 | 22 | 28 | N |
| | Phosphorus as $P_2O_5$ | 19 | 27 | 27 | 26 | 28 | 24 | 22 | > 12% |
| | Magnesium as MgO | 11 | 13 | 15 | 14 | 14 | 14 | 13 | N |
| | Total organic carbon | 1 | 2 | 2 | 2 | 2 | 1 | 1 | N |
| Heavy metals (mg/kg) | Aluminium | 31.9 | 22.0 | 49.8 | 6.64 | 7.83 | - | - | N |
| | Arsenic | - | 0.43 | 0.15 | 0.60 | 0.48 | 0.18 | 0.18 | 40-60 |
| | Cadmium | 0.02 | 0.02 | 0.01 | - | - | - | - | 3-50 |
| | Chromium | - | - | 0.15 | - | - | - | - | 2-670 |
| | Copper | - | - | 4.91 | - | - | - | - | 400-780 |
| | Iron | 313 | 220 | 260 | 96.5 | 120 | - | - | N |
| | Lead | 0.48 | - | 0.30 | - | - | - | - | 100-200 |
| | Mercury | 0.07 | 0.07 | 0.09 | - | 0.01 | - | - | 1-5 |
| | Nickel | 0.72 | 0.92 | 1.12 | - | - | - | - | 50-120 |
| | Zinc | 16.3 | 31.2 | 61.0 | 9.65 | 12.10 | 5.86 | 12.60 | 1100-3300 |

* = Dosed
Regs.▲ = Fertiliser regulations for non-EU countries and EU countries[61–63]
- = Below limit of detection
N = No standards defined

The minerals recovered by *B. antiquum* and *B. pumilus* encapsulated in MNE media were consistent with past studies under laboratory conditions both in morphology and mineralogy[36,47]. They also remain below heavy metal concentration standards set out by the EU and other nations[62] and have no compounds present of potentially hazardous micropollutants.

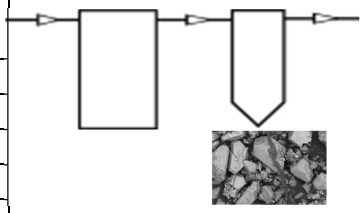
#### 5.3.4.4 Mass balance and longevity

A mass balance of each of the three operational conditions has shown that when the nutrient load was increased 1.5-fold, MNE-Ba removed double the $PO_4$-P as struvite, increasing from 0.05-0.12 kg P/$m^3$.d (Figure 5-6). This was also observed in the load of Mg removed in precipitates increasing from 0.02-0.07 kg Mg/$m^3$.d. In MNE-Bp, the magnitude of Ca removed increased from 0.03-0.1 kg Ca/$m^3$.d. (Figure 5-6). This suggests the difference of biomineralisation mechanism of *B. antiquum* and *B. pumilus* is a control on the magnitude of nutrient removal as bio-struvite. Mass balances coupled with the results discussed previously, has shown that BCM was more robust compared to BIM, actively removing $PO_4^{2-}$, $Mg^+$ and $NH_4^+$ has reduced the competition from other ions so bio-struvite can be biomineralised unhindered which can be seen in the mass and purity of minerals recovered and mass balance in Figures 5 and 6 and Table 5-4.

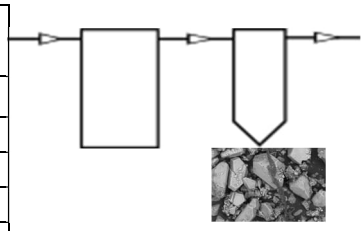
Dosing the system with Mg and carbon has led to an increase in the $PO_4$-P load removed through mineralisation in both MNE-Ba and MNE-Bp (Figure 5-6). The load of $PO_4$-P removed as minerals was 0.51 kg P/$m^3$.d and 0.36 kg P/$m^3$.d for MNE-Ba and MNE-Bp, respectively. Magnesium and Ca removed through mineralisation also increased 0.38 kg Mg/$m^3$.d and 0.37 kg Ca/$m^3$.d by MNE-Ba respectively, and 0.12 kg Mg/$m^3$.d and 0.27 kg Ca/$m^3$.d removed by MNE-Bp, respectively (Figure 5-6). Treated effluent still remain rich in $NH_4$, coupled with the low sCOD concentrations, biological treatment with denitrification would not be favourable. A possible secondary treatment for $NH_4$ is the use of zeolites which can recover $NH_4$ through chemically refreshing the zeolite[55,56]

There were no observations of MNE deterioration throughout the pilot study. Furthermore, continued observation and recovery of bio-struvite throughout the pilot study suggest that the

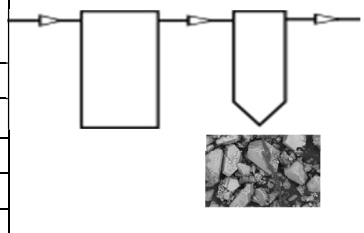
porosity of the MNE media was not reduced with prolonged exposure to SDL. Previous studies of other encapsulation methods have noted deterioration of alginate and hydrogel immobilisation technologies[24,64,65].

| **Influent 0.4 kg P/m$^3$.d** | | |  | **Effluent** | | | |
|---|---|---|---|---|---|---|---|
| | | | | MNE-Ba | MNE-Bp | MNE-Ba | MNE-Bp |
| | (mg/L) | (g/d) | | (mg/L) | (mg/L) | (g/d) | (g/d) |
| $PO_4$-P | 62 | 3.01 | | 40 | 42 | 0.3 | 0.3 |
| Mg | 22 | 1.12 | | 15 | 16 | 0.1 | 0.1 |
| Ca | 64 | 3.15 | | 63 | 59 | 0.4 | 0.4 |
| sCOD | 604 | 29.5 | | 91 | 82 | 0.6 | 0.6 |
| $NH_4$-N | 985 | 48.2 | Precipitate | 904 | 870 | 6.3 | 6.1 |

| | MNE-Ba | MNE-Bp |
|---|---|---|
| | (g/d) | (g/d) |
| P | 0.05 | 0.04 |
| Mg | 0.02 | 0.01 |
| Ca | 0.04 | 0.03 |

| **Influent 0.6 kg P/m$^3$.d** | | |  | **Effluent** | | | |
|---|---|---|---|---|---|---|---|
| | | | | MNE-Ba | MNE-Bp | MNE-Ba | MNE-Bp |
| | (mg/L) | (g/d) | | (mg/L) | (mg/L) | (g/d) | (g/d) |
| $PO_4$-P | 64 | 6.2 | | 48 | 48 | 0.5 | 0.5 |
| Mg | 16 | 1.9 | | 12 | 13 | 0.1 | 0.1 |
| Ca | 63 | 6.2 | | 63 | 63 | 0.6 | 0.6 |
| sCOD | 618 | 61.8 | | 86 | 92 | 0.9 | 0.9 |
| $NH_4$-N | 1275 | 128 | Precipitate | 1189 | 1211 | 11.9 | 12.1 |

| | MNE-Ba | MNE-Bp |
|---|---|---|
| | (g/d) | (g/d) |
| P | 0.12 | 0.11 |
| Mg | 0.07 | 0.02 |
| Ca | 0.08 | 0.1 |

| **Influent 0.5 kg P/m$^3$.d Dosed** | | |  | **Effluent** | | | |
|---|---|---|---|---|---|---|---|
| | | | | MNE-Ba | MNE-Bp | MNE-Ba | MNE-Bp |
| | (mg/L) | (g/d) | | (mg/L) | (mg/L) | (g/d) | (g/d) |
| $PO_4$-P | 51 | 5.1 | | 2 | 15 | 0.02 | 0.2 |
| Mg | 74 | 7.1 | | 11 | 20 | 0.1 | 0.2 |
| Ca | 34 | 3.4 | | 20 | 19 | 0.2 | 0.2 |
| sCOD | 796 | 79.6 | | 80 | 80 | 0.8 | 0.8 |
| $NH_4$-N | 1235 | 1235 | Precipitate | 1139 | 1101 | 11.4 | 11.0 |

| | MNE-Ba | MNE-Bp |
|---|---|---|
| | (g/d) | (g/d) |
| P | 5.1 | 3.6 |
| Mg | 3.8 | 1.2 |
| Ca | 3.7 | 2.7 |

**Figure 5-6 Pilot scheme mass balance of major precipitate ions at the operational conditions tested**

## 5.4 Conclusions

To conclude, enrichment of *B. antiquum* and *B. pumilus* in SBRs, using acetate and salt as selective pressure parameters with SDL as feed, was not successful, as the desired suspended microorganisms were washed-out and outcompeted withing a few days of operation. In the pilot-plant study with encapsulated microorganisms, MNE-Ba and MNE-Bp, the removal of nutrients and recovery of bio-struvite was observed at high P loading rates in open culture reactor fed with SDL. Nevertheless, the BOD was <40 mg/L limiting the reactor performance and P removals. Dosing the SDL to obtain a BOD 100-200 mg/L with serum albumin and adding magnesium resulted in a successful system obtaining a treated effluent with less than 3 mg $PO_4$-P/L consistently. Differences between MNE-Ba and MNE-Bp, clearly demonstrated that the type of biomineralisation mechanism becomes more pivotal as the loading rate was increased or when the availability of carbon and magnesium was increased with dosing, BCM used in MNE-Ba was able keep pace with the increased load of nutrients, producing a purer bio-struvite assemblage as it was able to out compete the abiotic mineralisation of calcium phosphate. The BIM mechanism in MNE-Bp was not as effective when the loading rate was increased or when the pilot was dosed with additional carbona and Mg, suggesting that the competing ions were reducing the efficiency of the BIM mechanism.

The results from this pilot-plant study have clearly demonstrated that encapsulation of microorganisms were able to proliferate in an open culture system fed with real wastewater and the same time recover minerals through biomineralisation, without the need to reseed. Importantly, the ratio of P:Mg remained below 1:2 and the influent concentration was never above 100 mg $PO_4$-P/L, successfully showing that the biomineralisation can recover bio-struvite in conditions not suitable for chemical recovery.

Next steps need to include optimising the nutrient loading on the system to maximise recovery, encapsulation media filling ratio, bio-struvite recovery and maintain low $PO_4$-P in treated effluents. Exploring the potential dosing reagents that are readily available to WWTP, such as fermented sludge and seawater, is also of interest. Nevertheless, the most interesting outcome of this study is the potential use of encapsulation to other desirable microorganism enabling full control of biological pathways in wastewater open systems, leading to turn-on/turn off plug and play intensified synthetic biology driven treatment processes by design.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Access Statement

Data shown in this review paper can be found through the resources in the following References list.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# 6. Public assessment and perception for wastewater-derived fertiliser use in agriculture and horticulture

R.E. Colston[a], D. Goodwin[a], H. Smith[a] A. Nair[b], P. Vale[c], T. Stephenson[a] & A. Soares[a]*

[a] Cranfield Water Science Institute, Cranfield University, College Road, Cranfield, MK43 0AL, UK

[b] Microvi Biotech, 26229 Eden Landing Rd, Hayward, CA 94545, United States

[c] Severn Trent Plc. Severn Trent Centre, 2 St John's Street, Coventry, CV1 2LZ

* Corresponding author: a.soares@cranfield.ac.uk

**Abstract**

The awareness of depleted nutrient sources has grown, leading to research into the recovery of nutrients to produce fertilisers for the growth of crops, closing the nutrient loop and ensuring food security. There are a wide number of recycling derived fertiliser (RDF) technologies available to recover nutrients and substitute into current agricultural and horticultural practices. This study builds on previous studies into the perceptions of RDFs and willingness to use or eat the produce grown from them following public surveys. The most common fertiliser type used was compound fertilisers at 44%. Plant types receiving the most fertiliser was 'flowering plants', 'fruit and vegetables' and 'house plants' accounting for 34% of fertiliser use. Up to 30% of respondents spent over £50/year on fertilisers for their horticultural needs. Willingness to use RDFs and eat produce grown from RDFs was high at 80% of the surveyed population. When given characteristics of three RDF products farmers would choose a bio-based mineral fertiliser (like struvite produced through bio-mineral process) over chemical fertilisers available. The results of this study clearly demonstrate that there is a willingness amongst the public and farmers to use RDFs despite concerns for possible pollutants there is confidence that RDFs will be a successful substitute. The study highlights that the public and farmers are highly receptive to use recovered fertilisers, a practice that can decrease the demand for nutrient imports and decrease supply security issues. The need for collaboration between producers of RDFs, suppliers, advisors, and governing bodies to incorporate recovered nutrients into the fertiliser market is evidenced.

**Keywords:** *Recycling derived fertilisers, public perceptions, circular wastewater treatment*

## 6.1 Introduction

The development of agriculture is key to ceasing global food shortages and will be pivotal in achieving the United Nation's Sustainable Development Goals (SDGs). The global population is estimated to have surpassed 8 billion at the time of writing, and is set to reach 10.4 billion by 2050[1], this makes the sustainable development of agricultural practices all the more important. In addition to this, agriculture is crucial for economic growth and employment across the globe. There are many factors influencing the sustainable development of agriculture, the availability and feasibility of fertilisers is becoming more necessary as farming intensifies to meet the demands of growing populations and globalisation. The scarcity of raw resources of key nutrients for fertilisers, such as phosphorus (P), potassium (K) and magnesium (Mg), the growing cost of their extraction and geo-political issues is putting pressure on the supply of synthetic mineral fertilisers and increasing their price[2–4]. The agriculture industry must diversify its sources of its nutrient supply and find alternatives to synthetic mineral fertilisers so that it becomes resilient to these factors, a solution for this is implementing a circular economy.

The transition to a circular economy is accelerating the development and roll out of resource recovery techniques, the most commonly used and simple recovery of nutrients for fertilisers are from spreading raw-biomass such as animal manure, sewage sludge and food waste[5]. However, scepticism of the efficacy and concerns for the accumulation of heavy metals and other micropollutants through the application of raw biomass has resulted in poor uptake across many EU nations[6]. The development of recovery technologies that process biomass and/or wastewater to produce recycling-derived fertilisers (RDFs)[7] has been shown to provide more bio-available nutrients whilst also limiting nutrient leakage which causes eutrophication[8–10]. Thus, increasing the agricultural value of these recovered products[11,12]. The loss of P within Europe and the UK include municipal wastewater discharge and landfill, estimated to be

221,000 ton of P and 13,600 ton P respectively[13,14]. By incorporating P recovery techniques such as struvite recovery into wastewater treatment plants (WWTP), these losses can be limited and meet synthetic mineral fertiliser demands or offset their need.

The drive and development of nutrient recovery technologies is no longer the limiting factor for incorporating them into waste/wastewater treatment streams, legislature, awareness and acceptance by end-users of RDFs are holding the technologies back[15]. Until recently the EU fertiliser regulation (2003/2003) would not accept secondary raw materials into the production of fertilisers, SAFEMANURE was a study led by the European Commission's Joint Research Centre put forth criteria for the safe use of manure-derived RDFs. This has helped push for revisions, leading to the EU Fertilising Products Regulation (EU 2019/1009) which accepts the use of several secondary sources for fertiliser production including, sewage sludge and dairy effluents provided that they follow the same regulations and limits as synthetic mineral fertilisers[16,17].

To develop a market for RDFs it is imperative that the current fertiliser uses, their requirements and concerns for RDFs are understood so that knowledge can be improved and the willingness to transition to RDFs can idetified[7,18,19]. A few surveys have been designed to assess the willingness of end-users to use RDFs and food consumers on their willingness to use and eat food grown from RDFs as a whole and specific RDF products[7,20,21]. These surveys have focussed on agricultural users and advisors across several European Nations, to surmise end-users' concerns were for nutrient content, organic matter, ease of use and product cost. In addition to this the interest in using RDFs was much greater than the current use between 33% and 66% interest versus 9% and 19% current use of processed manure and urban waste-derived fertiliser[7,22]. In addition to willingness of end-users, surveying food consumers is key for the development of RDFs into the food cycle. A survey focussing on the use of urine-derived

fertilisers showed clear cultural differences in student populations from a variety if countries in their willingness to consume food grown with those fertilisers[20] which need to be overcome if RDFs want to be implemented globally.

Many surveys typically limit end-users to the farming industry and survey the public as food consumers only[7,20,23,24]. There are surveys assessing the growth of private gardening by the public focussing reasons for taking part in the activity, impact on water use and the development of private fruit and vegetable production in urban areas[25–27]. These surveys have clearly show that the uptake of private gardening is on an upward trend, with motivations for gardening including a form of exercise, improving bio-diversity, and produce cheaper food alternatives to supermarkets[25–27]. One study found that gardening role models, typically within the family were the most common reasons for starting gardening, in cases where there was no role model new food cultures influenced their decision to start gardening[26]. The opportunity to diversify markets for RDFs into horticulture will potentially have great uptake as it supports organic gardening and supports sustainable development. In the UK private gardening by the public is less understood and therefor the willingness of them to use RDFs is not well-known. The aim of this study was to improve the understanding of how the UK public use fertilisers, current perceptions of the use of raw biomass and RDFs, willingness to use RDFs and consume food grown using RDFs. Additionally, farmers and advisors were invited to complete a questionnaire to establish the variables considered when choosing and applying fertilisers and their willingness to use RDFs.

## 6.2 Materials and methods

### 6.2.1 Survey designs

The public survey was designed to improve the understanding of the public use of fertilisers, what they grow and their perceptions of RDFs and their willingness to switch to them (Table

6-1). The survey was open to members of the public who garden and those who do not, if the respondent answered they do not grow plants the survey skipped forward to the perception of RDFs (Table 6-1). The survey covered topics including general demographics, the characteristics of gardening types, present day fertiliser uses, perceptions ad concerns for RDFs. The perception responses in the public survey and farmers questionnaire were categorised to a Likert scale[28]. All responses were collected anonymously as per the ethical approval of this research.

**Table 6-1 Topic and question criteria for public survey and farmer questionnaire**

| Topic | Question range | Question criteria | Response types |
|---|---|---|---|
| **Public survey** | | | |
| **Respondent Demographics** | 1-3 | Establish age, gender and where in the UK respondents are from | Single choice of bucketed values and geographical area |
| **Types of hobby-gardening/horticulture** | 4-8 | Understand the variety of publicly used spaces for horticulture, their sizes, and reasons for growing plants | Single and multiple-choice answers of predefined options |
| **Present day fertiliser use** | 9-19 | Understand what plant types are commonly grown using fertiliser, the type of fertiliser used (inorganic or organic), their form, quantity used, and money spent per year. | Single, multiple-choice, and open-ended questions using ranges of criteria set out by the Royal Horticultural Society for plant and fertiliser types. |
| **Perceptions** | 20-32 | Identify public confidence in where the fertilisers they use come from, the considerations made when choosing a fertiliser, concerns, and willingness to change to RDFs and the likelihood of paying for organic bio-based RDFs | Single and multiple-choice questions using a Likert scale |
| **Farming questionnaire** | | | |
| **Respondent Demographics** | 1-3 | Establish age, gender, and farming experience | Single choice of ranges and areas |
| **Present day farming practices** | 4-16 | Understand the range of farm sizes, the fertiliser(s) they use, the quantity they spread per year and the quantity of NPKMg they spread and in what form | Single and multiple-choice answers of predefined options, open-ended numerical answers |
| **Desired fertiliser qualities** | 17-23 | Explore the fertiliser qualities and attributes farmers find desirable and awareness of raw fertiliser resources | Single and multiple-choice answers using a Likert scale |
| **Sustainability and willingness to change** | 24-28 | Understand the perceptions farmers have towards sustainable development and incorporation of circular economies into farming. Assess the willingness to change to RDFs when key attributes of 3 RDF products are known. | Single choice questions, ranking of defined variables and 'blind' choice test of RDF products |

The farmer questionnaire followed the same topics as the public survey, with changes made to gather information on farm size, produce grown and desirable nutrient ratios for fertilisers (Table 6-1).

### 6.2.2 Survey distribution

The survey was created using Qualtrics XM. A sharable link was used to distribute the survey amongst staff and academics at Cranfield University, who were encouraged to share with their networks. The survey was also distributed through open, social media platforms and through regional farming and gardening groups. Both surveys were open for responses from January 2022 to July 2022. Farmers were approached through from biosolids focus groups organised by UK water utilities within the East Midlands and through social media to complete the farming questionnaire.

### 6.2.3 Data analysis

The responses were first analysed on Microsoft Excel, where key demographic data was compared and analysed. Google Earth was used to display geographical data of responses. Additionally, was analysed using Qualtrics XM data analysis tabs and cross-tabulation and SPSS version 26 (IBM Statistics). The data collected was analysed using cross tabulation to compare key variables and Pearson significance testing to determine the relationship of these variables to one another.

## 6.3 Results and discussion

### 6.3.1 Public survey

#### *6.3.1.1 Survey demographics and current gardening practices*

A total of 111 responses were recorded, with responses distributed across the whole of the United Kingdom (Figure 6-1). The dominant response locality was in the Southeast of England with 26% of the total response, this is due to some bias in the survey distribution within the

faculty of Cranfield University. However, through social media and networking the survey reached the whole UK (Figure 6-1). Responses from the Greater London and West Midlands regions were below the true proportion of the UK's population (Figure 6-1), these are some of the most built-up areas within the UK and this may have been a factor on the number of responses received by those areas. Whilst the response numbers were limited due to time limitations of this study and cannot be taken as a national representation, the results have been analysed discussed to provide an insight into the gardening practices and perceptions of the public and farmers and will provide a baseline for future national studies.

There was a 56% to 44% split in male to female respondents and all ages brackets had responses. The distribution in the age of respondents was 12%, 33%, 13%, 22% and 20%, for 18 to 25, 26 to 35, 36 to 45, 46 to 65 and 65+ years old, respectively. Within the survey population there was large range in spaces for gardening, 29% of respondents used a garden space to grow their chosen plant type (Figure 6-2), with 38% using a space < 100$m^2$, 28% between 100 $m^2$ and 250$m^2$ and 34% > 250$m^2$. Gardens were closely followed by potted plants and flower beds as the more common spaces to grow plants, 27% and 22%, respectively. The plant types grown using fertilisers varied, fruit and vegetables were the most common selection at 18% although this criterion does overlap with other plant types such as trees and climbers when considering fruit (Figure 6-2). The growth of house plants was second-most popular at 14%, less common plant types shown as 'other' in Figure 6-2 include cacti and succulents, heathers, and ferns, accounting for 10% of the responses. Respondents were asked for their motivations to grow plants, when given the choice there was a near even distribution between 'To eat', 'To encourage wildlife', 'To socialise/mental health' and 'Wider environmental benefits'. The motivations for gardening by the respondents are similar to those of previous studies and aligns with their conclusions that the growth of private gardening by the public will be through education and influences on changing food practices[26,27].

| Region | Percent | Difference to true UK population |
|---|---|---|
| **Scotland** | 14 | 6 |
| **Northern Ireland** | 1 | -2 |
| **Northeast** | 7 | 3 |
| **Northwest** | 13 | 2 |
| **Yorkshire and The Humber** | 7 | -1 |
| **West Midlands** | 3 | -6 |
| **East Midlands** | 9 | 2 |
| **Wales** | 2 | -3 |
| **East England** | 15 | 6 |
| **Greater London** | 5 | -8 |
| **Southeast** | 26 | 12 |
| **Southwest** | 6 | -2 |
| **Total count** | **111** | |


**Figure 6-1 Public survey distribution and comparison to UK population proportions to regions**

This survey covers a board demographic within the UK, geographically and socially. In comparison past surveys have focussed on key end users i.e., farmers-only, at various scales from large commercial farms to rural subsistence farmers[7,15,21,23], or specific social groups such as, students and rural communities[20,24]. Whilst these surveys cover a larger survey population from 300 up to 4000 respondents, they focus on specific groups. This survey does not reach the same survey population size but can provide a more holistic overview of the attitudes towards the UK public and farming population to provide clear context for RDFs use within horticulture and agriculture.

#### *6.3.1.2 Present-day fertiliser use*

Within the population of respondents 'potted plants' and the 'garden' were the main spaces for gardening, at 27% and 29%, respectively (Figure 6-2). 'Flower beds' made up 22% of

gardening spaces, with greenhouses and allotments making up the remainder of spaces at 13% and 9%, respectively (Figure 6-2). When cross-tabulated with the age of respondents 45% of all gardeners using greenhouses/polytunnels were over the age of 65, compared to 6% of users between 18- and 25- years old. This was true for 'gardens' and 'allotments', where older respondents made up the higher proportion of users. However, 'potted plants' were utilised by a higher proportion of 26- to 35-year-olds compared to those > 65 years old, at 34% of the users compared to 12% > 65 years old. This is potentially due to socio-economic circumstances, where the older population has the resources and time to commit to tending greenhouses and allotments, and typical migration of the youthful populations into cities which reduces the space for horticulture, hence the dominance of 'potted plants' as used spaces by 26- to 35-year olds[29]. It must be noted that the living space i.e., urban versus rural will play a significant role in what spaces are available for the public to garden in and should be accounted for in larger studies.

There was a large variety of plants grown by the respondents, to simplify analysis and make characterising the data easier some plant types were bucketed from the RHS' original list. The dominant plant types receiving fertilisers by the public were 'flowering plants' (bulbs, annuals, and perennials, but not fruit or vegetables) at 27%, 'fruit and vegetables' at 18% and 'woody plants' (trees and shrubs) at 17% (Figure 6-2). Respondents were asked to choose the types of fertilisers they use as defined by the RHS, 41% of total responses stipulated the use of compound fertilisers, a fertiliser which contains 2 to 3 key nutrients in typically NPK. The use of slow-release fertilisers accounted for 32% of responses, followed by respondents not being sure (12%), controlled-release (11%) and straight fertilisers (5%) (Figure 6-2). The use of compound and slow-release appears to be the dominant fertiliser types chosen by the public, this is beneficial for mineral RDFs such as struvite which is a compound and slow-release fertiliser. This is a promising indicator that the substitution mineral RDFs into public use would

be easy due to the similarities in form making using them easier. In addition, the plant types receiving the most compound and slow-release fertiliser are the most popular to grow amongst the surveyed population, fruit and vegetables and flowering plants (excluding fruit and vegetables) receive 35% of all fertilisers spread (Figure 6-2).

To understand the fertiliser habits of the survey population more they were asked to select a bucketed value for the mass of fertiliser they use and how much they spend on them per year (Figure 6-3). The most abundant mass of fertiliser used was <5 kg/year (but not 0) at 55%. This was followed by > 20 kg fertiliser used per year at 18%. When the plant types were cross tabulated with mass of fertiliser use, ‘fruit and vegetables’ and ‘flowering plants’ were most common to receive > 20 kg of fertiliser/year (Figure 6-3). ‘Fruit and vegetables’ and ‘house plants’ were the most common plant types to receive <5 kg of fertiliser per year also (Figure 6-3).

When asked how much they spent on fertilisers per year, 30% of respondents spent over £50. The next bracket of expenditure on fertilisers per year was £11 to £20 and £5 to £10, at 24% and 18% respectively (Figure 6-3). Figure 6-3 b) shows the breakdown in plants which respondents will spend more money on, ‘flowering plants’ and ‘woody plants’ are the most associated plant types for which the surveyed population will spend > £50 on for fertiliser use all accounting for 7% and 9% of the total responses respectively. Whilst ‘fruit and vegetables’ are amongst the most popular plant to be grown (Figure 6-2), respondents would spend typically between £5 and £20 (Figure 6-3 b).

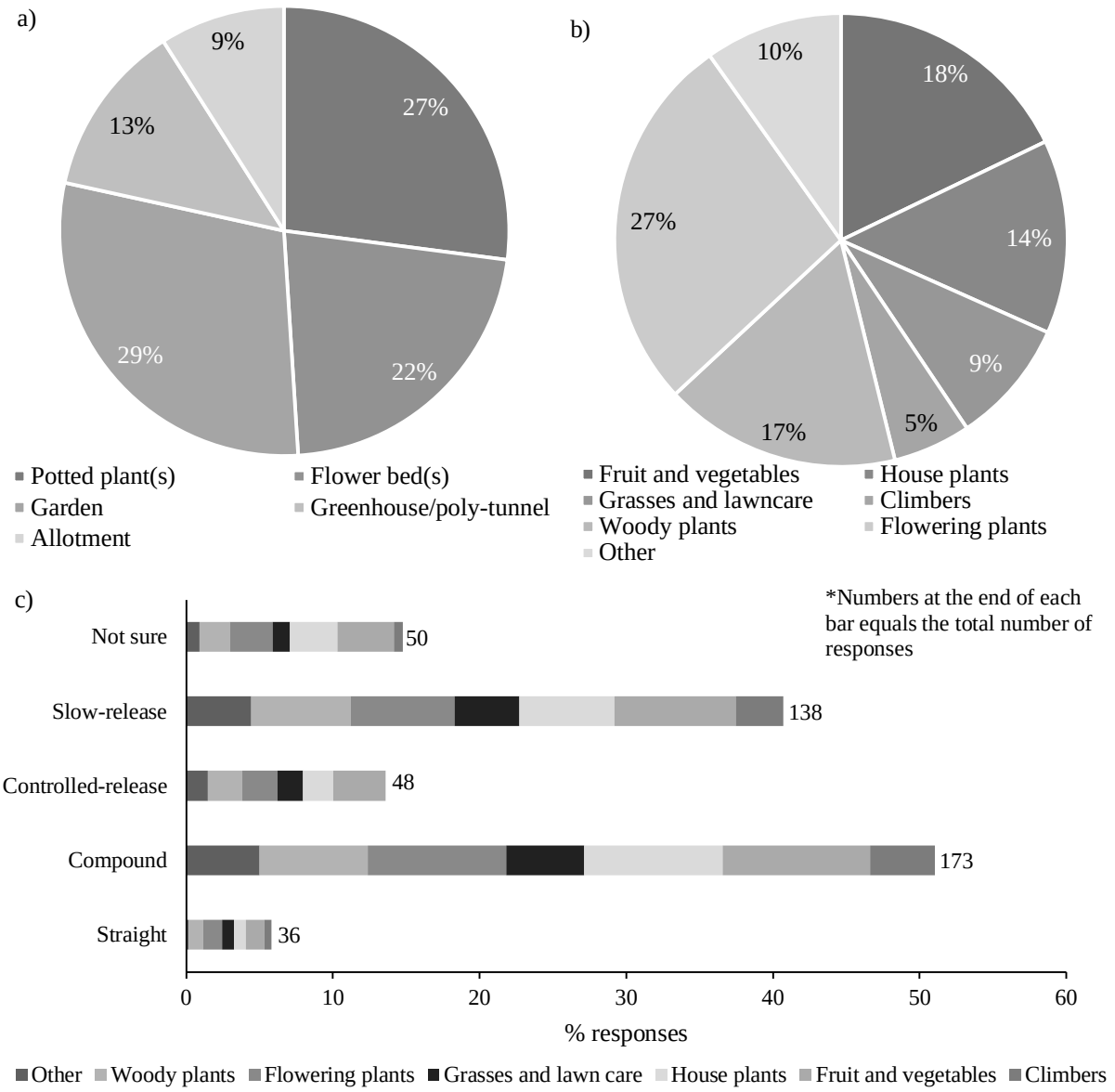


**Figure 6-2 Common spaces for gardening amongst surveyed population a) Range of plant types receiving fertilisers by respondents, 'other' includes ferns and cacti and succulents and 'Flowering plants' exclude fruit and vegetables b) Fertiliser types used compared to defined by plant types grown c)**

These findings suggest that when considering the marketing for RDFs it may be necessary to highlight their applicability to the more common plant types which the public are willing to spend money on. In comparison to a survey of 100 private gardeners, whose average spend was $300 (AUS) per year or £170/year on gardening, this included the cost of renting space for gardening (i.e., allotments) and some respondents who grew vegetables privately to diversify their income[26]. Furthermore, a survey into willingness to pay for decentralised wastewater treatment in South Korea identified that local communities would be willing to spend $12 (£10)

per year for the RDFs produced from the treatment system for use in subsistence farming and their horticulture[24].

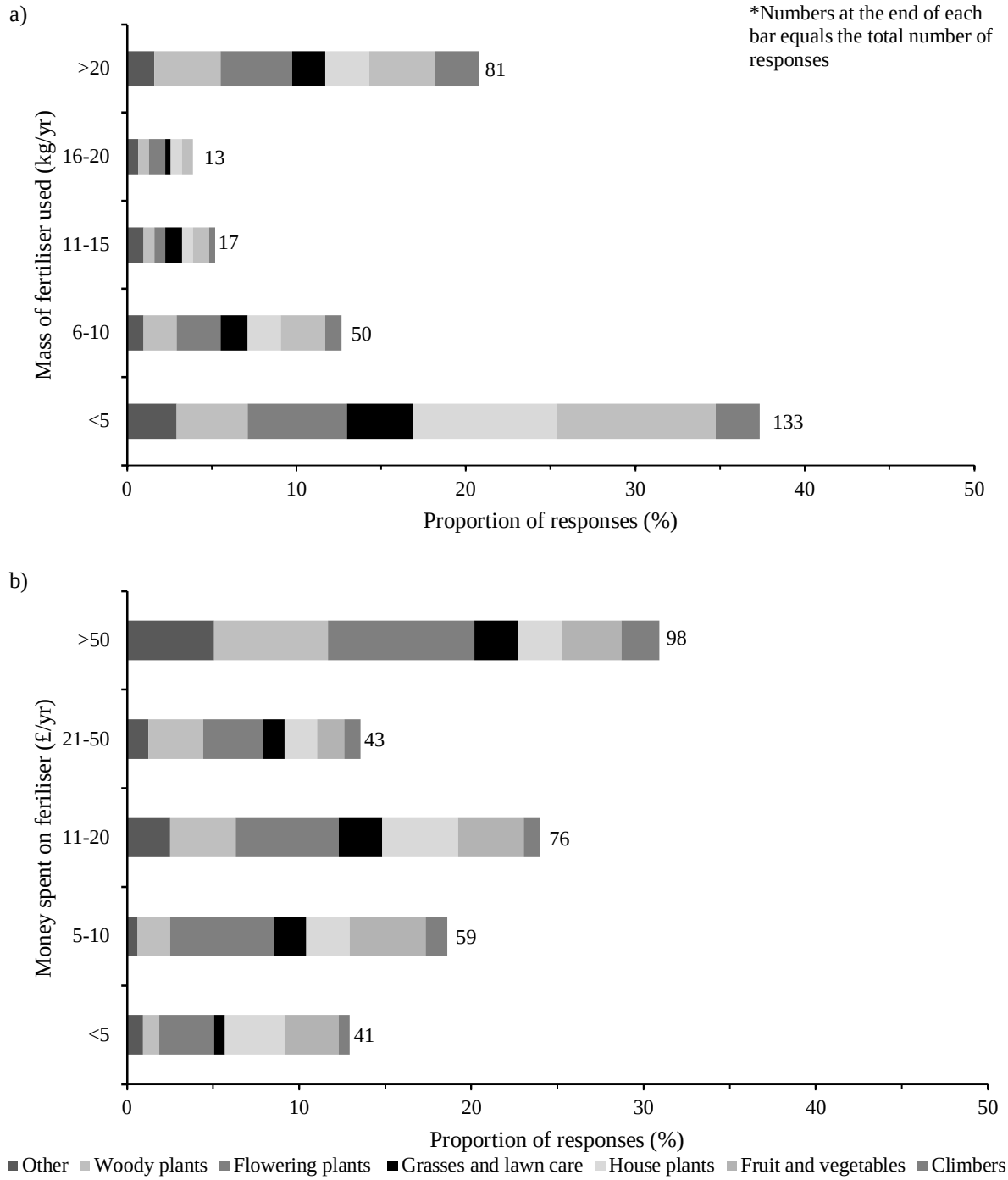


**Figure 6-3 Mass of fertiliser used per year and plant type a) Expenditure on fertilisers per year and plant type b)**

This is positive for the marketability of RDFs to the public in general, as it provides insight into what would be spent and the potential savings they could make. Such as 69% of the survey population already spend more than £10 per year on fertilisers in the UK, so cheaper RDF

alternatives may improve the uptake in private gardening by the public especially if cost savings could improve profit on produce, they grow to sell. Alternatively, the costs of RDF could be kept similar or higher than existing fertilisers. This would aid on the investment and operational costs needed to recover the nutrients from waste streams as the public might be willing to pay the same for utilising a product with green and sustainable sourcing. This public behaviour is seen in other products from clothing to electronics[30].

In addition, Pearson's significance testing gave an $r^2$ value of 0.03, when comparing age of respondents and kg of fertilisers per year, meaning that it is more likely that those that are older will use more fertiliser on average in a year. The same significance is not true when considering how much is spent on fertilisers per year. This suggests that whilst older respondents may use more, they have the time and resources to recycle more nutrients, therefore can produce their own fertilising resource giving them better access to more quantities. This is supported by responses showing no respondent below the age of 26 composted themselves or used manure/compost to fertilise their plants.

#### *6.3.1.3 Perceptions of RDFs*

The survey population was asked to rank the 9 factors identified as concerns in past reports on the use of RDFs[7,8,20], these included those concerns of industrial end-users and advisors, where cost, purity and nutrient release were the main concerns[7]. The results indicated that the surveyed population were most concerned about the presence of heavy metals in RDFs, pathogens and pharmaceuticals were the next concerning variables (Figure 6-4). The distribution in responses of pathogens and pharmaceuticals suggest there is discontinuity shown by the extended lower quartiles (Figure 6-4). However, the spread of the data suggests heavy metals remain the most concerning (Figure 6-4). The cost, lifespan, consistency, and supply of RDFs were equally the least concerning factors on average with purity and nutrient

release averaging in the middle of all tested variables (Figure 6-4). This suggests there is a difference in concerns between the public and industry end-users when variables for concern are considered, a survey of the most concerning RDF variables for farmers were the least concerning of this surveyed popualtion[7]. This may be due to media exposure and reporting of pollutants in wastewater has increased over the past decade leading to a higher social awareness of these issues. Whereas economic factors remain a higher concern for industrial end-users and advisors when considering RDFs.

This is important when considering messaging around RDFs when they are used in industry and marketed for public use to assure consumer confidence that these pollutants are not present in RDFs. Whilst RDFs need to remain economically viable for industrial end-users and not compromise on quality.

Through cross tabulation the cause for concern responses can be compared against the likelihood or the respondent eating produce from RDFs and using them (Figure 6-5). Overall, 80% of the surveyed population would eat produce grown by RDFs and use them in their gardening practices, this is towards the higher end of willingness when compared to a multinational survey of students[20], the multinational average was 59%, western countries willingness was 55% and the most willing student population was in France at 80%, which is equal to the willingness of respondents in this survey.

Eleven percent wanted more information before deciding to make the switch to RDFs, indicating that there are likely other factors of more concern for this population, i.e., whether or not a premium would be paid for these RDF products/produce. Between 3- 9% of the survey population would be unlikely to eat produce grown from RDFs or use RDFs. Whilst the proportion of respondents that are unlikely to eat produce grown from or use RDFs was low, cross tabulation indicates that the most associated cause for concern with unlikelihood of eating

or using RDFs was ‘pathogens’, with 100% of all respondents who would be unlikely to use or eat produce grown from RDFs ranking it as the most concerning factor (Figure 6-5).

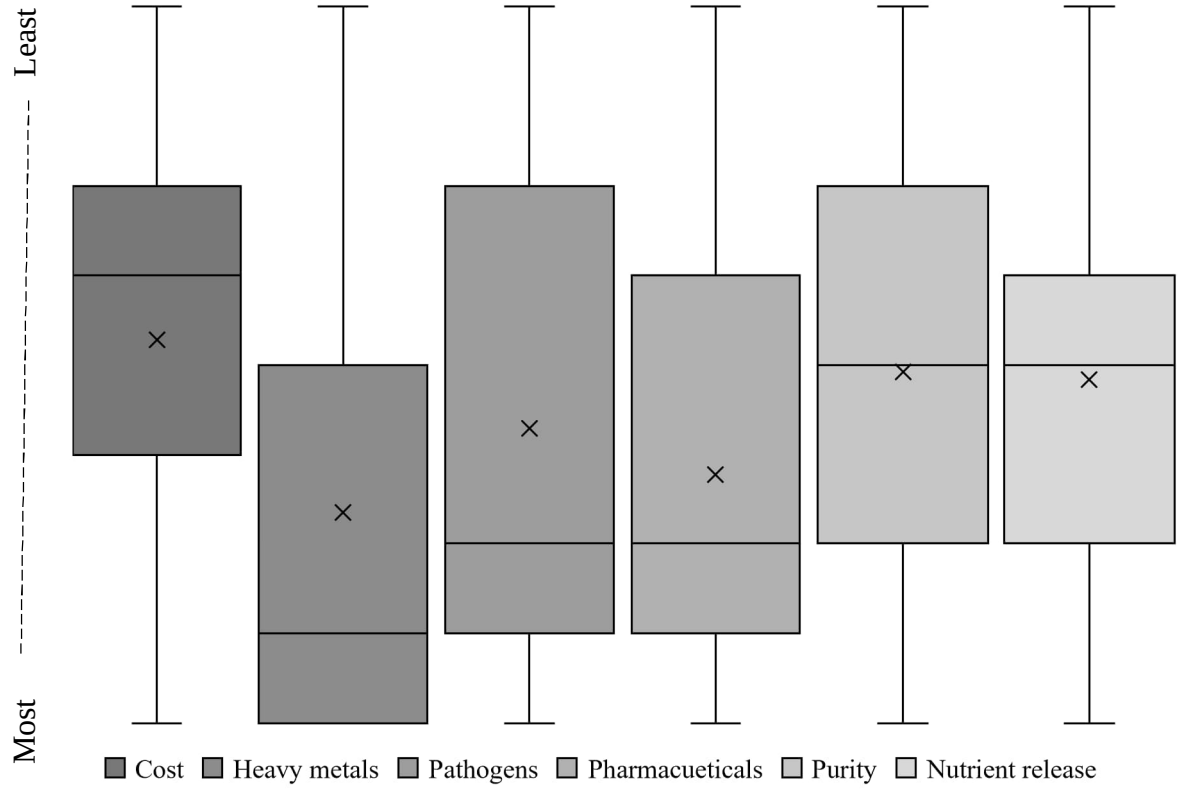


**Figure 6-4 Relative cause for concern of key variables associated with RDFs.**

This was followed by the presence of pharmaceuticals then heavy metals, even though heavy metals were ranked the most concerning factor of RDFs there is less indication heavy metals would factor in the survey population’s decision to use or eat produce grown from RDFs.

The presence of pathogens and heavy metals in RDFs has been researched in several RDF products including struvite, dewatering cake and urine-derived fertilisers[31–33]. Results have shown that RDFs with a drying stage inactivate pathogens and parasitic eggs to achieve a low moisture content[31]. The chemistry of the initial waste or wastewater is an important factor to be considered when producing RDFs, studies have shown that the heavy metal concentration in urine and municipal wastewater is several orders of magnitude lower than in commercial fertilisers, in addition to heavy metals having a higher affinity for the liquid phase of

wastewater than chemically or biologically recovered struvite in a phosphate recovery processes[32,34].

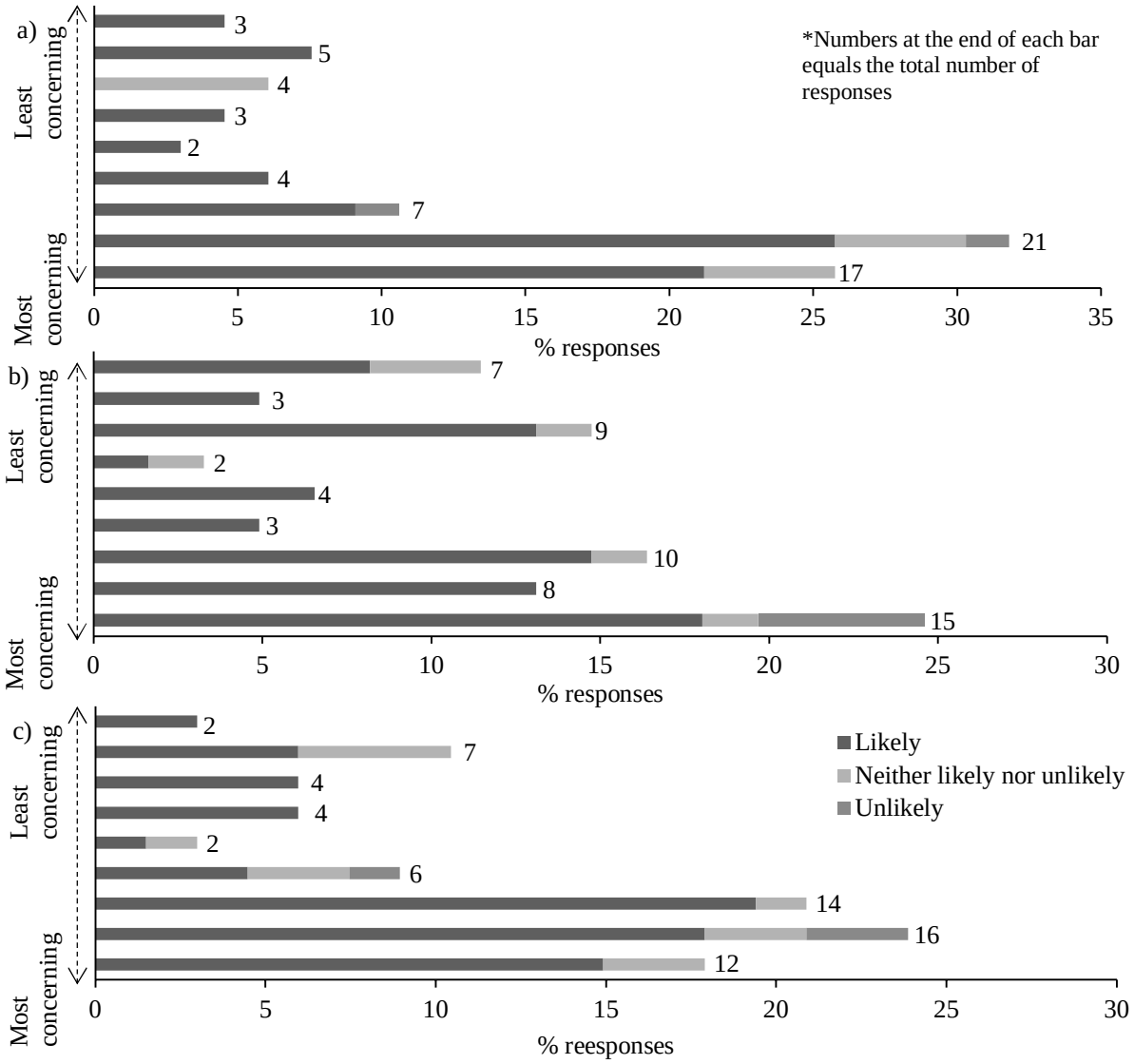


**Figure 6-5 Top three concerning factors of RDFs cross tabulated by likelihood of using RDFs and eating their produce. Heavy metals a) Pathogens b) Pharmaceuticals c)**

### 6.3.2 Farmer questionnaire

The uptake on responses to the farmer questionnaire was low, given the limited time of the survey being open and coinciding with busier periods of crop maintenance. A total of 3 farms were led through the questionnaire, produce grown covered grassland and roughage (< 50 hectares), arable crops and fruit, both 101-200 hectares in size. The experience of these farmers was either < 5 years or > 25 years. None of these farms used organic fertilisers, and anywhere

between 4 tonnes and 77 tonnes of inorganic fertiliser could be used in a year. Grassland and forage required the least amount of fertiliser at 4 tonnes, the fruit and arable farms required between 60 and 77 tonnes of fertiliser per year. These farmers used straight and/or slow-release fertiliser, in a granular form. Phosphorus was not a major nutrient they added to their crops, nitrogen, magnesium, and potassium were the sought-after nutrients and anywhere between 4 tonnes and 17 tonnes of each nutrient was needed per year. The data supplied by the questioned farmers provides an insight into the specific nutrient requirements for 3 different food groups grown by farmers and can allow basic mass balances to understand the volume of wastewater or waste required to produce enough RDF to meet their demands. For example, using lab-based recovery percentages for the biomineralisation of struvite from sludge dewatering liquors (92%), and the flux of phosphorus (P) through UK WWTP (46 kt P/year.) the potential yield of bio-struvite (an RDF under research) can be estimated assuming removal efficiency is maintained throughout and average phosphorus content does not fluctuate[13,34]. This basic mass balance estimates a bio-struvite yield of 43 kt/year. This would be enough to satisfy the nitrogen and magnesium requirements of 361 UK farms between 101 and 200 hectares that grow fruit or arable crops like those who completed the questionnaire or up to 1400 farms < 50 hectares requiring nitrogen for the growth grassland and roughage. Although this calculation has made some assumptions, such as the recovery yields of bio-struvite and assuming these farmers represent the average for their crop type farmed, it is a powerful indicator for the scope RDFs have in replacing inorganic fertilisers. The selection of struvite for this example was also based on its similarity to the inorganic fertilisers (granular and slow release) used by the approached farmers, so could make the easiest substitution into their current fertiliser use.

All participating farmers found it difficult to find fertilisers that were good value for money or sustainable and were not concerned or found the process easy to find fertilisers with their needed nutrient content and without heavy metals, pathogens, or pharmaceuticals. The farmers

were asked to rate the same factors asked in the public survey (Figure 6-4) from least concerning to most concerning. Cost was ranked the most concerning, followed by purity, consistency, and supply. Heavy metals, pathogens and pharmaceuticals were the least concerning for farmers when asked, this is the inverse of what has been gathered from the public survey and aligns with previous surveys of farmers concerning the use of RDFs[7]. This reiterates the importance of how RDFs need to be developed into public or industry markets to ensure confidence in the product and repeated use.

Finally, the farmers were given the basic characteristics of three RDFs produced at WWTP (Table 6-2) to choose from or continue with their current inorganic fertilisers. All farmers opted for bio-based fertiliser 2, this beneficial for WWTP when considering applying RDF technologies to their treatment systems, so they produce a product that is preferred possible farmer end-users. This finding has built from previous surveys which provided clear evidence for positive motivations towards RDFs and changing to them[7,15,21], to clearly show that a readily available RDF product would be chosen over chemical/inorganic fertilisers by the questioned farmers.

**Table 6-2 Attributes of 3 RDFs from wastewater**

| **Attributes** | **Bio-based fertiliser 1** | **Bio-based fertiliser 2** | **Chemically recovered fertiliser** |
|---|---|---|---|
| **Price relative to manufactured fertiliser** | <50% cheaper | Cheaper | Same price/ more expensive |
| **Form** | Solid and liquid | Solid (granular) | Solid (granular) |
| **Nutrient content variability** | 10% uncertainty | <5% uncertainty | Certain NPK content |
| **Organic carbon** | With organic carbon | <5% organic carbon | Without organic carbon |
| **Pests and diseases** | Some association | None associated | None associated |
| **Speed of nutrient release** | Fast | Slow | Slow |
| **Other attributes** | Organic | Organic and up to 15% Mg + 2% K | Inorganic and 8% Mg |

### 6.4 Conclusions

The results from this study corroborates with previous studies that many aspects of private gardening in the public are not interlinked, and that the main motivations for gardening include producing food and benefits to the environment. This study has identified that the more aged population surveyed used allotment spaces to garden and there was a significant link between the age of a gardener and how much fertiliser they used. The fertilisers used by the those surveyed were dominantly compound and slow release, which makes recovered mineral fertilisers like struvite more attractive to continue with a similar product. Finally, this survey has shown that there is a large willingness within the public to use RDFs and consume products grown using RDFs, this should provide confidence to RDF producers and the introduction of RDF recovery technologies into nutrient waste streams, such as water utilities and agriculture. There is the possibility for the development and growth of an RDF market within the UK population and in the global private gardening sector based on the results of past studies. Furthermore, whilst there were more concerns for pollutants in RDFs rather than the cost or purity, this is not enough to dissuade the public from using RDFs.

The farmers questioned about their fertiliser use and perceptions of RDFs provide a key insight into the opportunity there is for substituting mineral RDFs such as struvite, that will satisfy their nutrient requirements, and fit into their current practices. Additionally, farmers have difficulty finding sustainable and cost-effective fertilisers, by improving the development and market of RDFs farmers will be able to choose a more sustainable fertiliser product and will not experience the same cost issues as with inorganic fertilisers whose price can fluctuate due to global socio-economic issues.

The development of RDFs now requires collaboration between the producers of RDFs such as water utilities, end-users such as farmers and advisors, institutes, and governing bodies, to

improve the understanding and access to RDFs from suppliers through to end-users. This is so that the UK and further abroad can develop a circular economy within the farming and water industries, simultaneously improving its fertiliser resilience and food security.

### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data Access Statement

Data shown in this review paper can be found through the resources in the following References list.


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# 7. Discussion and economic assessment of bio-struvite production

The aim of this PhD was to develop a biomineralising technology to remove and recover nutrients from urine and sludge dewatering liquors in open systems. During this process suspended and encapsulated cultures of bio-mineral forming microorganisms were tested, and the precipitates characterised as struvite, here called bio-struvite due to its biological nature. The public and farmer acceptance of recovered fertilisers from wastes was also investigated.

## 7.1 Discussion of key findings

### P removal in open systems

In chapter 3, five selected microorganisms were incubated suspended in source-separated urine under open culture conditions. At the end of a 7-day incubation period with an average P:Mg ratio of 1:0.5, all selected microorganisms had removed at least 50% of the initial $PO_4$-P, 30% more than controls. *Brevibacterium antiquum* removed the most $PO_4$-P, at 70% of the initial concentration during this incubation period. When dosing $Mg^+$, to reach a solution P:Mg ratio of 1:1, resulted in 98% $PO_4$-P removal in *B. antiquum* inoculations (Figure 3-2). The results from chapter 3 and past studies provided the rationale for the selection of *B. antiquum* and *B. pumilus* for encapsulation trials, along with comparing biologically controlled mineralisation (BCM, *B. antiquum*) and biologically induced mineralisation (BIM, *B. pumilus*).

Chapter 4 trialled immobilisation techniques and encapsulation in MNE media (MNE-Ba and MNE-Bp, *B. antiquum* and *B. pumilus* respectively) incubated in synthetic media and SDL in pure and open culture conditions. *Bacillus pumilus* cells remained viable when immobilised in hydrogel and encapsulated in MNE media, this was observed as the same removal of sCOD and increased $PO_4$-P removal in MNE (Figure 4-1). Although, the hydrogel structure was not

stable, the same was observed in alginate immobilised *B. antiquum* when incubated in pure culture SDL.

In SDL batches comparing pure culture conditions with open culture conditions, MNE-Ba removed 70% of the initial $PO_4$-P in both pure and open culture SDL, MNE-Bp removed 50% (Figure 4-2). There was a lag in the removal of $PO_4$-P over the first 6 hours incubation (Figure 4-2), this was hypothesised to be due to *B. pumilus* using BIM for struvite production, which was slower slightly by the MNE media. Repeated cycles of the same MNE media in fresh SDL repeatedly removed $PO_4$-P to the same levels as when the MNE media was first incubated in SDL (Figure 4-3). It was calculated that the MNE media had an adsorption capacity of 1.28 mg $PO_4$-P/ mf MNE media (Figure 4-4), this is beneficial for initiating the biomineralisation process by supplying $PO_4$-P to the encapsulated microorganisms rather than the native communities.

Chapter 5 trialled the encapsulation by MNE media in a continuous pilot-scale reactor, at varying operational conditions where the loading rate of P was controlled. The removal from both MNE encapsulated microorganisms was between 20% and 30% (Figure 5-4). It was hypothesised that this was due to the very low BOD of the SDL, leading to a limiting carbon source and that Mg was limiting the removal of P through biomineralisation. Dosing external carbon and Mg increased removal of $PO_4$-P by MNE-Ba to 98% on average, a higher removal rate from most chemical methods[1] and achieving a treated effluent of <3 mg $PO_4$-P/L. In the pilot-scale reactor with MNE-Bp the average P removal was 61% but fluctuated between 36% and 91% removal. This suggested that the biomineralisation mechanisms of *B. pumilus* (BIM) was a factor in the removal and recovery of P.

**Bio-struvite production in open systems with and without encapsulated microorganisms**

In chapters 3, 4 and 5 minerals were recovered during and after incubation in open system wastewaters, source-separated urine, and SDL. These were recovered using gravity settling and filtration to collect minerals >10 µm. Using optical microscopy and SEM-EDS the mineral assemblage was identified (Figures 3-6, 3-7, 4-1, 4-5 and 5-5). This identified two mineral morphologies, tabular and prismatic, and amorphous, using SEM-EDS element abundances were associated with the morphologies, identifying the tabular, prismatic minerals as struvite and the amorphous minerals as calcium phosphate. There were no minerals recovered from controls of SDL throughout experimentation and only calcium phosphates recovered from control batches of urine (Figure 3-6 and Figure 3-7). The recovery of bio-struvite from SDL was only possible in pure culture conditions when suspended cells of selected microorganisms were inoculated, none was recovered from SDL in open culture conditions. The bio-struvite recovered was consistent with past experiments using these microorganisms and that bio-struvite was well-formed (euhedral) indicating ideal conditions were achieved for its growth[2–4].

In chapter 3 geochemical modelling showed that the saturation index of struvite was below 0, indicating that abiotic mineralisation was not thermodynamically favourable. Whereas for hydroxyapatite and other calcium phosphate minerals the saturation indices were above 0, in favour of their mineralisation (Figure 3-5). The mineral assemblages shown in Figure 3-7 show that the selected microorganisms were able to use their biomineralisation mechanisms to overcome the thermodynamic constraints of the system to mineralise bio-struvite[2]. This observation was consistent across all SDL inoculations.

In chapter 4 the ability of selected microorganisms encapsulated or immobilised in different media was tested in batches of growth media and/or SDL. In chapter 5 only those selected

microorganisms encapsulated in MNE media were tested at pilot-scale and compared with SBR enrichment of *B. antiquum.* Immobilising media was able to hold the selected microorganisms within a framework and them remain viable, however the structure deteriorated with prolonged exposure to synthetic media and SDL meaning bio-struvite mineralised was contaminated with media fragments.

Encapsulation within the MNE media kept selected microorganisms viable, confirmed by maintaining the same removal of $PO_4$-P and sCOD as suspended cells of the same microorganism (Figure 4-1). The minerals recovered from synthetic batches was confirmed as bio-struvite using morphological characteristics and SEM-EDS (Figure 4-5), yields were less than suspended microorganisms, 400 mg/L compared to between 600 and 1000 mg/L in suspended cell controls and previous studies, a reduction of up to 50%[5]. During the same incubation period bio-struvite minerals were on average 25% smaller after incubation of MNE media in synthetic solutions and pure culture SDL (Figure 4-5 and 4-6), this may be due to lag caused by the time taken for nucleated bio-struvite to leave the MNE media. It was proposed that this is beneficial for continuous reactors as the delay could provide more consistent seeding of bio-struvite when influent characteristics fluctuate. Bio-struvite recovery from suspended cells was not possible when incubated in open culture SDL likely to out-competition from native species, there was no difference in yield between pure and open culture batches with MNE media.

In chapter 5 bio-struvite was recovered in all operational conditions implemented on MNE-Ba and MNE-Bp. The yield was between 21 mg/L and 54 mg/L. The mineral assemblage from both MNE encapsulated microorganisms was primarily bio-struvite with secondary calcium phosphate minerals. The mineral assemblages from MNE-Bp were relatively consistent across all operational conditions, 45% to 55% was bio-struvite and 45% to 55% was calcium

phosphate. The mineral assemblages from MNE-Ba during the different operational conditions fluctuated, at 0.4 kg P/$m^3$.d 61% was bio-struvite, increasing the loading rate to 0.6 kg P/$m^3$.d raised the proportion of bio-struvite to 71%. This proportion came back down 61% when dosing was applied to a loading rate of 0.5 kg P/$m^3$.d. It was hypothesised that this difference is due to the mechanism of biomineralisation, *B. pumilus* uses BIM whereas *B. antiquum* uses BCM. Biologically controlled mineralisation appears to be able to overcome the thermodynamics of the system to concentrate the desired ions and mineralise its targeted biomineral.

**Technical readiness level of encapsulated microorganisms for P recovery**

The findings from chapter 5 have developed the understanding of the capabilities of the biomineralisation process for SDL resource recovery whilst encapsulated in MNE media. The addition of an external carbon source and Mg, increased the P-removal to 98% in the MNE-Ba pilot and a treated effluent quality < 3 mg $PO_4$-P/L on average which is within UK discharge limits[6] (Figure 5-6). This is improved compared to chemical struvite technologies which typically average at 80% removal and require further treatment or return of effluents to reach discharge limits[7,8]. Nearly 90% of the removed P was recovered as minerals, of which bio-struvite made up between 60% and 70% of the mineral assemblage (Figure 5-5).

The results of chapter 6 have identified that the appetite for a recovered mineral fertiliser or recycling derived fertilisers is present in the farming community and the public (Figure 6-4 and Figure 6-5). This gives the bio-struvite product more value as it can substitute into current fertiliser practices easily according to the surveyed public and questioned farmers (Figure 6-2 and Table 6-2). This coupled with contaminant analysis of bio-struvite recovered, including heavy metals, pathogens, and micro-pollutants (Table 5-4), means that it is unlikely further processing of the bio-struvite product will be needed.

The work presented in these chapters has shown proof of concept for the biomineralisation process of selected microorganisms in open culture conditions as suspended cells in source-separated urine and two of these microorganisms encapsulated in MNE media in SDL at batch scales (TRL 4). The encapsulated microorganisms have been trialled in continuous reactor at different operational conditions and has achieved commercially relevant removal and recovery efficiencies (TRL 5). These were not possible in SBR pilot studies using suspended cells of *B. antiquum*, as the biomass of *B. antiquum* was not maintained and other species dominated the reactor is just a few days of operation (Figure 5-2). The results from the continuous pilot-scale reactors are a strong indicator that a large-scale pilot (TRL 6) would be one of the next steps for the development of the biomineralisation process using encapsulated microorganisms. However, there are several operational conditions that remain to optimised that are presented in the Future Works section of this chapter.

## 7.2 Business case

### Capital expenditure and operational expenditure assessment

The capital expenditure (CAPEX) and operational expenditure (OPEX) were calculated for the biomineralisation technology to recover bio-struvite and a simplified chemical struvite recovery treatment stream. A single scenario was used to compare the two technologies, a WWTP with a 50,000-population equivalent (PE). The dewatering reduction from initial wastewater input (PE multiplied by wastewater production at 200 L/PE[9]) was used at 60% to calculate the SDL load to each recovery tehcnology[10,11]. This plant was based on the WWTP used for SDL used in pilot studies and MNE batch trails (Table 5-2, SDL2) whose treatment flow diagram is available in Appendix B. To calculate the CAPEX, and provide the comparison between the two recovery technologies, ‘reactors’, ‘piping, electricity for pumping and instrumentation’, ’chemical tanks’, and ‘driers’ were estimated (Table 7-1) and kept the same across both technologies. This was so that the comparison between the bio-struvite technology

can be simplified against the capital cost to set up a conventional, chemical struvite treatment process. This was with the exception of 'chemical tanks', where two were required for the chemical recovery technology compared to only one for the biomineralisation technology, using the findings from Chapter 5[12]. The design, project management and construction of both technologies was considered to be 25% of the CAPEX for materials[13]. The source of CAPEX values were from feasibility studies of chemical struvite recovery in Turkey[12] and economic life-cycle assessment of other WWTP technologies[14]. These were converted from Euros to Pounds Sterling and adjusted for the UK rate of inflation since the data was published, at 1.46% since 2010 and 1.26% since 2016[15] to be displayed as cost/$m^3$ SDL treated. The cost of MNE material, has been excluded from the calculations as this is protected intellectual property (Microvi© MNE™, San Francisco, USA), it would be anticipated this would increase the CAPEX for the biomineralisation technology.

**Table 7-1 CAPEX and OPEX assessment of struvite recovery technologies**

| | **Chemical struvite recovery** | **Bio-struvite recovery** |
|---|---|---|
| **Scale (PE)** | **50,000** | |
| **CAPEX (£$_{2022}$/$m^3$)** | **0.29** | **0.27** |
| Reactor[a] | 0.15 | 0.15 |
| Piping, electricity, and instrumentation[a] | 0.01 | 0.01 |
| Chemical tanks[a] | 0.03 | 0.02 |
| Drier[a] | 0.04 | 0.04 |
| Buildings and site perparation[b] | 0.05 | 0.05 |
| **OPEX (£$_{2022}$/$m^3$)** | **0.07** | **0.04** |
| Aeration energy[c] | 0.000 | 0.001 |
| Maintenance[c] | 0.01 | 0.01 |
| Dosing ingredients (Mg ± pH adjustment)[b] | 0.06 | 0.03 |
| **Revenue (£$_{2022}$/$m^3$)** | **0.04** | **0.05** |
| Struvite/Bio-struvite production (ton/year)[b] | 173 | 223 |
| **Payback time (yrs)** | **NF** | **19** |

[a] = Values calculated from Yetilmezsoy *et al.*[12]
[b] = Values calculated from Mills *et al.*[14]
[c] = Office for National Statistics quarterly energy prices April to June 2022[16]
Prices were adjusted to include cost of inflation since their original publication
**NF** = Not feasible

The operational costs for both recovery technologies includes, 'aeration energy', 'maintenance' and, 'dosing chemicals', aeration energy was calculated using an aeration efficiency of 1 kg $O_2$/kWh and target aeration of 8 mg $O_2$/L and the average UK energy price from April to June 2022 for the UK[16]. Maintenance costs were estimated at 3% of the CAPEX[14] and the mass and costs of dosing chemicals was calculated using the P:Mg for optimum removal and recovery as struvite for both technologies[12] and the findings from Chapter 5.

The revenue for chemical struvite recovery in this scenario was based on an 80% P-removal/recovery and using mass balances to estimate the yield of struvite[12] from the average $PO_4$-P concentrations of SDL (Table 5-2) to get the yield in tonnes/year (Table 7-1), and multiplying that by the average cost of struvite/tonne at £480/t[12].

The difference in CAPEX between a chemical and biomineralisation technology for the recovery of struvite was less 5%, this dependant on the cost for encapsulating the selected microorganisms, depending on the materials and processes for encapsulation the cost can vary[17]. The difference between OPEX of chemical and the biomineralisation technology was distinguished by the 'dosing chemicals', as the biomineralisation technology requires 77% less chemicals to treat the influent SDL and achieve better P-removal and recovery of minerals. The payback time of each technology has been calculated using the CAPEX, OPEX and potential revenue from the resale of recovered struvite. In this WWTP scenario the chemical recovery of struvite was not economically viable as plant would operate at a loss each year. This is similar to other studies that have found that chemical recovery of struvite needs high strength wastewater such as swine effluent, where the concentration of $PO_4$-P is above 100 mg/L[12,18,19]. The economic assessment for chemical recovery is limited by the source and cost of chemicals, for it to achieve a payback time less than 100 years the cost of Mg would need to be half. The feasibility assessment of the biomineralisation technology had a payback time of 19 years at a

WWTP with a 50,000 PE (Table 7-1). This period is greater than other studies for nutrient and struvite recovery which ranges between 6 and 10 years[19–21]. Savings can be made in the OPEX using combined heat and power technologies within the WWTP to run the technologies and cheaper Mg sources such as desalination brines, remain to be trialled with the biomineralisation technology[22]. There are additional savings to be made that have not been accounted for such as reducing the volume of sludge required for disposal and wider savings of preventing P pollution in the environment, which is estimated to cost £36/kg P released[23]. Further to this an important factor that could change the payback period is the fact that significantly lower concentrations of $PO_4$-P would be recirculated to the head of the WWTP, reducing aeration and pumping needs in the secondary BNR process.

**Purity and contaminants**

For resource recovery of P as struvite using the biomineral process to produce a marketable product, it needs to meet the same standards as chemically recovered struvite which are set out in the EU Fertilising Products Regulations[24]. The presence of certain heavy metals has legal limits set out across the globe which varies between nations (Figure 7-1), therefore bio-struvite products would benefit from remaining below all these limits. Previous batch experiments recovering bio-struvite has shown that heavy metal concentration remain below the variety of limits available (Figure 7-1)[25,26]. Further to this no micropollutants were found in bio-struvite samples collected during the pilot operation in Chapter 5. These results are in support of bio-struvite remaining viable as a fertiliser under several national regulations.

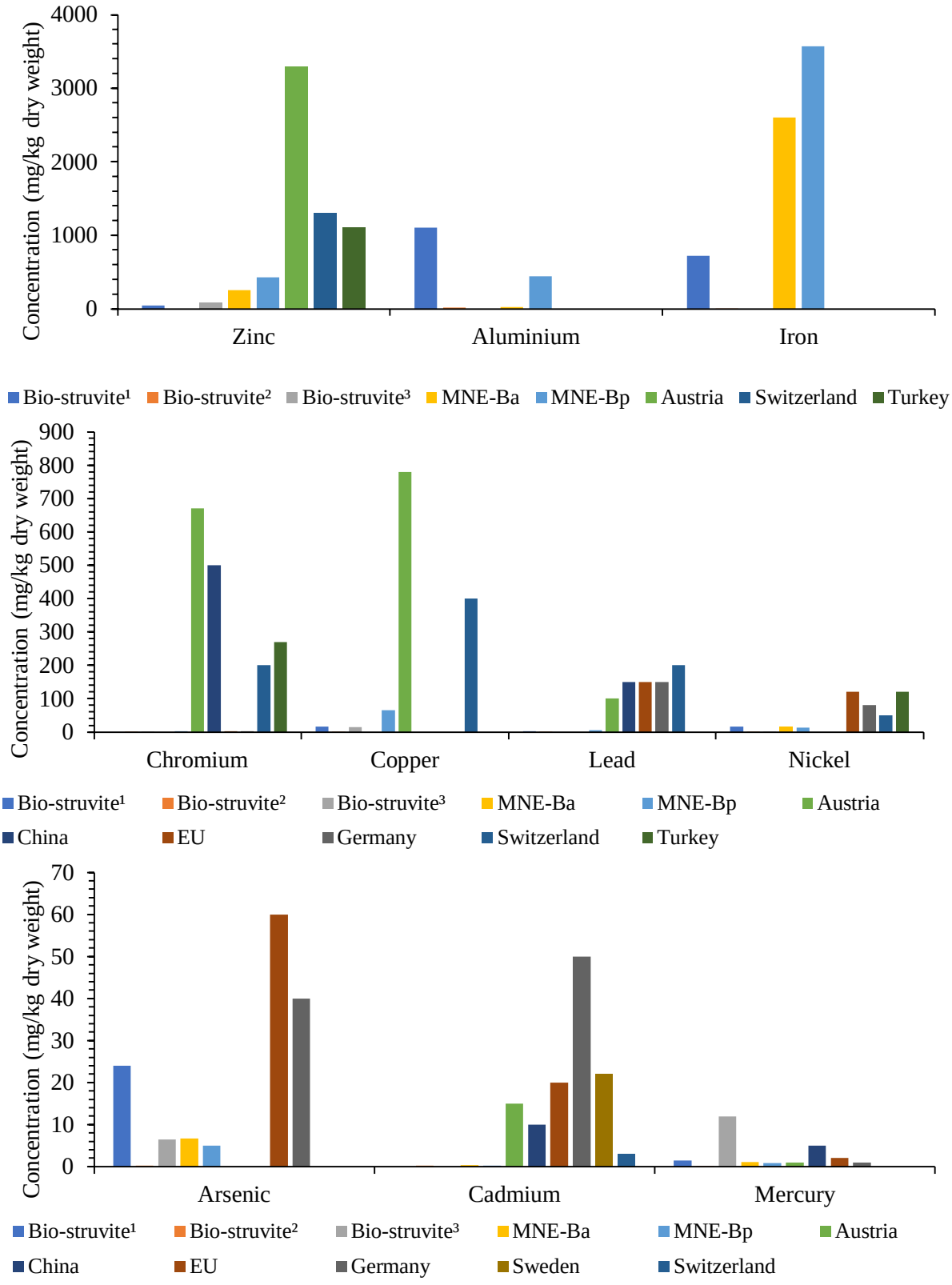


**Figure 7-1 Comparison between bio-struvite collected from this project and previous studies[3,25,27], and published heavy metal limits on fertilisers[26,28]. [1] – Simoes, et al, 2017[3], [2] – Yirong and Soares, 2022[25], [3] – Colston et al, 2023[27]**

## 7.3 Contribution to knowledge

Overall, this work has contributed to the understanding of how biomineralising microorganisms perform in open culture conditions in a variety of wastewater streams, whilst encapsulated in a biocatalyst media. The results have identified potential avenues for applying the biomineralisation to recover a viable fertiliser alternative in the form of bio-struvite. From the research of this study a flow chart of considerations and recommendations has been developed to provide clear guidance in addition to the summary of contributions to knowledge in Table 7-2 that this PhD has achieved. The knowledge gained from the recommendations in Figure 7-2 can feed back into the flowchart to provide a clear framework on how to implement biomineral recovery in industry.

## 7.4 Future work

This section summarises the suggestion of further work built on the findings discussed in Table 7-2 and highlights the key recommendations made in Figure 7-2.

**Investigate the use of encapsulated microorganisms in source-separated urine and other high-strength wastewater sources**

Chapter 3 highlighted the strength of source separated urine as a viable growth media for all selected microorganisms inoculated. Given the impact encapsulation had on the removal and recovery of P in open culture SDL, the use of these encapsulated microorganisms is a promising route for intensified P-removal with minimal and limit the loss of nutrient observed throughout the water treatment process as urine contains up to 80% of nutrients found in wastewater[29]. The treatment of source separated urine has huge economic benefits through more efficient treatment and less nutrient loading on WWTP[30] and is globally accepted as an RDF for produce[31]. The addition of biomineral recovery from urine may breakdown the social barriers associated with its direct reuse observed in some countries[31]. Other wastewater effluents that

have been investigated for chemical recovery of struvite include dairy and swine effluents, industry wastewaters including coking and textile wastewaters, which have $PO_4$-P concentrations up to 1000 mg/L[1].

**Explore low-cost carbon and Mg sources**

In chapters 3 and 5 the addition of a carbon source and/or Mg achieved near 100% removal of P, to below discharge limits in the UK. These laboratory grade chemicals are needed in chemical recovery, however the robustness of the biomineralisation process would indicate that the use of low-grade Mg such as seawater may be a feasible alternative[32,33]. In addition, the provision of internal carbon sources at WWT such as the recirculation of liquors from primary settled sludge which was identified and shown to be a good growth media for the selected mricobes[34,35].

**Optimise the reactor operational conditions**

The optimisation of the reactor operational conditions will bring the TRL level of the biomineralisation process for struvite recovery beyond 6. Firstly, this is necessary to test the production rate of encapsulated microorganisms using MNE technology ensuring product supply can meet the scale of wastewater treatment required. The loading rate and supply of additional carbon and Mg (established from earlier suggested work) can be optimised during the operation of the large-scale reactor and technology trialled for recovering biominerals, a large-scale pilot for struvite recovery from human urine, uses the density properties of struvite to recover minerals through sieving and a settling tank post- chemical reactor[36], this is technique used in Chapter 5 to recover minerals.

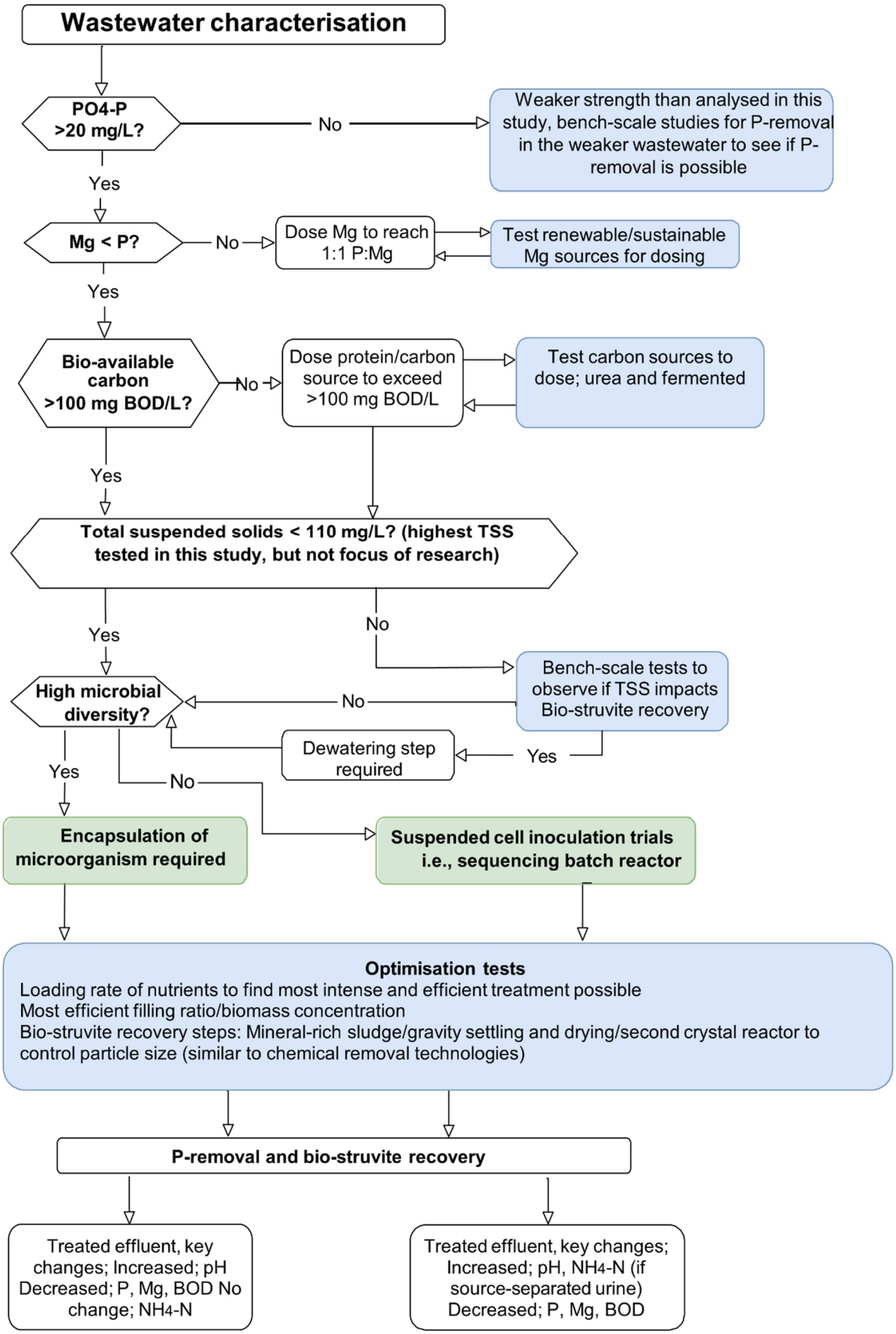


**Figure 7-2 Flow chart of considerations and recommendations for developing and implementing biomineral recovery of phosphorus treatment process**

**The potential of omic technologies**

A system biology approach using omics technologies is being used in developing biotechnologies and biomaterials[37]. Omic technologies characterise a range of fields within microbiology this includes, genomics, transcriptomics, proteomics, metabolomics and epigenomics, there are an array of commercially available omic technologies that can provide accurate and fast whole gene sequencing, targeted sequencing, and epigenome analysis such as Oxford nanopore sequencing, Illumina and Heliscopre[39]. The findings of these analyses can be utilised in the development of biotechnologies by providing a framework for ensuring microbes that have desirable attributes for commercial and industrial processes will be able to thrive in the conditions they are inoculated into prior to bench and pilot trials. Therefor streamlining and accelerating the testing phase. A clear example supporting this method is the development of several vaccines for Covid-19 in a relatively short time compared to historic vaccinations that have been developed[38].

In wastewater treatment, multi-omic approaches has led mutagenesis and genetic bioengineering of microalgae to alter metabolic pathways to improve their resistance in a wastewater treatment setting, facilitating an improved yield of high value bio-products such as biofuels[40]. The use of a multi-omic approach could provide data to inform microbe selection and/or genetic engineering to tailor treatment of various wastewater sources that can produce a high value bioproduct such as bio-struvite from phosphorus treatment.

### 7.5 Other considerations

Whilst the ability of MNE encapsulation to allow substantial biomass of a foreign microorganism to outcompete native microbes in open culture sludge dewatering liquors and recover biomineral precipitates has been demonstrated. The true cost, which was acknowledged in the business case (7.2) and longevity of the MNE media has not been accounted for directly.

Chapters 4 and 5 demonstrated that the MNE media maintained structural integrity and the advantage provided to the encapsulated microbes over repeated batches and continuous treatment for a period of 3 months. However, for industrial application the need to understand how often encapsulated microbes need to be replaced so that the cost for replacing the media can be accounted for.

Furthermore, whilst there is commercially available MNE encapsulated microorganisms, reactor design in industry must account for the need to recover biominerals, which may limit the design of the reactor compared to the commercially available nitrifying and denitrifying encapsulated microorganisms.

**Table 7-2 Contributions to knowledge achieved by the research work**

| | What has been confirmed | What has been developed | What has advanced knowledge |
|---|---|---|---|
| **Theoretical knowledge** | *Brevibacterium antiquum, B. pumilus, M. xanthus* and *H. salinarum* produced urease (Chapter 3)<br><br>*Idiomarina loihiensis* cannot produce urease (Chapter 3)<br><br>All the microorganisms grew under mesophilic temperature (22-34 °C) (Chapter 3)<br><br>Selected microorganisms can promote the mineralisation of bio-struvite (Chapter 3 and 4) | All microorganisms can utilise biomineralising mechanisms to produce recoverable (>10 µm) quantities of bio-struvite from open culture wastewaters (urine and SDL) (Chapter 3, 4 and 5)<br><br>The presence of heavy metals, organic non-polar and organic polar micropollutants was similar in both chemical and bio-struvite produced in a variety of open culture wastewater (Chapter 3 and 5) | Selected microorganisms remain viable and biomineralise struvite when inoculated in open culture source-separated urine (Chapter 3)<br><br>Encapsulation of selected microorganisms has no detrimental effect on the P removal in synthetic media when compared to pure culture inoculations (Chapter 4) |
| **Empirical evidence** | Formation of bio-struvite only occurred under aerobic conditions. (Chapter 3, 4 & 5)<br><br>Bio-struvite recovery was achieved in pure culture (sterilised) SDL inoculated with selected microorganisms (Chapter 4)<br><br>Farmers and their advisors are most concerned about how much bio-struvite (RDFs) will cost, the purity and ease of use (Chapter 6) | *B. antiquum* achieved 30% ureolysis using urease, in ambient conditions without the addition of chemicals, controls only saw 2% ureolysis on average (Chapter 3)<br><br>In B4.1 media a yield of 440 mg biostruvite/L was achieved in encapsulated microorganisms and selected microorganisms in suspended cultures (Chapter 4)<br><br>Growth and recovery of bio-struvite was not possible from SDL under open culture conditions with inoculated suspensions of pure cultures of selected microorganisms without the | A growth rate between 2.16 1/d and 4.32 1/d for selected microorganisms in source separated urine in open culture conditions was measured (Chapter 3)<br><br>A yield of 1000 mg bio-struvite/L urine was achieved without dosing Mg (Chapter 3)<br><br>In laboratory batches of source-separated urine close to 100% P removal was achieved when Mg:P was increased to 1:1 (Chapter 3)<br><br>A P-removal of up to 70% was achieved from SDL in open culture conditions when selected |

| | | | |
|---|---|---|---|
| | | supplementation of carbon or NaCl (Chapter 4)<br><br>A onetime inoculation of *B. antiquum* into an open mixed-culture sequencing batch reactor, cycling SDL with acetate and NaCl, followed by fresh SDL with acetate and no NaCl (with 30 min settling, and 90% volume replacement) did not conduct to the establishment of a stable bacterial community composed mostly of *B. antiquum* (Chapter 5)<br><br>Willingness to eat produce grown from and use RDFs by the public was in favour 80% (Chapter 6)<br><br>The presence of heavy metals, inorganic and organic micropollutants in bio-struvite was below EU and other governmental limits for fertiliser use and equal to or less than chemical struvite across laboratory and pilot studies (Chapter 5 and 6) | microorganisms were encapsulated in a biocatalyst media, without the supplementation of nutrients (Chapter 4)<br><br>Bio-struvite yields between 38 mg/L and 98 mg/L was achieved in a 5-day incubation period with selected microorganisms encapsulated in a biocatalyst media (Chapter 4)<br><br>Encapsulating biocatalysts have a P absorbance of 1.5 mg P/g biocatalyst (Chapter 4)<br><br>The P removal in a continuous reactor fed by SDL under open culture conditions by selected encapsulated microorganisms was on average between 20% and 30% regardless of changing the P-loading rate (Chapter 5)<br><br>A P-removal of 96% was achieved when sufficient BOD was present (100- 200 mg/L) and Mg to a ratio of 1:1 to 1:1.5, P:Mg with encapsulated *B. antiquum* (Chapter 5)<br><br>Bio-struvite yields of up to 115 mg/L SDL was achieved when carbon and Mg were no longer limiting (Chapter 5)<br><br>In a blind product questionnaire, farmers and advisors would switch to a bio-based mineral fertiliser over the |

| | | | |
|---|---|---|---|
| | | | inorganic fertiliser they use and other RDF products (Chapter 6) |
| **Methodology** | Techniques based on amplification of the 16S rRNA gene can be used to show the enrichment of Brevibacterium species in SDL (Chapter 5) | Flow cytometry can be used to identify intact cells to calculate growth rates of inoculated microorganisms in urine (Chapter 3)<br><br>Gravity methods can be used to separate bio-struvite from biomass and solids through differential density settling via centrifugation and gravity settling in a second tank (Chapter 3, 4 & 5) | Flow cytometry can be used to measure relative proportions of low to high nucleic acid to distinguish the growth of inoculated microorganisms in open conditions (Chapter 3) |

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# 8. Conclusions

### Contribution to knowledge

As discussed in Chapter 7, this work has contributed to further the understanding of bio-struvite production and the growth of the selected bacteria in sludge dewatering liquors and demonstrated the viability of the bio-struvite production process for the recovery of phosphorus from sludge dewatering liquors using encapsulating media. A CAPEX and OPEX comparison has been made utilising the data available from industrial chemical removal of phosphorus and recovery of struvite with the biomineralisation process showing that the savings made in chemical reagents required makes the biomineralisation process feasible compared to the chemical process which was not based on the conditions of sludge dewatering liquors used in this study which are typical of UK dewatering liquors from wastewater treatment plants. Figure 7-2 has summarised the findings of chapters 3, 4 and 5 to produce a flowchart of recommendations to guide future research and the application of the biomineralisation process into industry.

### Conclusions

The conclusions detailed are presented in respect to the project key objectives with the exception the literature reviewed in chapter 2 (Objective 1).

**Objective 2: Screen the five known bio-struvite microorganisms for pilot-scale experiments. Using laboratory experiments assessing their growth in; urine and wastewater without encapsulation.**

- The cell growth rate in pen culture urine inoculated by selected microorganisms was increased by 0.4 1/h on average.

- *Brevibacterium antiquum* was able to remove 70% initial $PO_4$-P and 98% when P:Mg in urine was increased to 1:1. Other selected microbes removed up to 50% initial $PO_4$-P.
- Between 300 mg and 1000 mg minerals/L was recovered by the selected microorganisms. The largest mass of minerals was recovered from *Bacillus pumilus* batches.
- Selected microbes were able to remove $PO_4$-P and mineralise recoverable yields of bio-struvite in pure culture SDL. They were not able to recover $PO_4$-P as bio-struvite in open culture SDL.

**Objective 3: Test microorganisms and encapsulation processes in lab-scale experiments, in synthetic media and pure versus open culture sludge dewatering liquors.**

- One-step encapsulation processes were able to maintain cell viability and measure removals in $PO_4$-P and sCOD associated with the selected microbes, but stable frameworks were not achieved in synthetic media or SDL.
- MicroNiche Engineering media was able to encapsulate microbes and improve the removal of $PO_4$-P in the first 6 hours of incubation in synthetic media relative to suspended cells. Achieving 35% removal after 6 hours.
- The sCOD and $PO_4$-P removal was equal in both MNE encapsulated tests in pure culture versus open culture. Reaching 70% $PO_4$-P removal in MNE-Ba batches.
- Bio-struvite was recovered in all MNE media batches, despite low concentrations of Mg. A recovery of 98 mg mineral/L SLD was achieved in open culture conditions with MNE media.

**Objective 4: Design and assemble a pilot-scale reactor to analyse the ability of encapsulated microorganisms to remove phosphate and recover as bio-struvite in a continuous reactor.**

- Phosphorus loading rates of 0.4 kg $P/m^3.d$ and 0.6 kg $P/m^3.d$ were trialled on MNE encapsulated *B. antiquum* and *B. pumilus.*

- $PO_4$-P removal was 32% and 24% with a loading rate of 0.4 kg P/$m^3$.d for MNE-Ba and MNE-Bp respectively.
- Removal remained unchanged in MNE-Bp but decreased in MNE-Ba at a loading rate 0.6 kg P/$m^3$.d.
- Supplementing a new carbon source and Mg increased $PO_4$-P removal to 98% in the MNE-Ba pilot and 68% in the MNE-Bp pilot.
- Average yields of bio-struvite were 64 mg/L at loading rates of 0.4 and 0.6 kg P/$m^3$.d and increased to 128 mg/L when supplemented with a carbon source and Mg.

**Objective 5: Assess the quality of bio-minerals recovered; purity of desired nutrients, contaminants (heavy metals, pharmaceuticals, faecal coliforms, etc.)**

- Dried bio-struvite recovered from source-separated urine had a purity of 26% $PO_4$ and 15.3% Mg
- Heavy metal concentrations were all below international fertiliser limits.
- The dominant mineralogy recovered from MNE media batches was classified as struvite using SEM-EDS.
- The quantity of minerals recovered from MNE batches, was 98 mg/L and 64 mg/L for MNE-Ba and MNE-Bp respectively.
- The yield of bio-struvite/L SDL from pilot studies increased with the higher P loading rates.
- Supplementing carbon and Mg to influent SDL increased the yield of bio-struvite relative to the mass of P removed, 70% for the MNE-Ba pilot and 63% for MNE-Bp.
- Analysed heavy metals and pharmaceuticals were all lower than international fertiliser limits of different countries and governing bodies or were below the limit of detection.

- No faecal coliforms or *E. coli* colony forming units were found in any of the minerals recovered from wastewater samples.

**Objective 6: Market research, breaking down perception barriers and development of an economic business case for bio-struvite recovery**

- The survey population dominantly used garden spaces and potted plants to garden in.
- Most common plant types to receive fertilisers were fruit and vegetables and, flowering plants.
- The average expenditure on fertilisers by the surveyed population was between £11 and £50/yr
- Questioned farmers and the surveyed population dominantly use slow-release and granular fertilisers.
- The majority of participants were willing to switch to a recycling derived fertiliser that is consistent with the properties of struvite.
- An assessment of CAPEX and OPEX for bio-struvite recovery treatment compared to chemical struvite recovery was for the SDL used in this study identified a payback time of 17 years for the bio-struvite treatment and a loss the same loss as CAPEX occurring every 4 years for chemical struvite.

# Appendices

## Appendix A Urine batch results

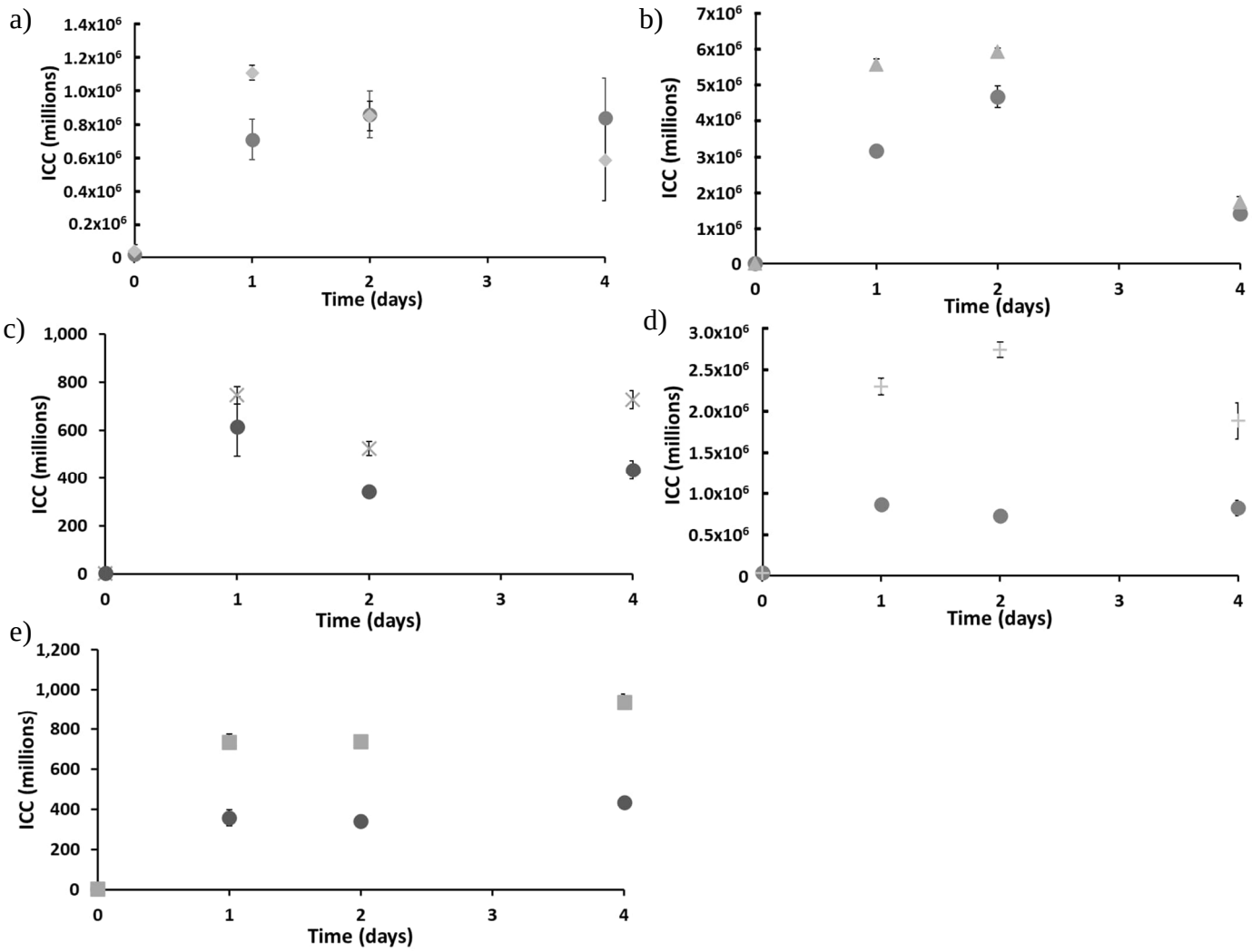


**Figure A1 Inoculated bacteria and control ICC data from flow cytometry analysis for a) *B. antiquum,* b) *B. pumilus,* c) *H. salinarum,* d) *I. loihiensis* and e) *M. xanthus* and their respective control (●)**

**Table A1 pH measurements of inoculated bottles and their respective controls**

| Time (days) | 0 | 1 | 2 | 4 | 7 | 10 |
|---|---|---|---|---|---|---|
| ***B. antiquum*** | **6.4** | **7.3** | **8.8** | **9.5** | **9.4** | **9.5** |
| | ±0.0 | ±0.0 | ±0.0 | ±0.0 | ±0.1 | ±0.1 |
| **UB1** | **6.4** | **7.0** | **7.3** | **7.4** | **8.3** | **8.8** |
| | ±0.0 | ±0.0 | ±0.0 | ±0.2 | ±0.3 | ±0.1 |
| ***B. pumilus*** | **6.1** | **6.3** | **6.8** | **7.6** | **8.5** | **8.8** |
| | ±0.0 | ±0.0 | ±0.0 | ±0.1 | ±0.1 | ±0.1 |
| **UB2** | **6.1** | **6.3** | **6.6** | **8.0** | **8.6** | **8.9** |
| | ±0.0 | ±0.0 | ±0.0 | ±0.1 | ±0.1 | ±0.1 |
| ***H. salinarum*** | **5.8** | **6.1** | **6.9** | **7.3** | **7.5** | **7.1** |
| | ±0.1 | ±0.0 | ±0.1 | ±0.0 | ±0.1 | ±0.0 |
| **UB3** | **5.8** | **5.8** | **5.9** | **6.5** | **6.9** | **7.4** |
| | ±0.0 | ±0.1 | ±0.1 | ±0.2 | ±0.1 | ±0.2 |
| ***I. loihiensis*** | **5.9** | **6.7** | **7.5** | **7.9** | **8.7** | **9** |
| | ±0.0 | ±0.3 | ±0.0 | ±0.0 | ±0.2 | ±0.0 |
| **UB4** | **5.9** | **6.3** | **6.4** | **6.7** | **7.2** | **7.3** |
| | ±0.0 | ±0.0 | ±0.1 | ±0.1 | ±0.2 | ±0.2 |
| ***M. xanthus*** | **6.1** | **6.8** | **7.0** | **7.4** | **7.9** | **8.3** |
| | ±0.0 | ±0.0 | ±0.0 | ±0.1 | ±0.0 | ±0.2 |
| **UB5** | **6.1** | **6.4** | **6.5** | **6.5** | **7.1** | **7.3** |
| | ±0.0 | ±0.0 | ±0.0 | ±0.1 | ±0.1 | ±0.1 |

## Appendix B WWTP flow sheet and micropollutant analysis

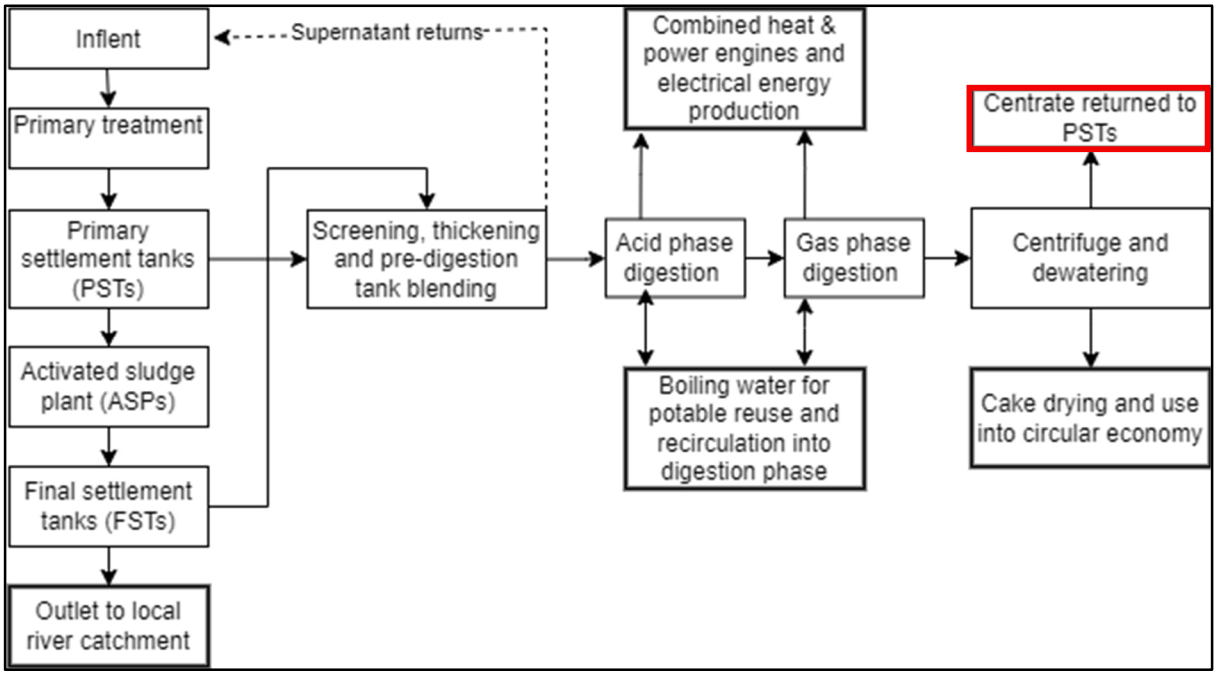


**Figure B1 Full-scale wastewater treatment plant flow diagram source for SDL used in continuous reactor, red box = sample point.**

99 Micropollutants were analysed using a 1290 series liquid chromatograph (Agilent, Santa Ana, USA) equipped with a Waters Acquity BEH (2.1×100 mm, 1.7 µm) kept at 21 °C. The mobile phase consisted of water (A) and methanol (B) mixed with 0.1% formic acid at a flow of 0.3 mL/min. Over a 15-minute run time the percentage of each mobile phase was adjusted to the sample following Table B-2. A 3-min post run was required for column equilibration and the injection volume was 20 µL.

**Table B1 Flow settings for liquid chromatograph**

| | Time | A | B | Flow |
|---|---|---|---|---|
| 1 | 0.50 min | 95.00 % | 5.00 % | 0.300 mL/min |
| 2 | 8.00 min | 5.00 % | 95.00 % | 0.300 mL/min |
| 3 | 10.00 min | 5.00 % | 95.00 % | 0.300 mL/min |
| 4 | 10.50 min | 95.00 % | 5.00 % | 0.300 mL/min |
| 5 | 15.00 min | 95.00 % | 5.00 % | 0.300 mL/min |

The liquid chromatography was connected to a 6540 accurate-mass quadrupole time-of-flight mass spectrometer (QTOF) (Agilent, Santa Ana, USA). Sample ionization was achieved with a jet stream electrospray operated in either positive or negative ion mode under the following conditions: sheath gas temperature 350 °C; nebulizer 40 psi; gas flow 8 L/min; gas temperature 250 °C; skimmer 65 V; fragmentor 175 V; nozzle 1000 V; octopole RF 750 V; and capillary 3.5 kV. Accurate mass spectra were acquired in scan mode (170 – 1700 m/z). Reference masses were 121.0509 and 922.0098 m/z with a resolution of 19546 at 922.0106 m/z. Data was acquired and analysed with MassHunter software.

**Table B2 Micropollutants found in wastewater sources, concentrations and analysed for in bio-struvite samples, Kosek et al, 2020.**

| Group of compounds | Identified compounds | Molar Mass | Highest concentration (µg/L) |
|---|---|---|---|
| Antidepressant agents | Citalopram | 324.4 | 840 |
| Antiepileptics | Gabapentin | 171.24 | 79.86 |
| Analgesics/anti-inflammatories | Tramadol | 263.37 | 59.05 |
| Antiretroviral agents | Lamivudine | 229.26 | 55.76 ± 5.48 |
| Antiretroviral agents | Zidovudine | 267.24 | 37.14 ± 2.56 |
| Antiretroviral agents | Efavirenz | 315.67 | 34 ± 2.8 |
| H2-receptor antagonists | Valsartan | 435.5 | 28.22 |
| Metabolites | *N*-acetyl-4-aminoantipyrine | 245.28 | 25.03 |
| Industrial chemicals | 4-Methyl-1H-benzotriazole | 133.15 | 24.3 |
| Analgesics/anti-inflammatories | Diclofenac | 296.1 | 23.5 |
| Artificial sweetener | Acesulfame | 163.15 | 22.5 |
| Industrial chemical | 1H-benzotriazole | 119.12 | 22.1 |
| Artificial sweetener | Sucralose | 397.6 | 18.8 |
| Angiotensin receptor antagonist | Irbesartan | 428.5 | 17.9 |
| Contrast media | Iopromide | 791.1 | 17.9 |
| Antiretroviral agents | Darunavir | 547.7 | 17 ± 0.55 |
| Contrast media | Iopamidol | 777.1 | 16.29 |
| Anti-anxiety agents | Bromazepam | 316.15 | 15.54 |
| Analgesics/anti-inflammatories | Naproxen | 230.26 | 14.4 |
| Analgesics/anti-inflammatories | Acetaminophen | 151.16 | 11.73 |

| | | | |
|---|---|---|---|
| Contrast media | Diatrizoate | 613.91 | 11.73 |
| Stimulants | Caffeine | 194.19 | 11.45 |
| Metabolites | Metronidazole-OH | 187.15 | 11.34 |
| Contrast media | Iomeprol | 777.1 | 11.25 |
| Antidiabetic drugs | Metformin | 129.16 | 10.35 |
| Diuretics | Furosemide | 330.74 | 9.96 |
| Analgesics/anti-inflammatories | Nimesulide | 308.31 | 9.73 |
| Metabolites | 4-Aminoantipyrine | 203.24 | 9.29 |
| Metabolites | 4-Methylaminoantipyrine | 217.27 | 9.25 |
| Analgesics/anti-inflammatories | Ibuprofen | 206.28 | 9.2 |
| Metabolites | Erythromycin-H2O | 733.9 | 7.84 |
| Anti-anxiety agents | Oxazepam | 286.71 | 7.43 |
| Contrast media | Diatrizoic acid | 613.91 | 7.03 |
| Metabolites | 4′-Hydroxy diclofenac | 700.7 | 7.02 |
| Beta-blockers | Metoprolol | 267.36 | 5.76 |
| Metabolites | Erythro/threo-hydrobupropion | 242.76 | 5.7 |
| Metabolites | o-desmethylvenlafaxine | 263.37 | 5.5 |
| Antidepressant agents | Venlafaxine | 277.4 | 5.5 |
| Analgesics/anti-inflammatories | Codeine | 299.4 | 5.27 |
| Analgesics/anti-inflammatories | Ketoprofen | 254.28 | 5.25 |
| Antibiotics | Cephalexin | 347.4 | 5.07 |
| Flame retardants | Tri-(2-chloroisopropyl)phosphate | 327.6 | 4.9 |
| Analgesics/anticonvulsant | Carbamazepine | 236.27 | 4.61 |
| Flame retardants | Tris-(2-butoxyethyl)phosphate | 398.5 | 4.6 |
| Sunscreen Agent | 4-Benzophenone | 262.96 | 4.31 |
| Preservative and anti-infective agent | Triclosan | 289.5 | 4.26 |
| Industrial chemicals | 2,4,7,9-Tetramethyl-5-decyne-4,7-diol | 226.35 | 4.2 |
| Antiepileptics | Lamotrigine | 256.089 | 4.12 |
| Diuretics | Theobromine | 180.16 | 4.01 |
| Antiretroviral agents | Lopinavir | 628.8 | 3.8 ± 0.35 |
| Metabolites | 10,11-Dihydro-trans-10,11-dihydroxy-carbamazepine | 270.28 | 3.6 |
| Metabolites | Carbamazepine-10,11-epoxide | 252.27 | 3.58 |
| Antiretroviral agents | Raltegravir | 444.4 | 3.5 ± 1.3 |
| Diuretics | Hydrochlorothiazide | 297.7 | 3.42 |
| Transformation product (oxidation) | Carboxy-Acyclovir | 239.19 | 3.4 |
| Beta-blockers | Sotalol | 273.37 | 3.33 |

| | | | |
|---|---|---|---|
| Antibiotics | Sulfamethoxazole | 253.28 | 3.25 |
| Bronchodilator | Theophylline | 180.16 | 3.17 |
| Lipid regulator | Bezafibrate | 361.8 | 3.12 |
| Metabolites | Cotinine | 176.21 | 3.1 |
| Beta-blockers | Atenolol | 266.34 | 2.87 |
| Angiotensin receptor antagonist | Telmisartan | 514.6 | 2.75 |
| H2-receptor antagonists | Cimetidine | 252.34 | 2.61 |
| Metabolites | Metoprolol acid | 267.32 | 2.51 |
| Antibiotics | Trimethoprim | 290.32 | 2.4 |
| Flame retardant | Tris(2-butoxyethyl)phosphate | 389.5 | 2.4 |
| Antibiotics | Penicillin G | 334.4 | 2.22 |
| Industrial chemicals | Tolyltriazole | 155.13 | 2.2 |
| Antibiotics | Levofloxacin | 361.4 | 2.19 |
| Analgesics | Salicylic acid | 138.12 | 2.18 |
| Angiotensin receptor antagonist | Candesartan | 440.5 | 1.99 |
| Antiretroviral agents | Nevirapine | 266.3 | 1.9 ± 0.68 |
| Metabolites | 10-Hydroxy-10,11-dihydrocarbamazepine | 238.28 | 1.9 |
| Psychoanaleptics | Desmethylvenlafaxine | 263.37 | 1.87 |
| Metabolites | Guanylurea | 102.1 | 1.86 |
| Antibiotics | Clarithromycin | 748 | 1.79 |
| Lipid-regulators | Simvastatin | 418.6 | 1.74 |
| Analgesics/anti-inflammatories | Aminopyrine | 231.29 | 1.68 |
| Anti-allergic agents | Fexofenadine | 501.7 | 1.61 |
| Metabolites | Benzoylecgonine | 289.33 | 1.6 |
| Metabolites | 4′-Hydroxy aceclofenac | 370.2 | 1.6 |
| Flame retardant | Tris(1-chloro-2-propyl)phosphate | 327.6 | 1.6 |
| Antiretroviral agents | Ritonavir | 720.9 | 1.50 ± 0.053 |
| Antibiotics | Norfloxacin | 319.33 | 1.5 |
| Phytosterols | Beta-sitosterol | 414.7 | 1.5 |
| Metabolites | *O*-Desmethyltramadol | 243.35 | 1.47 |
| Industrial chemicals | Methylindole | 189.21 | 1.42 |
| Beta-blockers | Labetalol | 328.4 | 1.4 |
| Solvents | 2-Butoxyethanol | 118.17 | 1.4 |
| Antibiotics | Erythromycin | 733.9 | 1.39 |
| H2-receptor antagonists | Ranitidine | 314.41 | 1.38 |
| Hormones | Progesterone | 314.5 | 1.34 |
| Metabolites | Carboxy-ibuprofen | 250.29 | 1.27 |
| Antihistamines | Cetirizine | 388.9 | 1.24 |
| Antiepileptics | Pregabalin | 159.23 | 1.24 |
| Flame retardant/plasticizer | Tris(2-chloroethyl)phosphate | 285.5 | 1.16 |
| Antibiotics | Ciprofloxacin | 331.34 | 1.08 |

| | | | |
|---|---|---|---|
| Angiotensin receptor antagonist | Eprosartan | 424.5 | 1.04 |
| Analgesics/anti-inflammatories | Lidocaine | 234.34 | 1 |