CRANFIELD UNIVERSITY

Francisco Adriano Pereira Simões

A new route to recover phosphorus from wastewater: biological struvite production

School of Water, Environment and Energy
Research Degree

PhD
Academic Year: 2013 - 2017

Supervisors: Dr. Ana Soares and Prof. Tom Stephenson
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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Struvite production mediated by bacteria has opened a new route for phosphorus (P) recovery but its application to wastewater sludge dewatering liquors (SDL) has not yet been explored. The aim of this PhD was to understand the key environmental conditions and factors responsible for biological struvite (bio-struvite) production towards delivering a process that could be applied in wastewater treatment plants (WWTPs).

The growth of *Brevibacterium antiquum*, *Bacillus pumilus* and *Halobacterium salinarum* and bio-struvite production was investigated in SDL at pHs from 5.7 to 9.1. The highest production bio-struvite production (135-198 mg/L) was observed at pH 7.3-8.3. Acetate, oleic acid, NaCl, NH_4-N, and Ca^{2+} influenced the growth rate of *B. antiquum* in SDL. Acetate and NaCl (1124 mg COD/L and 3% w/v, respectively) increased the growth rate of *B. antiquum*, from 0.91 to 3.44 1/d, but NaCl was found to hinder bio-struvite formation. Based on this information, the enrichment of *B. antiquum* in mixed-culture system was attempted but the relative abundance of *B. antiquum* declined from 96.4% to 0.9-1.5%, after 25 days.

B. antiquum was able to use 12.4 mg P/L of organic and condensed phosphorus and 13.2 mg P/L of phosphate to form 172 mg/L of bio-struvite. This demonstrated that *B. antiquum* was able to produce bio-struvite from P fractions that are not recovered through the chemical struvite precipitation process. Furthermore, bio-struvite had a higher purity (91%) than chemical struvite (50%). Only small differences were found on the growth of mustard (*Brassica nigra*) seedlings and on micropollutants content.

Overall this study clearly demonstrated the viability of the bio-struvite production process for recovering phosphorus from sludge dewatering liquors.

Keywords:

bio-struvite; biomineralization; *Brevibacterium antiquum*; sludge; dewatering; liquors; phosphorus recovery; growth rate; mixed-culture; enrichment; fertilizer value; P-recovery; nutrient recovery; centrate; reject liquors; return liquors, supernatant; digestate; bio-augmentation; *Bacillus pumilus*; *Halobacterium salinarum*.

ACKNOWLEDGEMENTS

I want to leave my Thank You to all that helped make this thesis possible, without their help my life would have been a bit less happy, and sometimes a bit more bleak.

First on the list is Dr. Ana Soares. Thank you for the opportunity, the guidance, the support, the patience, and the ambition, without which I believe would not have reached this far. Also thank you to Prof. Tom Stephenson for its key support and availability. To Dr. Pete Vale for the interest, advise and always prompt support. To Andy Blake, and David Thompson for having received me so well in Derby, and for having answered all the questions and requests. The same extends to Simon Oakden and Chris Borredaille, their willingness to help and availability made some long days quite a bit easier.

I would also like to thank Yoann, and Catarina, whose dedication gave me a very good help, but also for what they taught me. Caroline, Shirley, Ekwu, Sarah and André, and for all the others that were always available for a question or for a last minute request for material, thank you!

To my family, for their support, for their hopes, and for the patience listening of what is so important with phosphorus.

To my friends that listened to all the ramblings, and super smart things I say, André, Anish, Pablo, João, Gonçalo, Carla, António, and many others, I wish you all the best, I will always be grateful for your kindness, and for the happiness we've shared.

And to Alicia. My friend, my love, the best gift Cranfield brought me, thank you for being my partner in this cosmic adventure that is life, without you my life would be so much less – thank you for all.

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ADS	Anaerobically digested sludge
AOB	Ammonium oxidising bacteria
AS	Activated sludge
ATP	Adenosine triphosphate
BCM	Biologically controlled mineralization
BIM	Biologically induced mineralization
BioS-P	Bio-struvite phosphorus
BNR	Biological nutrient removal
CCD	Central composite design
CHES	Cyclohexylamino-ethane-sulfonic acid
COD	Chemical oxygen demand
CPR	Chemical phosphorus removal site
Cond-P	Condensed phosphorus
DNA	Deoxyribonucleic acid
EBPR	Enhanced biological phosphorus removal
EDX	Energy dispersive X-ray
EPPS	N-2-hydroxy ethylpiperazine-N9-propane-sulfonic acid
ESEM	Environmental scanning electron microscope
HMP	Hexametaphosphate
ICP-OES	Inductively coupled plasma optical emission spectrometry
MES	N-(morpholino)-ethane-sulfonic acid
NOB	Nitrite oxidising bacteria

NPR	No phosphorus removal site
ON	oxidised nitrogen
Org-P	Organic phosphorus
P	Phosphorus
PAH	Poly-aromatic hydrocarbons
PAOs	Phosphorus accumulating organisms
PB	Plackett-Burman experimental design
PCR	Polimerase chain reaction
PE	Population equivalent
PHA	Polyhydroxyalkanoate
PLFA	Phospholipid-derived fatty acid analysis
polyP	Polyphosphate
PSA	Path of steepest ascent method
PTFE	Polytetrafluoroethylene
QTOF	Quadrupole time-of-flight mass spectrometer
RNA	Ribonucleic acid
SBR	Sequencing batch reactor
SBRs	Sequencing batch reactors
SDL	Sludge dewatering liquors
SEM	Scanning electron microscope
TN	Total nitrogen
TP	Total phosphorus
TS	Total solids
TSS	Total suspended solids

UASB	Up-flow anaerobic sludge blanket reactor
WWTP	Wastewater treatment plant
WWTPs	Wastewater treatment plants

Chapter 1 Introduction

1.1 Project background

Phosphorus (P) is a non-replaceable, essential nutrient for all living organisms. It is a component of important bio-molecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP) and phospholipids, providing for the transport and trade of energy within the cells, and for the backbone of every cell wall membrane.

Phosphorus is a major limiting nutrient to crop production and to food security (Cordell et al*., 2009*; Ryan et al*., 2013). Cur*rent food production is dependent on P-rich fertilizers as unfertilized soils do not release nutrients fast enough to support the high growth rates of crop species (Jenks & Hasegawa, 2008; Kebreab *et al., 2012;* Bennett & Elser, 2011; Elser, 2012; Tian et al*., 2012).*

*Pho*sphorus demand in Europe is driven by agriculture and food production (91%), with the remainder used by non-food applications (van Dijk et al*., 2016*). Phosphorus is mainly extracted from rock phosphate, a natural mineral with 4.9-6.5% w/w of P content. However, rock phosphate has limited availability, with asymmetric world distribution (Desmidt *et al.*, 2015). Rock phosphate is one of twenty critical raw materials for the European Union (European Commission, 2014). Hence, there is a need to find ways to recover and recycle phosphorus from waste streams.

Wastewater treatment plants (WWTPs) are the main point-source for P emissions to surface waters, and the release of P to surface waters can compromise their quality and increase the risk for algal blooms and eutrophication (Comber *et al.*, 2013). However, WWTPs in the UK receive 125 ton P/day, in the form of raw wastewater with 3-10 mg P/L (DEFRA, 2012; ONS, 2012). Phosphorus fertilizer application on farm crops in 2016 was estimated to be 87700 ton P, corresponding to 234 ton P/day (DEFRA 2017). Existing processes for P-removal from wastewater accumulate phosphorus in the wastewater sludge, from where phosphorus can be recovered.

P-recovery from wastewater sludge is done via land application of the sludge or via extraction of P from sludge ash after incineration, or through chemical precipitation of struvite or calcium phosphate from sludge or sludge dewatering liquors. Struvite precipitation from sludge dewatering liquors is the most studied process, and has been implemented successfully in several WWTPs, including in the UK (Kleemann *et al.*, 2015; Mayer *et al.*, 2016). However, the chemical precipitation of struvite requires the use of chemicals or CO_2 stripping, to raise the pH to >8.5, the addition of a magnesium source to maximize the amount of recovered phosphate, and the use of complex reactors (Le Corre *et al.*, 2009). Thus, struvite precipitation has been reported to only be viable in streams with more than 100 mg P/L (Dockhorn, 2009), a concentration difficult to obtain in smaller WWTPs. Hence, there is a need to find economically viable processes for P-recovery from wastewater streams with less than 100 mg P/L.

Recently, the precipitation of struvite mediated by selected microorganisms was shown to be possible in settled wastewater with 7.5 mg PO_4-P/L, and in sludge dewatering liquors with 30.5 mg PO_4-P/L, without the use of chemicals. The application of *Bacillus pumilus* and *Brevibacterium antiquum,* on sludge dewatering liquors produced large struvite crystals (up to 250 µm) and removed 93 – 95% of the phosphate from the liquors (Soares *et al.*, 2014). This opened up a completely new way to recover phosphorus from wastewater derived streams.

The biological formation of minerals, biomineralization, is widespread in nature. Numerous microorganisms have been reported to promote biomineralization of very distinct minerals, both in natural environments, and also in synthetic culture media under sterile conditions (Mann, 2001). Reports range from the formation of minerals that lead to the entombment of the bacteria in calcium carbonate (Rodriguez-Navarro *et al.*, 2012), to the genetically controlled formation of pure magnetite crystals, which the magnetotactic bacteria use as a navigation compass to reach low-oxygen environments (Moisescu et al*., 2014*). Alongside with the species-specific, genetically controlled formation of silicate shells by diatom algae, which serve as a model for research in nano-fabrication, due to

their control over mineral shell pattern details at micro- and nano-scale (Gröger *et al.*, 2016).

The biomineralization mechanisms of the struvite forming bacteria are still poorly understood and process development and application to wastewater has not yet been explored. Basic understanding of how, why and what for bio-struvite is produced by biomineralizing bacteria is still a significant unknown. Furthermore is not understood if biologically produced struvite (bio-struvite) is different from chemically precipitated struvite.

The identification of minerals in the granules of granular wastewater treatment processes is a recent development that has been capturing research interest. Struvite, calcium phosphate, and calcium carbonate have been found in the cores of granules of different types of granular wastewater treatment processes (Juang *et al.*, 2010; Lin, Lotti, *et al.*, 2013; Li *et al.*, 2014; Liu *et al.*, 2016; Zhang *et al.*, 2016). The mineral cores at the centre of granules of aerobic granular sludge and of anaerobic granular sludge, were found to have tree trunk like ring structure, providing evidence for an evolving mineral deposition (Mañas, Spérandio, *et al.*, 2012). Struvite in the granules of enhanced biological phosphorus removal (EBPR) granular sludge was proposed to have been biomineralized via the effect of alginate-like polysaccharides present in the granules (Lin et al.*, 2012*). Recently, direct application to land of the granules has been suggested as a form of P-recovery of the phosphate minerals found at the core of the granules in granular Anammox processes (Johansson et al.*, 2017*).

Although these granular processes were not initially designed for biomineralization, or for the recovery of the bio-minerals, these cases offer tantalizing evidence in support of the potential for bio-struvite formation in wastewater treatment applications.

1.2 Aims and objectives

The aim of the PhD was to understand the key environmental conditions and factors responsible for biological struvite (bio-struvite) production and growth of selected bacteria in sludge dewatering liquors towards delivering a bio-struvite production process in WWTPs using sustainable and economical systems.

The project key objectives were to:

A. Understand the impact of pH variability on the ability of selected bacteria to grow and to produce bio-struvite

B. Identify the compounds that influence the growth rate of *Brevibacterium antiquum* and the production of bio-struvite in sludge dewatering liquors

C. Understand what fractions of phosphorus are used by *B. antiquum* to produce bio-struvite and predict the potential of various sludge dewatering liquors to apply P recovery processes

D. Understand the operational envelope in sequencing batch reactors (SBR) to obtain the enrichment of *B. antiquum* in mixed open cultures conditions

E. Understand and quantify the fertilizer value of bio-struvite produced from sludge dewatering liquors.

1.3 Thesis plan

Each chapter of this thesis was written in the format of a journal paper (submitted or in preparation). Figure 1-1 presents the general organization of the objectives and chapters.

Francisco Adriano Pereira Simões undertook all the planning, experimental work and data analysis. All chapters were written by the first author Francisco Adriano Pereira Simões with comments from supervisors Dr Ana Soares and Prof Tom Stephenson. Dr Peter Vale, from Severn Trent Water PLC, provided industrial supervision. Named authors, completed part of the experimental work in chapters 5, 6, and 7, under the supervision of Francisco Adriano Pereira Simões. Bacterial community profiling in Chapter 6, poly-aromatic hydrocarbons (PAH) and trace metal measurements, in Chapters 5 and 7, were completed by

an external commercial laboratory under the direction of Francisco Adriano Pereira Simões.

Chapter 2 reviewed the state of the art on bacterial biomineralization of struvite. This chapter identified extensive gaps in knowledge for the bacterial biomineralization of struvite, and on the ability of the bacteria to grow in sludge dewatering liquors. Knowledge gained in this chapter established the basis for the methodologies used in Chapters 3, 4, 5, 6, and 7.

Chapter 3 investigated the impact of the sludge dewatering liquors pH variability on the growth of selected bacteria and bio-struvite productivity. Insights gained in this chapter were used to select *B. antiquum* bacteria for further study in the remainder of the thesis and to plan all the following experiments. This chapter has been submitted to the journal *Scientific Reports*.

Chapter 4 aimed to identify components of sludge dewatering liquors that influence the growth rate of *B. antiquum*. Also, to elucidate how the components responsible for optimal growth of *B. antiquum* affect bio-struvite production. The outcomes of this paper were used to support the experimental planning of chapters 6 and 7. This chapter has been submitted to the journal *Environmental Technology*

Chapter 5 quantified phosphorus fractions in sludge dewatering liquors and investigated how *B. antiquum* used these forms of phosphorus to produce bio-struvite. Furthermore, an extensive characterization of liquors originating from various types of WWTPs with different phosphorus removal technologies to predict the potential the various liquor streams to apply recovery processes.

Chapter 6 investigated the enrichment of *B. antiquum* in sludge dewatering liquors in an open mixed-culture system, building form the knowledge developed in Chapters 2, 3, and 4.

Chapter 7 addressed the fertilizer value of the bio-struvite obtained from from sludge dewatering liquors. This chapter also provided a comparison on the characteristics of bio-struvite with struvite obtained by conventional chemical precipitation relative to purity, fertilizer value and occurrence of hazardous substances such as heavy metals and other micropollutants. This chapter builds on the knowledge developed in Chapters 2, 3, and 4.

Chapter 8 details and overall synthesis of the PhD outputs and discusses the contributions to knowledge and the areas for future work.

Chapter 9 brings together the overall conclusions of the project in relation to the initial objectives.

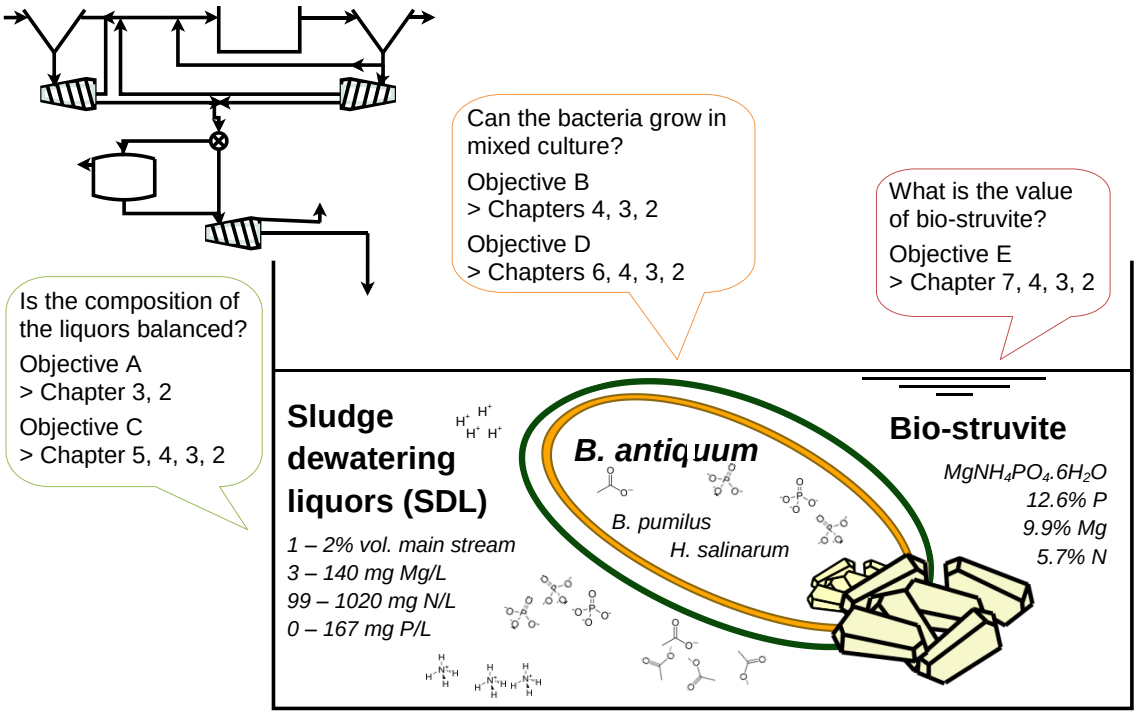

Figure 1-1 Schematic representation of the thesis structure.

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Chapter 2 Application of biomineralization for phosphorus recovery

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Abstract

Biomineralization refers to the processes by which microorganisms produce minerals. Several microorganisms and microbial communities have been found to be able to produce phosphorus (P) rich bio-minerals. Hence, biomineralization may have a role to improve phosphorus removal and recovery in wastewater treatment processes. The biological formation of struvite (bio-struvite) in synthetic media has been found to be relatively widespread amongst soil and aquatic bacteria. Nevertheless, the mechanisms of bio-struvite formation and the biochemical benefits of producing P-rich minerals are not yet fully understood. This review aims at scrutinising the advantages and barriers for the use of biomineralization processes in wastewater treatment plants for phosphorus recovery. Two main paths were identified: the direct application of bacteria to wastewater streams in order to produce bio-struvite; and the development of struvite or calcium-phosphate minerals within the granular sludge currently used in aerobic, anaerobic and Anammox processes.

Keywords

Biocrystalisation, bio-mineral, bio-struvite, struvite, sludge, dewatering liquors.

2.1 Introduction

Phosphorus (P) is an essential nutrient for all living organisms. It is a major constraint to crop output and food security (Cordell et al*., 2009*; Ryan et al*., 2013). Wor*ldwide phosphorus demand is driven largely by agriculture and the chemical industry (Gilbert, 2009). Phosphorus is extracted from rock phosphate, a P-rich mineral with 4.9 to 6.5% (w/w) as P. However, sources of concentrated, usable phosphorus are limited, and have a widely asymmetric world distribution. Hence, P is taken as a strategic resource by several countries (Cordell *et al., 2009*; Gilbert, 2009). Recovery and recycling of P from waste streams has been pointed out as possible route to address the source supply issues.

One of the main point source of P emissions is the discharge of final effluent of wastewater treatment plants (WWTP) to natural waters. In the UK, the estimated daily load of P per person in domestic wastewater is 2.06 g/(person.d) (Gilmour *et al.*, 2008). With 63.2 million inhabitants, and 96% of the population connected to sewers, the total daily quantity of P arriving to WWTP is estimated to be 125 ton/day (DEFRA, 2002; ONS, 2012).

Wastewater and sludge streams have been reported to have a P content of 3-10 mg/L in raw wastewater, 1-167 mg/L in sludge dewatering liquors, and up to 42 g/kg in sludge dry matter (Battistoni *et al.*, 2000; Fux *et al.*, 2002; Soares *et al.*, 2010; Herzel *et al.*, 2016).

[a] Soares *et al.*, 2010

[b] Jaffer e*t al.,* 2002, Galvagno et *al., 2*016

[c] calculated from 0.8-2.8 %TS as P_2O_5 (Kominko *et al.*, 2017) and assuming 5%TS and 1.02 specific gravity (Tchobanoglous *et al.*, 2003)

[d] Ebbers e*t al.,* 2015 and calculated from 346 mg P/L in un-thickened BNR secondary sludge (Acevedo *et al.*, 2012) assuming a thickening to 5%TS (Tchobanoglous *et al.*, 2003)

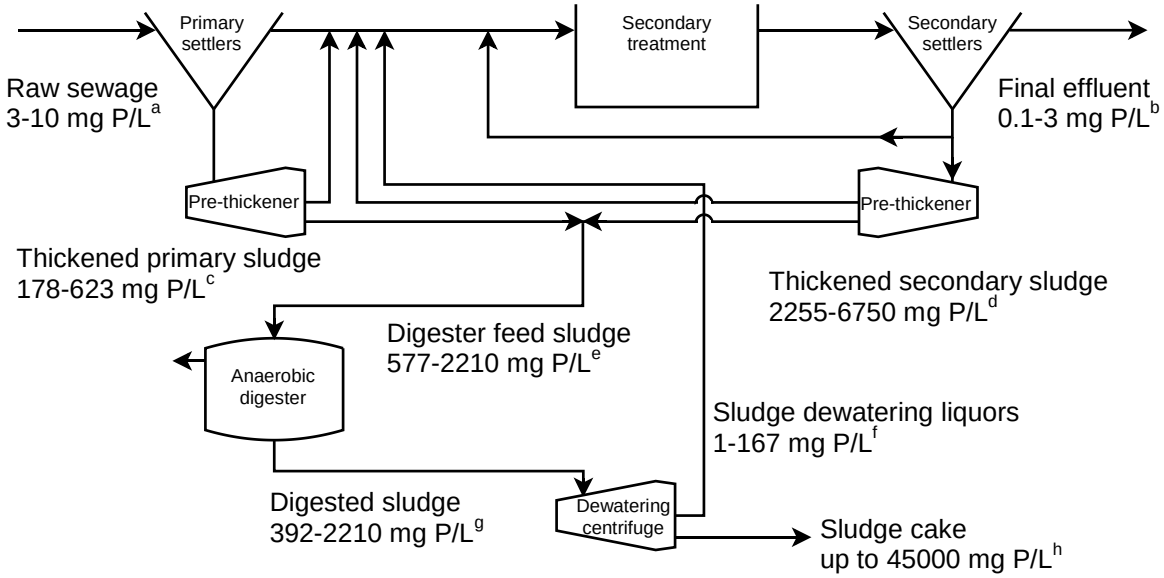
[e] Ebbers *et al.*, 2015

[f] Battistoni e*t al.,* 2000; Fux e*t al.,* 2002; Pastor, Mangin, e*t al.,* 2008

[g] Uysal e*t al.,* 2010, Ebbers e*t al.,* 2015

[h] calculated from 42 g P/Kg TS (Herzel *et al.*, 2016) assuming a sludge solids specific gravity of 1.4 and 20-23%TS in the sludge cake (Tchobanoglous *et al.*, 2003)

Figure 2-1 presents typical values of the concentration of phosphorus in a common WWTP flow-sheet.



[a] Soares *et al.*, 2010

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[g] Uysal e*t al.,* 2010, Ebbers e*t al.,* 2015

[h] calculated from 42 g P/Kg TS (Herzel *et al.*, 2016) assuming a sludge solids specific gravity of 1.4 and 20-23%TS in the sludge cake (Tchobanoglous *et al.*, 2003)

Figure 2-1 Typical wastewater treatment flow-sheet. Values at the marked streams indicate total phosphorus concentration.

Existing processes for P removal from wastewater involve chemical precipitation or enhanced biological phosphorus removal (EBPR). For the chemical precipitation of phosphorus, metal salts, as ferric chloride, are added to the wastewater in order to precipitate the phosphate. Flocculation and coagulation of the precipitated salts, and adsorption of further phosphorus substances to the produced particles are also said to play a role in P-removal. In EBPR systems, phosphate accumulating organisms (PAOs) take up phosphorus from the wastewater and accumulate intracellular polyphosphates. With both technologies phosphorus is removed from the main stream of

treatment with the sludge (Tchobanoglous et al*., 2003*). In both processes P-removal takes place by accumulation in the wastewater sludge.

Currently, there are three main routes to recover P from wastewater sludge: sludge recycling to land, chemical P precipitation in the form of struvite or calcium phosphate and P recovery from sludge ash (Desmidt *et al.*, 2015). In Europe, the proportion of total sludge produced that is recycled to agriculture has been reported to 37%, but application varies widely between member states, from 0.006 to 70% (Evans, 2012). However, the P content on sludge has been reported to be <1% (w/w) in P (Soares *et al.*, 2010), which is a low proportion when compared with conventional sources of P used for land application as rock phosphate (4.9-6.5 % w/w as P). Furthermore, sludge recycling to land has received mixed views. Reports on contamination of soils with hazardous pollutants, after sludge spreading to land, have made this route of P recovery rather problematic and constrained by legislation and social acceptance (Hospido *et al.*, 2010).

P recovery from sludge ash has the potential to recover 58-80% P inputs to the WWTP (

[a] Soares *et al.*, 2010

[b] Jaffer e*t al.,* 2002, Galvagno et *al., 2*016

[c] calculated from 0.8-2.8 %TS as P_2O_5 (Kominko *et al.*, 2017) and assuming 5%TS and 1.02 specific gravity (Tchobanoglous *et al.*, 2003)

[d] Ebbers e*t al.,* 2015 and calculated from 346 mg P/L in un-thickened BNR secondary sludge (Acevedo *et al.*, 2012) assuming a thickening to 5%TS (Tchobanoglous *et al.*, 2003)

[e] Ebbers *et al.*, 2015

[f] Battistoni e*t al.,* 2000; Fux e*t al.,* 2002; Pastor, Mangin, e*t al.,* 2008

[g] Uysal e*t al.,* 2010, Ebbers e*t al.,* 2015

[h] calculated from 42 g P/Kg TS (Herzel *et al.*, 2016) assuming a sludge solids specific gravity of 1.4 and 20-23%TS in the sludge cake (Tchobanoglous *et al.*, 2003)

Figure 2-1), (Jaffer *et al., 2002*; van Dijk et al., 2016*). How*ever, P recovery from sludge ash requires complex processes with sequential chemical treatment-precipitation steps, or thermal treatment at high temperatures (1000°C) to improve P availability (Schoumans *et al.*, 2015).

Chemical P precipitation in the form of struvite or calcium phosphate is done either from the liquors produced on dewatering sludge or directly from digested sludge, limited to WWTPs with EBPR. Precipitation from digested sludge has

been reported to recover 80-90% of P in sludge, but the produced struvite can have excessive organic contamination and needs washing in sand washers to reduce organic contamination to 0.5% (w/w) in total organic carbon (Desmidt *et al.*, 2015). Recovery from sludge dewatering liquors can provide 10-20% of P in the WWTP (

[a] Soares *et al.*, 2010

[b] Jaffer e*t al.,* 2002, Galvagno et *al., 2*016

[c] calculated from 0.8-2.8 %TS as P_2O_5 (Kominko *et al.*, 2017) and assuming 5%TS and 1.02 specific gravity (Tchobanoglous *et al.*, 2003)

[d] Ebbers e*t al.,* 2015 and calculated from 346 mg P/L in un-thickened BNR secondary sludge (Acevedo *et al.*, 2012) assuming a thickening to 5%TS (Tchobanoglous *et al.*, 2003)

[e] Ebbers *et al.*, 2015

[f] Battistoni e*t al.,* 2000; Fux e*t al.,* 2002; Pastor, Mangin, e*t al.,* 2008

[g] Uysal e*t al.,* 2010, Ebbers e*t al.,* 2015

[h] calculated from 42 g P/Kg TS (Herzel *et al.*, 2016) assuming a sludge solids specific gravity of 1.4 and 20-23%TS in the sludge cake (Tchobanoglous *et al.*, 2003)

Figure 2-1) (Jaffer *et al., 2002*; van Dijk et al., 2016*) with* an end product reported to be applicable without further processing (Schick *et al.*, 2009; Gell *et al.*, 2011).

Sludge dewatering liquors are a by-product of processes aimed at reducing the volume of sludge such as sedimentation, centrifugation and filtration. Table 2-1 summarizes typical characteristics of sludge liquors in published studies.

Table 2-1 Typical characteristics of sludge liquors according to existing literature.

Parameter	Median	Minimum	Maximum
Phosphate (PO_4-P, mg P/L)	44	1 [a,b]	167 [c *]
Ammonium (NH_4-N, mg N/L)	545	99 [b]	1020 [d]
Magnesium (mg/L)	34	3 [d]	64 [b]
Calcium (mg/L)	126	29 [d]	456 [b]
Potassium (mg/L)	75	36 [e *]	241 [c *]
Total nitrogen (TN, mg N/L)	1025	490 [f *]	1280 [d]
Chemical oxygen demand (COD, mg/L)	462	308 [g *]	1762 [f *]
Alkalinity (mg/L as $CaCO_3$)	2205	53 [a]	4156 [d]
Total suspended solids (TSS, mg/L)	239	28 [h *]	2430 [d]
pH	7.8	7.0 [b]	8.3 [d]

[a] sludge digester supernatant from WWTP sludge (Fux *et al.*, 2002)

[b] ADS belt press filtrate from a non EBPR/BNR WWTP (Battistoni *et al.*, 2000)

[c] ADS supernatant from sludge of EBPR WWTP with separate pre-digester thickening for primary and secondary treatment sludges (Pastor, Mangin, *et al.*, 2008)

[d] ADS supernatant produced sludge of non EBPR/BNR WWTPs (Doyle *et al.*, 2000; David, 2007)

[e] ADS supernatant mixed with supernatant from the pre-thickening of secondary treatment sludge from EBPR WWTP (Pastor, Mangin, *et al.*, 2008)

[f] ADS supernatant from sludge of a BNR municipal WWTP (Ahn and Choi, 2006)

[g] ADS supernatant from a BNR WWTP (David, 2007)

[h] ADS belt press filtrate from a BNR WWTP (Battistoni *et al.*, 2006)

* sludge liquors from a WWTP with EBPR or BNR process in the main treatment sequence.

Other sludge liquors considered were the ADS belt press filtrate of a WWTP with nitrification and denitrification (Battistoni *et al.*, 1997), the dewatering filtrate from the ADS of a BNR WWTP (Ueno and Fujii, 2001), and the supernatants of ADS of non EBPR/BNR WWTP with and without iron or aluminium dosing (Jaffer *et al.*, 2002; Le Corre *et al.*, 2009).

ADS, anaerobically digested sludge;

BNR, biological nutrient removal process;

EBPR, enhanced biological phosphorus removal process;

WWTP, wastewater treatment plant.

Sludge dewatering liquors contain significant concentrations of P (up to 167 mg P/L, Table 2-1) in a limited volume (Jaffer *et al.*, 2001), making them ideal to install specialised processes for P-recovery.

Phosphorus recovery through precipitation of phosphate minerals from sludge liquors is being successfully applied in full-scale sites throughout the world

(Mayer *et al.*, 2016). Although calcium phosphate is often mentioned as an alternative to struvite in order to recover phosphorus from wastewater, the large majority of existing commercial processes has struvite as an end product (Desmidt *et al.*, 2015).

Struvite is a hydrated salt of magnesium, ammonium and phosphate ($MgNH_4PO_4.6H_2O$), frequently found as scaling on WWTP pipes, particularly in anaerobically digested sludge lines (Parsons and Doyle, 2004). Struvite can be used as an agricultural fertilizer without further processing (Schick *et al.*, 2009). The combination of high phosphorus content (up to 12.6% w/w as P), significant quantity of ammonium, and low solubility, has been reported to make struvite valuable as a slow release fertilizer (Talboys *et al.*, 2016).

Several factors have been reported to affect struvite precipitation from wastewater derived streams (Table 2-2). However, the chemical struvite precipitation process requires complex reactors, expensive chemical addition and pH adjustments, which limits its application to large sites, with sludge liquors with high P concentrations (>100 mg/L), in order to be economically viable (Dockhorn, 2009; Le Corre *et al.*, 2009).

Table 2-2 Main factors that affect the chemical struvite precipitation process (adapted from Le Corre e*t al., 2009*)**.**

Factor
pH
Supersaturation
$Mg:PO_4:NH_4$ 1:1:1 molar ratio
Presence of crystal nucleation sites
Mixing energy
Temperature
Presence of foreign ions (*e.g.*: Ca^{2+},K^{+},CO_3^{2-})
Ionic strength

Recently, a study tested the ability of selected commercially available bacteria: *Myxococcus xanthus*, *Bacillus pumilus*, *Halobacterium salinarum*, and

Brevibacterium antiquum to produce phosphorus bio-minerals from settled wastewater and from sludge dewatering centrifuge liquors. All selected bacteria were confirmed to promote struvite formation in synthetic media and *B. pumilus* and *B. antiquum* were capable of producing phosphate minerals within 10 days in both sludge dewatering liquors and settled wastewater (Soares *et al.*, 2014).

2.1.1 Microorganisms associated with struvite formation

The connection between struvite and microorganisms was first proposed by Robinson in 1889 (Beavon and Heatley, 1963). Since then, several microbial species have been reported to produce phosphorus bio-minerals as struvite, and calcium phosphate, in synthetic media. Furthermore, in natural environments, the occurrence of struvite in renal stones has also been linked with microbial infections (Prywer and Torzewska, 2010). Table 2-3 lists microorganisms which have been identified to produce struvite in synthetic media.

Table 2-3 Microorganisms able to precipitate struvite in synthetic media in published literature.

Microorganism	Strain	Taxonomic domain	Reference
Acinetobacter sp. *	-	Bacteria	[a, b]
Aeromonas sp.	-	Bacteria	[b]
Alcaligenes sp. *	-	Bacteria	[b]
Arthrobacter sp. *	-	Bacteria	[a, b]
Bacillus pumilus	-, GB 34	Bacteria	[c, d]
sp. *	-		[a, b]
Brevibacterium antiquum	DSM 21545	Bacteria	[e, d]
sp.	-		[a]
Chromohalobacter marismortui	ATCC 17056	Bacteria	[f]
Corynebacterium sp. *	-	Bacteria	[a, b]
Flavobacterium sp.	-	Bacteria	[a]
Halobacillus halophilus	ATCC 35676	Bacteria	[g]
Halobacterium salinarum	DSM 671	Archaea	[e, d]

Microorganism	Strain	Taxonomic domain	Reference
Halomonas aquamarina	ATCC 14400	Bacteria	[g, h]
canadiensis	ATCC 43984		[g, h]
cupida	ATCC 27124		[g, h]
elongata	ATCC 33173		[g, h]
eurihalina	ATCC 49509		[h]
halmophila	ATCC 19717		[g, h]
halodenitrificans	ATCC 13511		[g, h]
halodurans	ATCC 29686		[g, h]
halophila	CCM 3662		[h]
marina	ATCC 25374,		[g, h]
meridiana	ACAM 246		[g]
pacifica	ATCC 27122		[g, h]
pantelleriensis	DSM 9661		[g, h]
salina	ATCC 49509		[g, h]
subglaciescola	ACAM 12		[g, h]
variabilis	DSM 3051		[g, h]
venusta	ATCC 27125		[g, h]
Halorubrum distributum	VKM-1739	Archaea	[e]
Idiomarina abyssalis	ATCC BAA-312	*Bacteria*	[i]
baltica	DSM 15154		[i]
loihiensis	DSM 15497		[i]
sp.	MAH1		[i]
Kurthia sp. *	-	*Bacteria*	[a, b]
Leclercia adcarboxglata	JLS1	*Bacteria*	[j]
Listeria sp.	-	*Bacteria*	[a]
Marinobacter hydrocarbonoclasticus	ATCC 49840	*Bacteria*	[g, h]
Marinomonas communis	DSM 5604	*Bacteria*	[g, h]
vaga	ATCC 27119		[g]
Micrococcus sp. *	-	*Bacteria*	[b]
Murraya sp. *	-	*Bacteria*	[b]
Myxococcus coralloides	D	*Bacteria*	[k, l]
xanthus	CECT 422		[m, k, n, o, p, q, d]
Paramecium tetraurelia	51s	*Eukarya*	[r]
Plesiomonas sp.	-	*Bacteria*	[b]
Proteus mirabilis	-	*Bacteria*	[s, t, u]
Pseudomonas halophila	DSM 3050	*Bacteria*	[g]
sp. *	-		[a, b]
Salibacillus salexigens	DSM 11483	*Bacteria*	[g]
Salinivibrio costicola	NCIMB 701	*Bacteria*	[g, h]
Staphylococcus aureus	-	*Bacteria*	[v]
sp.	-		[b]

Microorganism	Strain	Taxonomic domain	Reference
Streptomyces acidiscabies	E13	*Bacteria*	[w]
Trypanosoma cruzi	Maracay	*Eukarya*	[x]

* multiple species of the same genera reported as being able to form struvite; sp., actual specific name of the species not detailed in original publication; a, Rivadeneyra *et al.* 1983; b, Rivadeneyra *et al.* 1992; c, Nelson *et al.* 1991; d, Soares *et al.* 2014; e, Smirnov *et al.* 2005; f, Rivadeneyra, Martín-Algarra, *et al.* 2006; g, Rivadeneyra, Delgado, *et al.* 2006; h, Sánchez-Román *et al.* 2007; i, González-Muñoz *et al.* 2008; j, Han *et al.* 2016; k, Omar *et al.* 1996; l, González-Muñoz *et al.* 1993; m, Omar *et al.* 1995; n, Omar *et al.* 1998; o, González-Muñoz *et al.* 1996; p, Jimenez-Lopez *et al.* 2007; q, Da Silva *et al.* 2000; r, Grover *et al.* 1997; s, McLean *et al.* 1991; t, Chen *et al.* 2010; u, Prywer and Torzewska, 2010; v, Beavon and Heatley, 1963; w, Haferburg *et al.* 2008; x, Adroher *et al.* 1988.

Rivadeneyra *et al.* (1983), found 20 bacterial strains able to produce struvite, out of 96 strains of soil bacteria previously reported to precipitate calcite. In a later work, 94 out of 161 strains of soil and fresh water bacteria, were able to form struvite in synthetic media, but no relationship was found with the taxonomic genera of the bacteria tested (Rivadeneyra *et al.*, 1992). Nevertheless, of a total of 148 microorganisms, found in literature, reported to form struvite in synthetic media, 144 belong to the domain *Bacteria*, 2 belong to the domain *Archaea* and 2 to the domain *Eucarya* (Table 2-3).

Precipitation of struvite was reported for 22 strains of moderately halophilic bacteria in synthetic marine salts media with >7.5% salt concentration. Struvite formed after carbonate precipitation which suggested that the formation of struvite required a decrease in the calcium concentration in the media. (Rivadeneyra, Delgado, *et al.*, 2006). Seventeen of the same strains, and 2 other strains, were tested in synthetic media with 7.5% NaCl (w/v) at different magnesium to calcium ratios. Struvite precipitated in the tests with more than 1.63 Mg:Ca ratio and the crystals were visible in the media up to 15 days sooner, than in the tests without calcium (Sánchez-Román *et al.*, 2007). These works highlighted that calcium concentration in the media influenced the biomineralization of struvite but also that struvite was able to form in the presence of salt concentrations >7.5%.

A common reference in these works was the description of the microorganisms as aerobic, and chemoorganotrophic (Rivadeneyra, Martín-Algarra, *et al.*, 2006; Sánchez-Román *et al.*, 2011). This fits the description of both *B. pumilus* and *B. antiquum*.

2.1.2 Biomineralization mechanisms

The occurrence of struvite in synthetic media has been hypothesized to be a consequence of the microbial degradation of the nitrogenous organic matter in the media. The degradation of nitrogenous substances leads to production of ammonia which combines with the magnesium and phosphate present, to form struvite (Beavon and Heatley, 1963; Rivadeneyra *et al.*, 1983).

In medical and veterinary research, struvite biomineralization is said to be a consequence of the ureolytic activity of infectious microorganisms. Urea hydrolysis produces ammonia, and increases the pH of urine, thus creating conditions for the precipitation of struvite. But no complete conclusion has been made for the existence of additional intervention in the formation of struvite stones by the infectious microorganisms (Prywer and Torzewska, 2010).

Lowenstam & Weiner (1989), defined two main mechanisms for biomineralization, depending on the level of control exerted by the intervening organism: biologically induced mineralisation (BIM), and biologically controlled mineralisation (BCM). In BIM, the precipitation of minerals is a collateral consequence of the reaction between extracellular ions and metabolic products extruded across, or into the cell wall. The mineral products of BIM are expected to have a heterogeneous range of crystal and chemical properties, and may be found closely associated with the cell wall. The sulphate-reducing bacteria *Leclercia adcarboxglata* JLS1 was found to precipitate struvite from synthetic media after driving the pH from 7.2 to 8.6 (Han *et al.*, 2016). Uranium precipitates formed in the periplasmic space of *Geobacter metallireducens* cells when uranium (VI) was used by the bacteria as electron acceptor (Gadd, 2010).

The mechanism of struvite biomineralization in synthetic media proposed by Beavon and Heatley, 1963, fits the description of BIM, where the formation of struvite occurs as a collateral consequence of the production of ammonia by the microorganisms.

On the other side, biologically controlled mineralisation occurs through specialised regulation of mineral deposition. BCM is expected to result in functional materials with reproducible, homogeneous, species-specific properties as: particle size, structure and composition, spatial organization, complex morphologies, controlled aggregation and texture, preferential crystal orientation, high-order assembly, and hierarchical structures (Mann, 2001). Magnetotactic bacteria and diatom algae fit under this mechanism description. Unicellular diatom algae produce species-specific mineral shells of amorphous silica deposited in a matrix of specialised proteins, creating intricate micrometer and nanometre scale patterns (Hedrich *et al.*, 2013). The magnetotactic bacteria produce crystals of iron oxide magnetite (Fe_3O_4) or iron sulphide greigite (Fe_3S_4) in specialised organelles called magnetosomes. The produced crystals have uniform, species-specific, size and composition, which the bacteria use as a compass to guide their movement in search of low-oxygen environments (Rahn-Lee and Komeili, 2013).

It is not clear which biomineralization mechanisms lead to the formation of struvite when incubating *B. pumilus*, and *B. antiquum,* in settled wastewater, and in sludge dewatering liquors. Given the presence of high quantity of ammonium in sludge dewatering liquors (629 mg NH_4-N/L), well in excess of phosphate (30 mg PO_4-P/L) and magnesium (39 mg/L), an increase in ammonium concentration does not seem the likely trigger for the struvite precipitation. This suggests that the microorganisms had a greater role than increasing ammonium in the culture media, and hints of a biologically controlled mechanism of biomineralization.

2.1.3 Functional role of biomineralization

Microorganisms have been pointed in literature to make bio-minerals for very distinct reasons. In some cases, the formation of bio-minerals can be

accidental, and does not seem to benefit the microorganism, including causing their own entombment and death, within the minerals formed in their cell wall (Obst *et al.*, 2009; Uad *et al.*, 2015). This seems be the result of catastrophic biologically induced mineralisation. Other microorganisms have been reported to have means to protect themselves from occlusion. These microorganisms are reported to have nucleation points in their cell wall where crystal formation can start (Hallberg and Ferris, 2004; Bontognali *et al.*, 2008; Li *et al.*, 2013). In opposition, the occlusion of *Halobacterium salinarum* within halide crystals has been proposed to be a form of spore-like resting state of this Archaea (Fendrihan *et al.*, 2012).

The shells of unicellular diatom algae are said to offer protection and structural integrity to the cell (Gröger *et al.*, 2016). The magnetite crystals produced by magnetotactic bacteria are used as a compass to orient their movement (Rahn-Lee and Komeili, 2013).

The formation of a nickel-struvite mineral ($NiNH_4PO_4.6H_2O$) by *Streptomyces acidiscabies* has been postulated to be a resistance factor that allows the bacteria to withstand high concentrations of nickel (30 mM $NiCl_2$) (Haferburg *et al.*, 2008). Calcite precipitation by bacteria in calcium-rich environments has been hypothesized to be a detoxification process where cells expel excess intracellular calcium to reach homeostasis (Cacchio *et al.*, 2015; Maciejewska *et al.*, 2017). For *H. salinarum* and *B. antiquum,* the accumulation of insoluble magnesium phosphate salts has been hypothesised to render phosphate into an osmotic-inert form or, in the case of *B. antiquum*, to provide a nitrogen reserve (Smirnov *et al.*, 2005).

Struvite, calcium carbonate and calcium phosphate have been found inside biomass granules of granular bio-reactors for wastewater treatment (Mañas *et al.*, 2011; Lin *et al.*, 2012; Johansson *et al.*, 2017). These studies mostly focused in aerobic reactors, with or without nutrient removal and on Anammox reactors, but cases of mineral formation in granules of anaerobic reactors have also been reported. The presence of minerals within the granules is said to strengthen the granules and increase their settleability (Mañas *et al.*, 2012).

In other cases the advantage of biomineralization is yet more exotic. A fungus in dead wood of broad-leaf trees, provisionally identified as *Exidiopsis effuse*, has been found to promote hair-like shape water ice formations, during cold days at temperatures slightly below 0°C. These ice formations were found to scour the wood, and in doing so, facilitate access to the interior of the wood (Hofmann *et al.*, 2015).

The reasons for bacteria as *B. pumilus*, and *B. antiquum*, to make struvite in sludge dewatering liquors, and settled wastewater are not yet fully understood. Insights gained on this subject have the potential to enable designing conditions where the struvite production can be maximized, or to enable the growth of the bacteria in open mixed-culture conditions.

2.1.4 Biomineralization in wastewater treatment

The identification of minerals inside the granules of aerobic, anaerobic and anaerobic wastewater treatment has only recently become a topic of research interest (Juang *et al.*, 2010; Lin *et al.*, 2013a; Li *et al.*, 2014; Liu, Lan and Zeng, 2016; Zhang *et al.*, 2016). Bio-minerals inside the granules of aerobic granular sludge process have been reported to have a ring like structure, similar to tree trunk rings (Mañas *et al.*, 2011). Struvite, at the core of granules of aerobic granular sludge, was found to have been biomineralized with the contribution of alginate-like exopolysaccharides present in the granules (Lin *et al.*, 2012; Lin *et al.*, 2013b). These reports have indicated that mineral deposition can possibly be controlled and optimised. In one study, struvite accounted for 44% of the total phosphorus within the granules of a granulating sequencing batch reactor aerobic EBPR process (Lin *et al.*, 2012). On another study, calcium-phosphates contributed to 46% of the total phosphorus within the granules (Mañas *et al.*, 2012). Direct application to land of the granules has been suggested to recycle the mineralised phosphorus found at the core of a granular Anammox (Johansson *et al.*, 2017).

The fact that selected commercially available bacteria such as *B. antiquum* and *B. pumilus*, were able to mediate the formation of struvite in wastewater derived

streams, demonstrates an alternative way to recover phosphorus from wastewater. Nevertheless further research is needed to:

- understand the bacteria compatibility with the wastewater streams
- elucidate the bacteria growth requirements
- understand the struvite biomineralization mechanisms
- assess the quality of the struvite formed
- gain insight over the economic feasibility of using a biological struvite process for P-recovery.

2.1.5 Potential for the implementation of a bio-struvite production process from wastewater streams

Although the possibility of bio-struvite production from wastewater derived streams has been demonstrated, only one sample of primary effluent and one sample sludge dewatering liquors has been tested. Wastewater streams have notoriously variable composition from site to site, and even within the same sampling point, as such, further trials are necessary to understand the range of variation where these selected bacteria can still produce bio-struvite.

B. pumilus and *B. antiquum* were shown to promote bio-struvite formation in sterile settled wastewater and in sterile sludge dewatering liquors. Sterilization was used to isolate the effect of the bacteria from potential interfering chemical precipitation (Soares *et al.*, 2014). However, biological processes for the treatment of wastewater streams are done under open mixed-culture conditions. An ideal bio-struvite process should also be done under open mixed culture conditions. To accomplish this, the selected bio-struvite producing bacteria need to be kept growing as a stable culture within a bio-reactor.

The unknowns that can present a barrier for implementation of a bio-struvite process are extensive.

Table 2-4 summarizes the advantages and the barriers for a successful implementation.

Table 2-4 Summary of the advantages and barriers to implementation for the development of a bio-struvite production process

	Advantages	Barriers to implementation
Known	**Strengths** Large crystals observed Struvite recovered from wastewaters with low (<100 mg PO_4-P/L) P content (7.5, and 30.0 mg PO_4-P/L in settled wastewater and sludge dewatering liquors, respectively) No chemicals were needed Low phosphorus content in the effluent (<2.1 mg PO_4-P/L) Struvite formation with 2 bacteria Tested in 2 types of wastewater	**Weaknesses** Slow growth rates observed (<0.1 1/d) Struvite producing microorganisms were aerobic, suggesting a process with aeration costs Tested in 2 sources of wastewater
Unknown	**Opportunities** The findings of Soares *et al.*, 2014, (reported above as strengths) extend to a wide range of wastewater sources including: - Low-P sludge dewatering liquors not viable for use with chemical struvite precipitation - Other wastewater streams not typically used for P-recovery, as settled wastewater	**Threats** Wastewater sludge liquors composition differences and variability can impact the growth of the selected bacteria and the formation of the bio-struvite. The impact of pH variability on the ability of the selected bacteria to grow and to produce bio-struvite is not understood. The differences in composition of different types of sludge liquors, sourced from different wastewater treatment processes, are not known.

	Advantages	Barriers to implementation
Unknown		The influence of the composition of the sludge liquors in terms of the struvite ions (PO_4, Mg^{2+}, and NH_4), and of interfering ions as calcium; and in terms of the presence of carbon sources to sustain the growth of the selected bacteria have not been assessed. It is not known which fractions of phosphorus can be used by the selected bacteria to produce bio-struvite. Hence the potential of various types of sludge liquors to apply P recovery processes is not understood. The selected bacteria have not been tested in open mixed culture conditions. The operational conditions necessary to obtain the enrichment of the selected bacteria in mixed open cultures conditions are not known. The value of the bio-struvite has not been assessed in terms of its use as a fertilizer, presence of micropollutants, and purity. The biomineralization mechanisms are not known, nor the functional benefits for the bacteria of the struvite formation.

2.2 Conclusions

Phosphorus recovery from wastewater sludge dewatering liquors has the potential to contribute for the recovery of 10-20% of the phosphorus arriving to the WWTP. However, existing P recovery processes have limitations to their applicability.

The production of bio-struvite from sludge dewatering liquors using selected bacteria has the potential to be applicable in wastewaters not suitable for conventional struvite recovery processes, without the need for addition of chemicals while producing an effluent with low P content.

Several microorganisms have been reported in association with struvite formation and that confirms that the ability for struvite biomineralization may be available in a wide range of microorganisms. However, the potential reasons for the formation of struvite and the underlying mechanisms of biomineralization are not yet known.

The use biomineralization in wastewater treatment has the potential to change the way in which wastewater is treated, particularly in what regards phosphorus removal and recovery.

2.3 References

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Chapter 3 The role of bulk pH on the biological struvite production in digested sludge dewatering liquors

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Abstract

Struvite production mediated by bacteria has opened up a new route for phosphorus recovery from wastewater streams but its application to digested sludge dewatering liquors is not yet well understood. This study investigates the growth and biological struvite production of selected bacteria in liquors with pHs between 5.7 to 9.1. The growth pH was assessed through flow cytometry. *B. pumilus* and *H. salinarum* remained viable at pHs between 7.3-9.1 and *B. antiquum* was able to grow at pHs between 7.3-7.8. Further analysis allowed the identification of crystals as struvite in tests between pH 7.3 to 8.3. All strains were capable of producing struvite at a range of pHs, but the highest production of 135-198 mg/L was observed for pHs between 7.3-8.3. At pHs >8.3, precipitation of struvite and calcium compounds was observed in inoculated and non-inoculated tests. This study demonstrates that biological struvite production can occur at a wide range of pHs, hence significantly different from chemical struvite precipitation that occurs at pH >8.3, making it a potentially viable process for phosphorus recovery as struvite from wastewater streams and sludge liquors without strict pH control.

Keywords

Biomineralization; centrate; phosphorus recovery; wastewater.

3.1 Introduction

Phosphorus (P) is an essential element in all organisms and a key nutrient in agriculture limiting food production. However, in contrast, P can also act as a pollutant causing eutrophication if released into the natural environments in excess (Elser, 2012). Wastewater treatment plants (WWTPs) are control points for P discharge to the environment where P is removed from the wastewater by chemical and/or biological processes. Nevertheless, because P is a limiting nutrient, there is growing interest in processes and technologies that allow P recovery from wastewater streams (Shu *et al.*, 2006; Qiu and Ting, 2014).

Phosphorus recovery from sludge dewatering liquors lines has been implemented in several WWTPs through chemically induced struvite precipitation (Le Corre *et al.*, 2009). Sludge dewatering liquors are a by-product of processes aimed at concentrating solids in the sludge. As a result, these liquors only represent a relatively small volume (usually 1-2% of the wastewater flow) but in turn are a nutrient rich stream with high ammonium (99 to 1020 mg N/L) and phosphate (1 to 167 mg P/L) (Battistoni *et al.*, 2002; Doyle and Parsons, 2002; Fux *et al.*, 2002; Pastor *et al.*, 2010). Consequently when the liquors are returned to the head of the WWTP, there is an increase on the P load to the secondary treatment that can be as high as 10-40% resulting in a constant cycling of nutrients (Battistoni *et al.*, 2002). Chemically induced struvite precipitation in the liquor lines has the advantage of reducing the return of P to the head of the works, as well as decreasing operational costs by minimising scale formation (calcium phosphate and struvite) in the pipelines and pumps. Furthermore, this process allows for the recovery of P as struvite which can be used as an agricultural fertiliser (Schick *et al.*, 2009). Nevertheless, chemical struvite precipitation requires chemicals to increase the pH to values above 8.5, and to correct the amount of available magnesium to molar concentrations above or equal to the available phosphate (Le Corre *et al.*, 2009). The increase in operational costs due to chemical consumption can sometimes result in difficulties justifying the implementation of a sidestream reactor for chemical struvite production. Taking advantage of the potential for high pH variability of sludge liquors and to minimise costs associated with the addition of chemicals,

pH increase through CO_2 stripping through increased agitation or aeration is also used in full scale processes for liquors from biological nutrient removal (BNR) sites (Doyle and Parsons, 2002).

As an alternative process to chemical struvite precipitation, a recent study demonstrated the ability of selected bacteria to form bio-struvite in activated sludge liquors and in dewatering liquors, opening a completely new route for P recovery via biomineralization (Soares *et al.*, 2014). Biomineralization is a widespread phenomenon in nature. Examples of bio-minerals range from the bones and teeth in vertebrates, to the silicate shells in diatom algae and the crystalline magnetite in magnetotactic bacteria (Mann, 2001). The most widespread bio-minerals are calcium phosphate and calcium carbonate, but others such as struvite, iron oxides, and silicates have been reported, as well as crystalline magnetite produced by magnetotactic bacteria (Weiner, 2003). The most common function of bio-minerals is to provide structural support in the form of skeletons, protection in the form of shells and to provide storage and a source of nutrients.

Several microorganisms have been investigated for the ability to form biological struvite (bio-struvite), amongst other bio-minerals using synthetic media (Sánchez-Román *et al.*, 2007). Precipitation of bio-struvite was shown to be frequent in strains capable of producing calcite precipitates (Rivadeneyra *et al.*, 1983). No relationship was found between the taxonomic status of a given strain and its ability to form bio-struvite (Rivadeneyra *et al.*, 1992) as this property has been reported in aerobic, chemoorganotrophic, halotolerant and halophilic bacterial strains (Sánchez-Román *et al.*, 2007). Bio-struvite forming microorganisms have been pointed to bring about a local pH increase through the release of ammonia produced from the degradation of nitrogenous organic matter. The local pH increase and the released ammonia combine with the magnesium and phosphate ions present to induce the precipitation the struvite crystals (Rivadeneyra *et al.*, 1983; Soares *et al.*, 2014).

In WWTPs, the occurrence of struvite within aerobic granular sludge has been reported as being induced by alginate-like exopolysaccharides isolated from

extra polymeric substances collected from the granules (Lin *et al.*, 2012). Recently, the cultivation of *B. pumilus* and *B. antiquum* on sludge dewatering liquors showed promising results, producing bio-struvite crystals reaching 250 µm in size within 10 days (Soares *et al.*, 2014). However, bio-struvite production and bacterial growth have not been investigated in detail in sludge dewatering liquors. This work aims at investigating the impact of pH variability in digested sludge dewatering liquors on the ability of selected bacteria, *B. pumilus*, *H. salinarum*, and *B. antiquum*, to grow and produce bio-struvite towards bringing bio-struvite production through biomineralization to WWTPs.

3.2 Materials and Methods

3.2.1 Source of microorganisms and sludge dewatering liquors

Three pure microbial strains were purchased from commercial culture collections: *Bacillus pumilus* (GB 43, LGC Standards, Middlesex, UK), *Halobacterium salinarum* (DSM 671, German Resource Centre for Biological Material, Brunswick, Germany), and *Brevibacterium antiquum* (DSM 21545, German Resource Centre for Biological Material, Brunswick, Germany).

Sludge dewatering liquors were collected from a full-scale WWTP with a 500,000 population equivalent, with biological nutrient removal (BNR) applied as a secondary treatment. Primary and secondary sludge produced onsite and imported from nearby municipal WWTP (40% v/v) were stabilised in anaerobic digesters. After digestion, the sludge was stored in a holding tank, from 10 to 27 days, before dewatering. A horizontal centrifuge decanter was used to dewater the sludge from typical values of 7% solids to 22% solids content. Cationic polymer, anti-scaling and antifoaming agents were used to aid the centrifugation process. The sludge dewatering liquors were collected at the liquid line from the centrifuge.

3.2.2 Microorganisms cultivation

Starter cultures of the selected bacteria were produced for the experiments completed in sludge dewatering liquors. The selected bacteria were inoculated at a ratio of 10% (v/v) in B41 synthetic media with pH 7.7 (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°C), under agitation at 150 rpm (Stuart SSL1, Fisher Scientific, Loughborough, UK) for 4 days.

In order to measure the bacterial growth and isolate the effect of the selected bacteria towards the bio-struvite production, the sludge dewatering liquors were sterilised. Two different sterilisation techniques were used: autoclave (121°C, 20 min), and filter sterilisation method (disposable 0.45 µm vacuum filtration set-up, Nalgene, Fisher Scientific, Loughborough, UK); to assess the impact of different sterilisation techniques on the pH of the dewatering liquors.

3.2.3 Growth of selected bacteria in sludge dewatering liquors

Autoclaved digested sludge dewatering liquors were inoculated with selected bacteria from starter cultures and incubated in conical flasks, at room temperature (20-22°C), under agitation at 150 rpm (Stuart SSL1, Fisher Scientific, Loughborough, UK). Samples of 10 mL were collected immediately after inoculation and after 2, 4, 5, 6, 7, and 10 days for ammonium (NH_4), phosphate (PO_4), magnesium (Mg^{2+}), and pH analysis. At the end of the incubation, the solid precipitates produced were collected by filtration, dried and identified. All cultures were carried out in duplicate. Control tests with autoclaved sludge dewatering liquors and no added bacteria were also monitored.

3.2.4 Effect of pH in the bacterial growth and precipitate production

To assess the impact of pH in the growth of the selected bacteria and the production of precipitates such as struvite, the sludge dewatering liquors were buffered at 5 different pH values. Three buffer compounds, reported to not affect bacterial growth (Thiel *et al.*, 1998), were used: N-(morpholino)-ethane-sulfonic acid (MES), N-2-hydroxy ethylpiperazine-N9-propane-sulfonic acid (EPPS), and cyclohexylamino-ethane-sulfonic acid (CHES) (Fisher Scientific, Loughborough, UK). Buffers were added at a 10% (v/v) ratio to 54 mL of magnesium-supplemented sludge dewatering liquors autoclaved in capped

serum bottles. The quantities and concentrations of the chemicals used to control the pH of the sludge dewatering liquors are summarised in Table 3-1.

For the inoculum, bacterial cells were centrifuged from 5 mL of the starter cultures (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 5 min) and resuspended in 5 mL sterile 0.9% NaCl solution. The tests were completed in triplicates in sacrificial serum bottles under the same conditions described above.

Bacterial growth was assessed by flow cytometry using a live/dead cells staining method that provides the number of cells with intact membranes (Gatza *et al.*, 2013). Intact cells counts were measured using a SYBR Green I and propidium iodide dye mixture with an incubation period of 10 min at 37°C (Lipphaus *et al.*, 2014). Measurements were taken using a BD Accuri C6® flow cytometer equipped with a 488 nm solid-state laser (Becton Dickinson U.K. Ltd., Oxford, UK). When necessary, samples were diluted with filter sterilised Evian mineral water (0.1 µm) (Evian, Évian-les-Bains, France). Specific fixed regions of the density plots of green fluorescence (533 nm), and red fluorescence (>670 nm) were used for distinction between the stained intact microbial cells and instrument noise or sample background (Gatza *et al.*, 2013). Data were processed using the BD Accuri C6® software.

Table 3-1 Buffer and acid or base additions to control the pH of sludge dewatering liquors.

Buffer	Concentration (mM)	pH correction	pH
MES	50	1200 µL of 2 M HCl	5.7
EPPS	10	180 µL of 2 M HCl	7.3
EPPS	10	60 µL of 2 M NaOH	7.8
EPPS	10	300 µL of 2 M NaOH	8.3
CHES	50	1200 µL of 2 M NaOH	9.1

MES, N-(morpholino)-ethane-sulfonic acid; EPPS, N-2-hydroxy ethylpiperazine-N9-propane-sulfonic acid; CHES, cyclohexylamino-ethane-sulfonic acid.

3.2.5 Bio-struvite production rate

To follow the formation of bio-struvite over 10 days of incubation period, sacrificial bottles were prepared by autoclaving 45 mL of the sludge dewatering liquors in 120 mL glass serum bottles. The pH of the sludge dewatering liquors was controlled by two different processes: half of the bottles were autoclaved with a cotton-plug to keep sterile conditions and pH corrected using sulphuric and hydrochloric acids, at 2 N concentration to control the pH to values between 7.9 and 8.2 (Experiment A); the other half of the bottles were kept closed with rubber stoppers during autoclaving and incubation to limit degassing of CO_2 allowing for stable pH values between 7.8 and 8.2 (Experiment B). For the inoculum, bacterial cells were centrifuged from 5 mL of the starter cultures (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 5 min) and resuspended in 5 mL sterile 0.9% NaCl solution, in order to avoid the addition of PO_4, NH_4 and Mg^{2+} present in the B41 media.

Each sacrificial bottle was tested for pH, PO_4, NH_4, Mg^{2+} and the precipitates formed were collected by filtration, dried and identified. In Experiment B, each bottle was also tested for total phosphorus (TP) and dissolved oxygen. To maximise the potential for struvite production, the sludge dewatering liquors were supplemented with magnesium, making phosphate the limiting ion for struvite formation. Magnesium sulphate was added to a final concentration of 60.0 mg/L as Mg^{2+}.

3.2.6 Precipitates isolation, quantification and identification

After incubation, the media was vacuum-filtered through a previously dried (2 hours, 37°C) and weighted 10 µm aperture nylon mesh sheet (Plastok Associates, Birkenhead, UK). The solid precipitates recovered were washed with a small quantity of washing water (deionised water adjusted to pH 10 with 1M NaOH) and allowed to dry at 37°C for 2 hours prior to being weighed. The precipitates were identified using high resolution scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDX) (scanning electron microscope XL 30 SFEG, Phillips, The Netherlands).

3.2.7 Analytical methods

The concentrations of NH_4, PO_4, TP, and chemical oxygen demand (COD), were measured using Merck cell test kits according to the manufacturer instructions. Mg^{2+} was analysed using an atomic absorption spectrophotometer (Analyst 800, Perkin Elmer Ltd, Beaconsfield, UK) equipped with an air/acetylene burner system. The pH was measured with a Fisherbrand hydrous 300 pH meter (Fisher Scientific, Loughborough, UK) immediately after sampling. Values presented details of the mean and standard deviation of duplicate or triplicate tests.

3.3 Results and Discussion

The sludge dewatering liquors used in this study were analysed for typical wastewater quality parameters (Table 3-2). Nutrients were measured at concentrations of 825 ± 66 mg NH_4-N/L, 44.5 ± 2 mg PO_4-P/L, and 15.2 mg Mg^{2+}/L with a pH of 7.8. The characteristics of the studied sludge dewatering liquors were similar to others originating from biological nutrient removal sites (Ueno and Fujii, 2001; Pastor *et al.*, 2010) and thus, it can be considered standard.

Table 3-2 Characteristics of the sludge dewatering liquors collected from a full scale WWTP.

pH	**COD** (mg/L)	**NH_4** (mg N/L)	**PO_4** (mg P/L)	**Mg^{2+}** (mg/L)
7.8	455 ± 3	825 ± 66	44.5 ± 2	15.2

In order to assess the production of bio-struvite in the sludge dewatering liquors, the first test completed was to grow the selected bacteria in the autoclaved sludge dewatering liquors, as previously described by Soares *et al.* (2014). Nevertheless, after 10 days of incubation, no significant differences were found between inoculated and non-inoculated controls, as no bacterial growth was observed and similar precipitate formation was observed in all the bottles. A detailed investigation indicated that the pH in the sludge dewatering

liquors had increased from 7.8 to 9.8 due to CO_2 degassing (see Appendix A). The solid precipitate collected after incubation were similar both in the control and inoculated tests, and did not display crystalline forms typical of struvite (Figure 3-1). Given the amorphous nature of the solids collected and the pH of the sludge dewatering liquors measured at 9.8 ± 0.1, it was hypothesised that the precipitates formed were amorphous calcium compounds (Song *et al.*, 2001). Liquors from biological nutrient removal sites have been shown to have a high propensity to form scaling in the pipes as a result of high concentrations of phosphate together with pH variations caused by degassing of CO_2 after anaerobic digestion in turbulent flow regions (Loewenthal *et al.*, 1995; Doyle and Parsons, 2002).

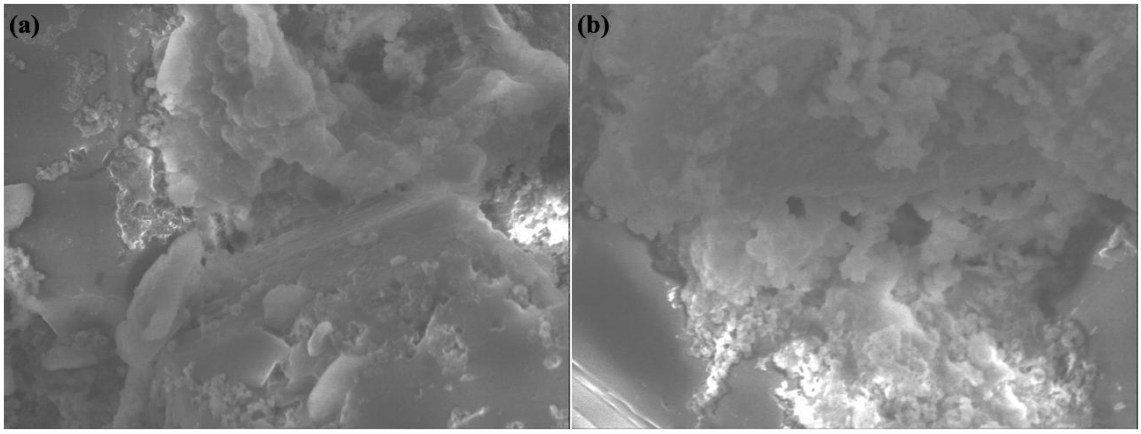

Figure 3-1 Electron microscope images of the solid precipitate collected at the end of incubation of sludge dewatering liquors without pH control at 2000x magnification: (a) *B. antiquum*; (b) non-inoculated control.

The degassing of CO_2 from sludge dewatering liquors has been described previously as a suitable method to increase the pH of dewatering liquors, but this is not applicable to all WWTPs (Battistoni *et al.*, 2002; Pastor *et al.*, 2010). The sludge dewatering liquors used in this study were obtained from a BNR WWTP where the digested sludge was kept in a holding tank for a long period (10-27 days). Assessing previous studies and the results obtained, it is clear

that the source of sludge and the type of post anaerobic digestion treatment can influence dissolved CO_2 concentrations and consequently impact on the pH sludge dewatering liquors. Therefore, pH is identified as a relevant factor that requires careful consideration prior to implementation of P recovery processes such as chemical or biologically induced struvite production.

3.3.1 Impact of pH in the bacterial growth and bio-struvite production

In order to understand the impact of pH on the growth of selected bacteria and the ability to produce minerals such as struvite, the pH of the sludge dewatering liquors was corrected to different target values: 5.7, 7.3, 7.8, 8.3, and 9.1 using buffers. The results obtained indicate that all bacteria were able to remain viable in sludge dewatering liquors at pH values of 5.7 to 9.1 (Figure 3-2). However, the final intact cell counts for *H. salinarum* and *B. pumilus* after 6 days of incubation ($9.6\times10^7 \pm 4.6\times10^6$ cells/mL for *H. salinarum* and $1.2\times10^8 \pm 2.5\times10^6$ cells/mL *B. pumilus* at pH 7.3) were slightly below the initial count ($1.2\times10^8 \pm 3.1\times10^6$ cells/mL for *H. salinarum* and $2.0\times10^8 \pm 8.6\times10^6$ for *B. pumilus*). This suggests that sludge dewatering liquors limited the growth of these bacteria at the studied pH values. Nevertheless the bacterial cell counts remained at the initial values at pHs tested between 7.3- 9.1. For *B. antiquum* the initial cell count was $4.7\times10^8 \pm 7.5\times10^7$ cells/mL and increased to 5.4×10^8 cells/mL at pH 7.3. and 4.8×10^8 cells/mL at pH 7.8 (Figure 3-2). As such, from the three selected bacteria, *B. antiquum* seems to be better adjusted to grow in sludge dewatering liquors and the optimal pH for growth was 7.3-7.8. Published studies on *B. antiquum* have reported growth in synthetic media at pH values between 6.0 and 7.0 (Smirnov *et al.*, 2005; Mounier *et al.*, 2007) and between 5 and 9 (Shivaji *et al.*, 2011). These published values are in good agreement with the results described in this study and suggest that pH is not a major limiting factor for *B. antiquum* growth in sludge dewatering liquors. From the three investigated bacteria, *B. pumilus* was expected to have the widest pH interval for growth as 16 isolates from a coastal environment were reported to be able to grow at pH values between and 5 and 11 (Parvathi *et al.*, 2009). For *H.*

salinarum, growth has been reported at pH between 6.0 and 7.0 in synthetic media (Smirnov *et al.*, 2005; Wende *et al.*, 2009) but optimum growth was reported at pH 7.0 (Mormile *et al.*, 2003).

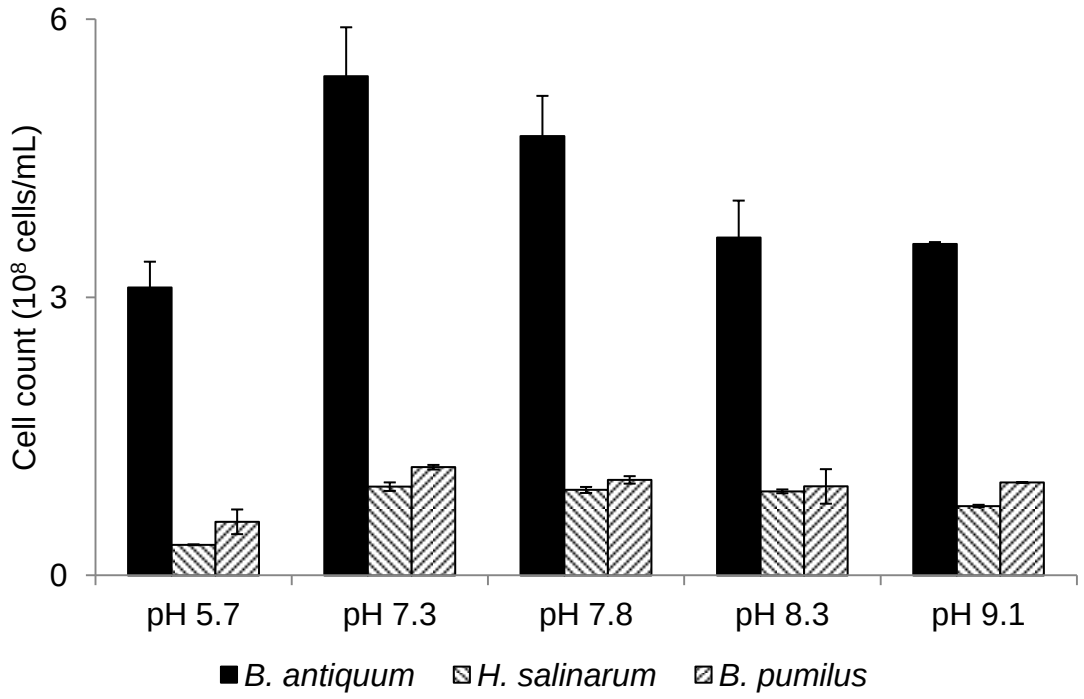

Figure 3-2 Flow cytometry live cell counts after incubation of the selected bacteria at different pH values after 6 days of incubation.

Regarding solid precipitate formation, further analysis using SEM and EDX allowed the identification of crystals as struvite in tests between pH 7.3 to 8.3 (Figure 3-3). The bio-struvite produced displayed a orthorhombic shape typical of struvite crystals (Le Corre *et al.*, 2009), with sizes between 0.10-0.40 mm (Figure 3-3). Some of the crystals seemed to result from the aggregation of two crystals resulting in an X-shaped crystal with sizes on the higher values of the observed range of sizes, and also rhombohedral crystals with sizes in the lower values. Both these forms have been described as typical crystal habits of struvite (Babic-Ivancic *et al.*, 2002). Bio-struvite production was measured taking in consideration the values measured in the un-inoculated control and the maximum values reached of: 112 mg/L for *B. pumilus* in the test with initial pH

of 7.8, 107 mg/L for *H. salinarum* in the test with initial pH of 7.3 and 77 mg/L for *B. antiquum* in the test with initial pH of 7.3.

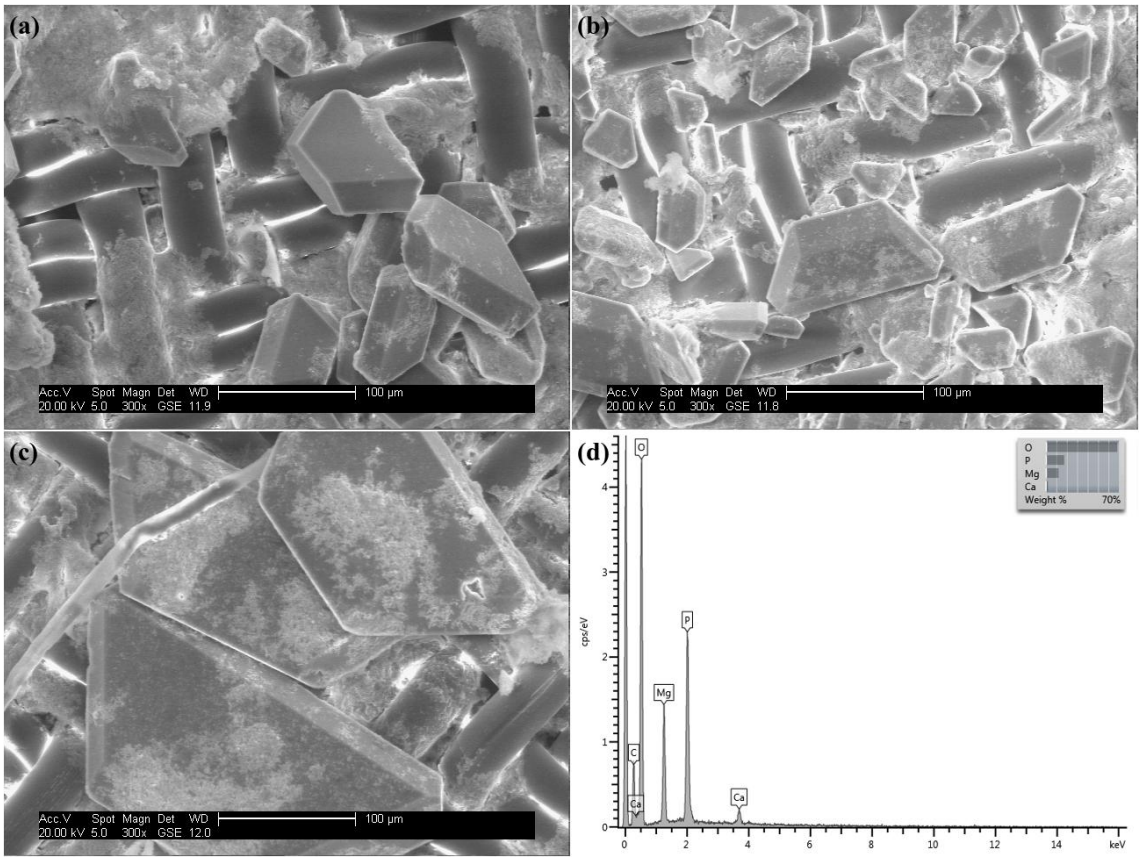

Figure 3-3 Electron scanning microscope images of the solid precipitate collected at the end of the 10 days of incubation for *B. pumilus* (a), *H. salinarum* (b), and *B. antiquum* (c) at pH 7.3 in sludge dewatering liquors at 300x magnification, and (d) example of a energy dispersive X-ray (EDX) microanalysis of the crystals for *B. antiquum* (c) with weigh percentage of the elemental components of the analysed crystal that allowed the identification of the crystal as bio-struvite. EDX was also completed for the crystals produced by *B. pumilus* (a), *H. salinarum* (b) and present overlapping results to figure (d).

At pH of 5.7 no statistical differences were identified between the inoculated group and the control tests. At pHs above 8.3 the solid precipitate formation was higher in the controls, reaching a maximum of 173 mg/L (Figure 3-4). pH values above 8.5 are known to be optimal for chemical struvite precipitation

(Suzuki *et al.*, 2002; Nelson *et al.*, 2003; Le Corre *et al.*, 2009), hence, it was expected that solid precipitate formation was high in the controls due to the formation of precipitates through both the biological and chemical routes. At pH 9.1, an increased formation of solid precipitate was observed in the inoculated tests relative to the controls, with the highest difference observed for *B. antiquum*, with 74.2 mg/L above the control. The precipitates recovered in the control had an amorphous nature and it was hypothesised that the precipitates formed were mainly amorphous calcium compounds (Song *et al.*, 2001). Nevertheless, the precipitates recovered from the inoculated tests, showed a mix of amorphous and crystalline structures indicating the influence of bacteria at a high of pH >9.1.

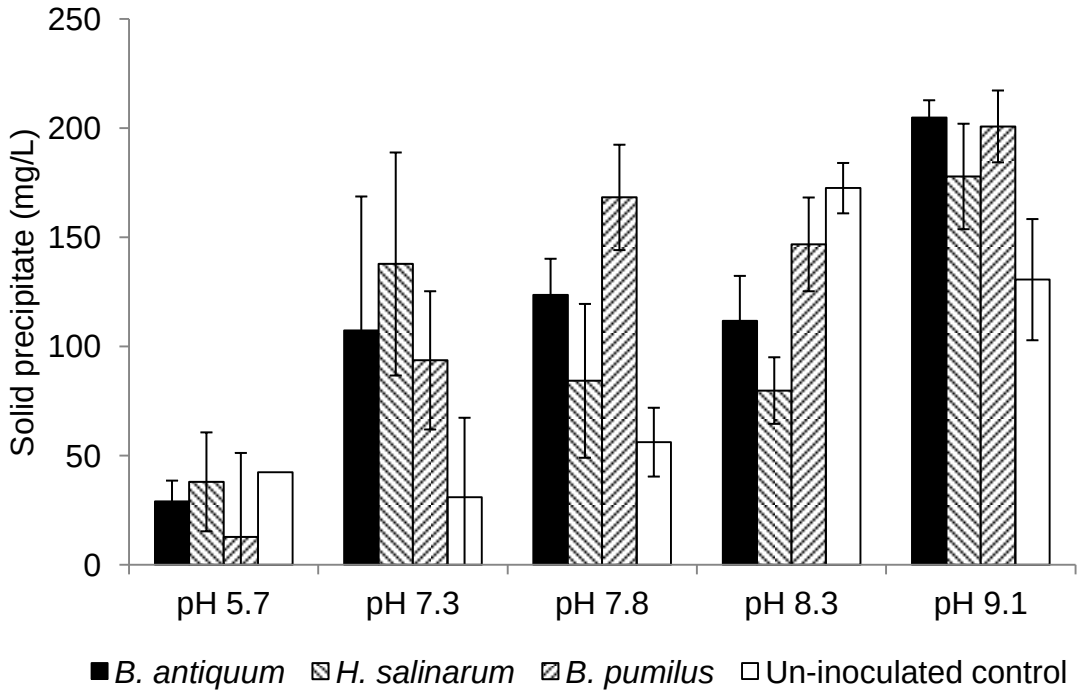

Figure 3-4 Solid precipitate collected after 6 days of incubation of pure cultures of the selected bacteria in magnesium supplemented sludge dewatering liquors at different initial pH values. Error bars represent standard deviation obtained from triplicate tests.

3.3.2 Bio-struvite production rate

Bio-struvite formation over a 10-day period was assessed using sacrificial bottles in order to understand the production rate over time. In these tests, the pH was controlled either through pH correction with chemicals (Experiment A, pH of 7.9), or by limiting CO_2 degassing in closed serum bottles (Experiment B, pH of 7.8). Both strategies were tested with the aim of reducing chemical changes in the composition of the sludge dewatering liquors. In both tests, the headspace was 75 mL allowing for dissolved oxygen concentrations being always above 5 mg/L in both Experiments A and B. As a result, no oxygen depletion was observed in any of the tests.

Inoculated and control tests presented clearly different results as limited amounts of solid precipitates were recovered in the non-inoculated controls (Figure 3-5). In the inoculated bottles, the precipitates produced were analysed with SEM and EDX which allowed the identification of crystals as bio-struvite with the same characteristics as the crystals presented in Figure 3-3. The concentrations of PO_4, NH_4 and Mg^{2+} were measured at the end of the 10 days incubation in both Experiments A and B (Table 3-3). Considering the struvite stoichiometric ratio of Mg:NH_4-N:PO_4-P; 1:1:1, the content of PO_4 recovered as bio-struvite in Experiment A represented 92, 102, and 94% of the initial PO_4. However, the removal of PO_4 was only 51, 47, and 52% of the initial PO_4 measured at 39.1 mg P /L. In Experiment B, the content of PO_4 in the recovered bio-struvite represented 69, 71, and 35% of the initial PO_4 (35.5 mg P /L) for *B. pumilus*, *H. salinarum*, and *B. antiquum*, respectively, whilst the overall removal of PO_4 from the sludge dewatering liquors was only 16, 16, and 23% of the PO_4 available initially. Final PO_4 concentrations for Experiment A were 19.1, 20.7, and 18.9 mg P /L, for *B. pumilus*, *H. salinarum*, and *B. antiquum*, respectively, and in Experiment B they were 20.8, 21.0, and 24.0 mg P /L, for *B. pumilus*, *H. salinarum*, and *B. antiquum*, respectively (Table 3-3). These final concentrations of PO_4 were one order of magnitude higher than the final concentrations observed in the first report of bio-struvite formation, where *B. pumilus* and *B. antiquum* lowered the PO_4 concentration to 2.1, and 1.5 mg P /L, respectively (Liang *et al.*, 2015). These results suggest that the bacteria

were able to use other forms of P present in the sludge dewatering liquors, such as organic P and polyphosphates. Others have reported that during anaerobic digestion of BNR biomass, polyphosphate or its hydrolysed forms are released into the liquors, thereby contributing to the phosphorus total concentration (Jardin and Pöpel, 1994). Further work is necessary to understand the types of P used by the bio-struvite producing bacteria and their favoured sources in order to predict the potential for bio-struvite production in WWTP. Another challenge is to identify the optimal conditions and factors that can enhance the growth of the bio-struvite producing bacteria in the sludge dewatering liquors mixed cultures and thus increase the potential for application of this process at full-scale. Comparing the solid precipitate formed in the experiment A at 5 days (Figure 3-5a) with the results after 6 days when testing different pH values at pH of 7.8 (Figure 3-4), shows that the differences observed for *B. antiquum* and for the control tests were not significant (p-value of 0.8899, and 0.3149, respectively). The differences for *H. salinarum* (p-value of 0.0836) and *B. pumilus* (p-value of 0.1035) can be considered significant at a significance level of 10%. These differences were likely the result of using different methods of correcting the sludge liquors pH. In experiment A, the pH correction was done with an initial addition of hydrochloric acid when preparing the sludge liquors. On testing the bacteria at different pHs, a buffer compound (10 mM EPPS for pH 7.8) was used to compensate pH changes throughout the experiment. Although EPPS and the Good family of buffers are not expected to affect bacterial growth, effects on enzyme activity have been reported (Thiels *et al.*, 1998) and this can be the cause for the differences observed for *H. salinarum* and *B. pumilus*.

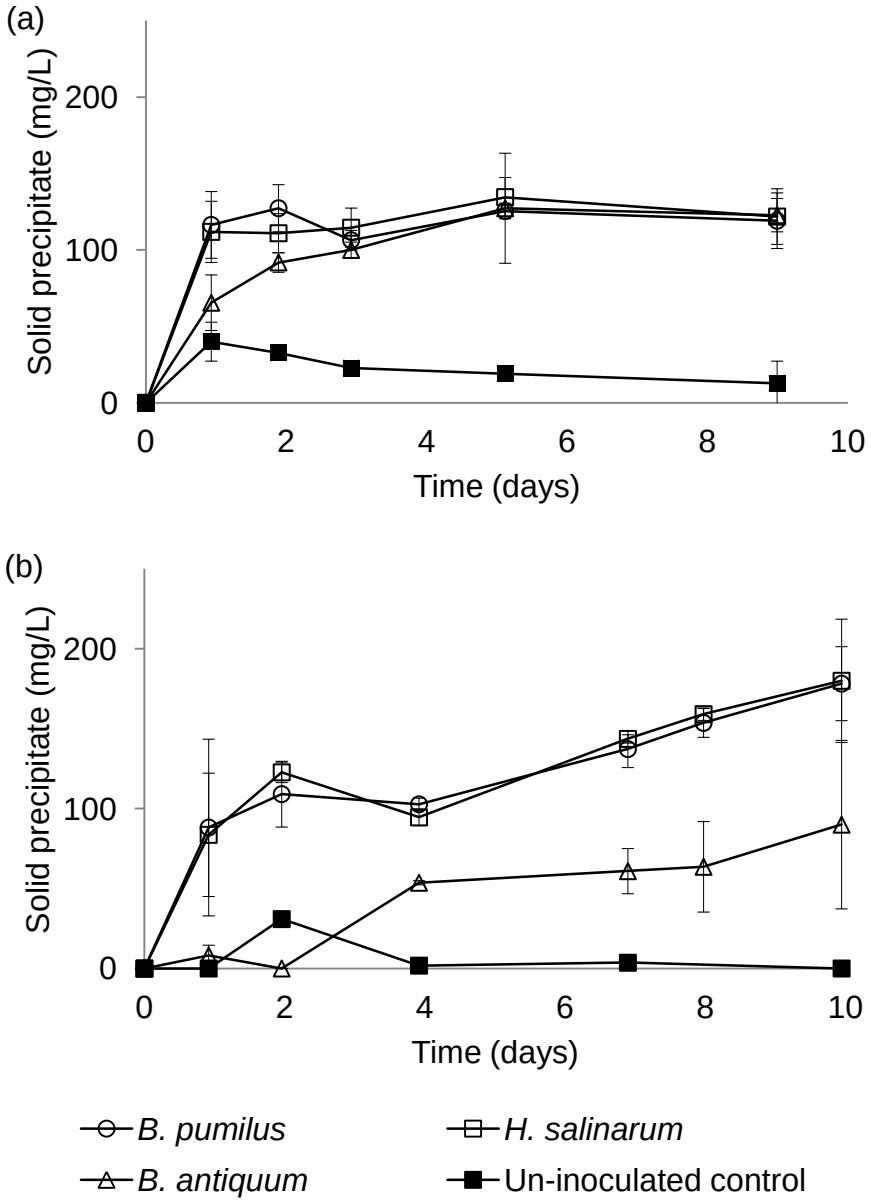

Figure 3-5 Solid precipitate production when incubating the selected bacteria in: Experiment A: pH controlled through acid addition; Experiment B: pH controlled by limiting degassing in closed serum bottles. Controls tests were not inoculated with bacteria. Error bars represent standard deviation obtained from triplicate tests.

Table 3-3 Initial and final concentrations of PO_4, NH_4, and Mg in sludge dewatering liquors before and after 10 days of incubation with selected bacteria. In Experiment A the pH was controlled through acid addition and in Experiment B the pH controlled by limiting degassing in closed serum bottles.

		PO_4 (mg P /L)	NH_4 (mg N /L)	Mg (mg/L)
	Experiment A			
Initial		39.1 ± 0.5	708 ± 6	51.7 ± 0.8
Final	*B. antiquum*	18.9 ± 1.3	711 ± 11	34.1 ± 1.3
	B. pumilus	19.1 ± 0.2	704 ± 3	35.8 ± 1.6
	H. salinarum	20.7 ± 0.4	711 ± 3	36.4 ± 1.0
	Experiment B			
Initial		35.5 ± 1.0	887 ± 42	47.5 ± 0.1
Final	*B. antiquum*	24.0 ± 2.8	827 ± 16	30.7 ± 1.2
	B. pumilus	20.8 ± 1.1	814 ± 6	28.8 ± 0.5
	H. salinarum	21.0 ± 2.8	814 ± 20	29.2 ± 1.0

In Experiment A, the bio-struvite production was similar for all inoculated tests reaching 131 ± 20, 134 ± 20, and 135 ± 12 mg/L for *B. pumilus*, *H. salinarum*, and *B. antiquum*, respectively (Figure 3-5a). In Experiment B, using closed serum bottles, bio-struvite production was similar for *B. pumilus* and *H. salinarum*, reaching 196 ± 25 and 198 ± 42 mg/L, respectively. *B. antiquum* demonstrated a lower production of bio-struvite, 99 ± 58 mg/L, although it is not clear why it was lower than in experiment A.

Maximum productivities in Experiment A were observed after the first day for all bacteria: 138, 132, and 77 mg/L.d, for *B. pumilus*, *H. salinarum*, and *B. antiquum*, respectively. In Experiment B, *B. pumilus* and *H. salinarum* reached 106, and 100 mg/L.d within 1 day, and *B. antiquum* required 4 days to reach a maximum productivity of 15 mg/L.d. Overall, the results indicated that 75% of the bio-struvite production took place in the first 2 days of incubation in Experiment A. This is an important result to note when considering scaling up this process, as long retention times imply larger tanks and increased costs. A

retention time of 1-2 days is long in comparison to mainstream wastewater treatment processes such as activated sludge or membrane bioreactors, that allow for treatment of the wastewater in the order of hours (Tchobanoglous *et al.*, 2003). Retention times for chemical struvite precipitation in fluidised reactors range from 0.5-9 h (Ueno and Fujii, 2001; Le Corre *et al.*, 2009). Nevertheless, processes that deal with low volumes can be economically feasible, such as anaerobic digestion (retention time between 10-15 days) and other processes that deal with the treatment of sludge dewatering liquors such as Anammox processes with retention times of up to 2 days (Fux *et al.*, 2002).

3.4 Conclusions

Sludge dewatering liquors can be used to produce bio-struvite, however, pH can impact the growth of the selected bacteria. The bacteria investigated were able to grow in sludge dewatering liquors at a wide range of pHs from 5.7 to 9.1. Bio-struvite production was observed at pHs between 7.3 to 7.8 with productions between 135-198 mg/L above non-inoculated controls. At pHs above 8.3, chemical precipitation becomes significant and it is not possible to differentiate between chemical and biological struvite production. Results indicated that bio-struvite production processes are likely to be applicable in WWTP with sludge dewatering liquors with a neutral pH and low dissolved CO_2 concentrations.

3.5 Acknowledgements

The authors are grateful for the financial support and contribution from Severn Trent Water Ltd (Coventry, UK) and for the financial support from POPH/FSE as a PhD grant (SFRH/BD/84116/2012) awarded by the Fundação para a Ciência e Tecnologia (FCT, Portugal).

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Chapter 4 Improving the growth of Brevibacterium antiquum in sludge liquors for biological struvite production

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Abstract

Biological struvite (bio-struvite) production through biomineralization has been suggested as an alternative to chemically derived struvite production to recover phosphorus from wastewater streams. In this study, statistical experimental design techniques were used to find the optimal growth rate (μ) of *Brevibacterium antiquum* in sludge liquors. Acetate, oleic acid, NaCl, NH_4-N, and Ca^{2+} were all shown to affect the growth rate of *B. antiquum*. The growth rate reached 3.44 1/d when the bacteria were supplemented with 3.0% w/v NaCl and 1124 mg COD/L as acetate. However, NaCl was found to hinder the biomineralization of bio-struvite. A 2-Stage experiment was set-up where the Stage 1 promoted growth (using sludge liquors with NaCl and acetate) was followed by Stage 2 to produce bio-struvite with only acetate. Bio-struvite production was confirmed with X-ray spectroscopy and crystal morphology (prismatic, tabular, and twinned-crystal habit) through electron microscope analysis. The bio-struvite production was estimated by measuring phosphate content of the recovered precipitates, reaching 9.6 mg P/L as bio-struvite. Overall, these results demonstrated the optimal conditions required to achieve high growth rates as well as bio-struvite production with *B. antiquum.* The results obtained in this study could be used to develop a process for growing

B. antiquum in wastewater streams in mixed cultures and recovery of phosphorus rich products such as struvite.

Keywords

Biomineralization; centrate; phosphorus recovery; wastewater; mixed-culture.

4.1 Introduction

Phosphorus (P) is a mineral nutrient of limited availability but crucial for ensuring worldwide food security (Cordell *et al.*, 2009). Wastewater treatment plants (WWTP) receive and treat streams with significant P concentrations (2-15 mg P/L in wastewater and up to 200 mg P/L in sludge dewatering liquors) where P recovery can be instigated (Jaffer *et al.*, 2002). Phosphorus recovery from sludge dewatering liquors as struvite has been shown to be feasible at the commercial scale, however, this process is not always viable, requiring streams with high phosphate (PO_4-P) concentrations >70 mg PO_4-P/L (Le Corre *et al.*, 2009).

Biological struvite (bio-struvite) production through biomineralization has been suggested as an alternative to chemically derived struvite production. It is currently receiving significant interest from the scientific community around the world (Sánchez-Román *et al.*, 2007; Sinha *et al.*, 2014; Soares *et al.*, 2014; Li *et al.*, 2017). Biomineralization is a common process occurring in the natural environment in which living organisms are able to form minerals (e.g. calcium carbonate; magnetite, struvite; magnesium phosphate; calcium phosphate etc.) (Mann, 2001). Phosphorus recovery from wastewater and sludge liquors using biomineralizing bacteria has been shown possible, but the process is still poorly understood and it has not yet been optimised (Soares *et al.*, 2014). Nevertheless, some promising results have demonstrated that bio-struvite productivity by *Brevibacterium antiquum* can reach 200 mg in 1L of sludge dewatering liquors with an initial concentration of 44.5 ± 2 mg PO_4-P/L (Simoes *et al.*, 2017b). *B. antiquum* was isolated by Gavrish *et al.* (2004) from a

permafrost sample and was characterised as an aerobic heterotroph capable of growing at temperatures as low as 7°C (but not at 37°C), in high salinity environments (up to 18% NaCl). It was also shown to be capable of hydrolysing urea and gelatine. Furthermore *B. antiquum* was not able to use starch as a carbon source and it was not able to reduce nitrate or produce H_2S (Gavrish *et al.*, 2004). Following from the study of Gavrish *et al.* (2004) there is still a need to understand and characterise the growth requirements of *B. antiquum* in complex and substrate limited media such as sludge dewatering liquors. This information is crucial to developing a process with conditions where the growth of *B. antiquum* can be favoured in relation to indigenous organisms in sludge dewatering liquors, thus enabling the production of bio-struvite in open mixed-culture reactors. The use of pure culture systems is perceived as unpractical and too costly to be implemented in wastewater treatment plants.

Hence the optimal growth conditions of *B. antiquum* in sludge dewatering liquors needs to be investigated for the impact of factors such as electron donor and electron acceptor, nutrient requirements, impact of pH, salinity, etc. (Tchobanoglous *et al.*, 2003). Moreover, factors potentially important for bio-struvite formation should be considered. Such factors include the concentration and molar ratio of the struvite-ion species (i.e.: magnesium (Mg^{2+}), ammonium (NH_4-N), and PO_4-P); the pH, salinity, temperature, and the presence of foreign ions as calcium (Le Corre *et al.*, 2009).

Taking into consideration the factors described above, as well as the practicality of changing the sludge liquors characteristics and environmental conditions in an open reactor in a WWTP, the following parameters were selected for further investigation in this study: Mg^{2+}, NH_4-N, PO_4-P, Ca^{2+}, NaCl and two carbon sources, a readily available source – acetate and a more complex form of carbon – oleic acid. Statistical experimental design techniques are frequently used to streamline experimental complexity and assess the effect and interaction of several factors on microbial viability and growth (Sun *et al.*, 2007; Cheng *et al.*, 2011). The experimental sequence starts with a screening phase to identify the factors that have a significant impact on the response to being

optimised. The Plackett-Burman (PB) methodology is particularly efficient for the screening phase as it allows the test of N-1 factors with N experiments (where N is the number of experiments and is a multiple of 4) (Zhou *et al.*, 2011). The PB methodology produces a first order approximation of the design space that estimates the response variable, such as growth rate (μ), as a function of the factors with significant impact (Cheng *et al.*, 2011). The next stage is to optimise the response variable until a maximum is found using the path of steepest ascent method (PSA). Further testing is then required to refine the model of the response variable as a function of the factors investigated. This step is typically completed with an experimental design that minimises the number of tests whilst also providing an indication of the interactions factors. This is frequently accomplished with a central composite experimental design (Rhodes and Stanbury, 1997).

The application of statistical experimental design techniques has been demonstrated in the literature as Cheng *et al.* (2011) identified the variables and optimal conditions to increase the activity of lipase from *Bacillus subtilis* by 5 fold. In another study, the optimisation of phenol degradation by *Candida tropicalis* in synthetic phenol-rich effluent was investigated, resulting in 99% phenol degradation (Zhou *et al.*, 2011).

In this study, statistical experimental design techniques were used to investigate the optimal growth rate (μ) of *B. antiquum* in sludge dewatering liquors for factors such as Mg^{2+}, NH_4-N, PO_4-P, Ca^{2+}, NaCl and two carbon sources. Tests were also completed to help understand the impact of the factors responsible for optimal growth conditions of *B. antiquum* on bio-struvite production.

4.2 Materials and Methods

4.2.1 Microorganism and cultivation in sludge dewatering liquors

Brevibacterium antiquum (DSM 21545) was purchased from the German Resource Centre for Biological Material (Braunschweig, Germany). Starter cultures were grown in B41 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°C), under agitation at 150 rpm (Stuart SSL1, Fisher Scientific, Loughborough, UK) for 4 days. Inoculation of sludge dewatering liquors was carried out with *B. antiquum* cells centrifuged (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 5 min) from starter cultures and resuspended in the same volume of autoclaved 0.9% NaCl aqueous solution. This was performed in order to avoid the addition of PO_4, NH_4-N and Mg^{2+} present in the B41 media to the sludge dewatering liquors. In all the experiments, the sludge dewatering liquors were sterilised by autoclaving at 121°C for 20 min after controlling the pH to obtain a post-autoclave pH of 7.8.

4.2.2 Sludge dewatering liquors

Sludge dewatering liquors were collected from a full-scale WWTP with 500,000 population equivalent, with biological nutrient removal (BNR) as a secondary treatment. Primary and secondary sludge produced onsite and import sludge, from nearby WWTP (40% v/v), were stabilised in standard operated anaerobic digesters. After digestion the sludge was stored in a holding tank, from 10 to 27 days, before dewatering. A horizontal centrifuge decanter was used to dewater the sludge from typical contents of 7% solids to 22% solids content. Cationic polymer, anti-scaling and anti-foaming agents were used to aid the centrifugation process. Samples of sludge dewatering liquors were frequently collected directly from the horizontal centrifuge decanter and stored at 4°C before being used in the experiments.

4.2.3 Optimisation of *B. antiquum* growth using a statistical experimental design method

4.2.3.1 Screening of factors

A Plackett-Burman (PB) experimental design was used to screen 7 factors: Mg^{2+}, NH_4-N, PO_4-P, Ca^{2+}, NaCl acetate, and oleic acid at 2 different concentrations (Table 4-1). The concentration of NH_4-N in the sludge dewatering liquors was decreased by applying 8 g/L of Mesolite (NanoChem Pty Ltd, Sydney, Australia) to the sludge dewatering liquors and allowing it to equilibrate under agitation with a magnetic stirrer for 18 hours. Mesolite is an

ion exchange zeolite media that has been shown to be highly selective for NH_4-N when applied to sludge dewatering liquors (Thornton *et al.*, 2007).

Table 4-1 Factors and levels tested in the screening experiment.

Factor	Low level (-)	High level (+)
Acetic acid	(na)	890 µL/L from 99.8% Acetic acid (Acros Organics). Equivalent to1000 mg COD/L
Ammonium (NH_4-N)	510 mg N/L	839 mg N/L
Calcium (Ca^{2+})	(na)	340 mg/L from 99% anhydrous $CaSO_4$ (Acros Organics). Increased 100 mg Ca^{2+}/L
Magnesium (Mg^{2+})	66 mg/L	300 µL/L from a 365 mg/L solution of $MgSO_4.7H_2O$ (Fisher BioReagents). Final conc. of 78 mg/L
Oleic acid	(na)	387 µL/L from 97% Oleic acid (Acros Organics). Equivalent to1000 mg COD/L
Phosphate (PO_4-P)	13 mg P/L	1000 µL/L from a 77 g P/L stock solution of KH_2PO_4/K_2HPO_4. Final conc. of 90 mg P/L
Sodium chloride (NaCl)	(na)	6% (w/v) from 99.0% NaCl (Fisher BioReagents)

na, not altered, maintaining the original concentration in the sludge dewatering liquors.

Twelve tests were prepared in a 1 L conical borosilicate glass flask containing 300 mL of sludge dewatering liquors inoculated with *B. antiquum* at 1% v/v ratio, stoppered with foam polystyrene plugs and then incubated for 8 days (Table 4-2). Samples were taken daily to measure the growth rate of *B. antiquum* using flow cytometry. At the end of the incubation period, bio-struvite produced was filtered, identified and quantified.

Table 4-2 Plackett–Burman experimental design of the screening experiment detailing the high (–) and low (+) levels of the different factors tested.

Factor	Test											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Acetic acid	–	–	–	+	+	+	–	–	–	+	+	+
Ammonium (NH_4-N)	+	–	+	–	+	–	–	+	–	+	+	–
Calcium (Ca^{2+})	–	–	+	–	+	+	–	+	+	–	–	+
Magnesium (Mg^{2+})	–	–	+	+	–	+	+	+	–	–	+	–
Oleic acid	–	–	–	–	–	–	+	+	+	+	+	+
Phosphate (PO_4-P)	–	+	+	–	+	–	+	–	–	–	+	+
Sodium chloride (NaCl)	–	+	+	–	–	+	–	–	+	+	+	–

4.2.3.2 Optimisation of the screened growth factors

In order to optimise the factors that significantly influenced the growth of *B. antiquum*, the path of steepest ascent (PSA) method was applied, following from the first order model obtained in the PB experiment. Eleven tests were prepared with different levels of acetate and NaCl. For each step tested, the NaCl and acetate concentrations of the sludge dewatering liquors were increased with 1.5% w/v NaCl and acetate in chemical oxygen demand (COD) equivalent to 562 mg COD/L. Two control tests were prepared with sludge dewatering liquors, without any addition of acetate or NaCl. The tests were inoculated with *B. antiquum* at 1% v/v ratio, stoppered with foam polystyrene plugs and then incubated for 7 days. Samples were taken daily to measure the growth rate of *B. antiquum* using flow cytometry. At the end of the incubation period, bio-struvite produced was filtered, identified and quantified.

In order to assess for possible interactions between NaCl and acetate on the *B. antiquum* growth, the concentrations that resulted in higher growth rates in the PSA experiment (3.0% w/v NaCl and 1124 mg COD/L as acetate) were used as the central point for a circumscribed central composite design (CCD) experiment. In order to estimate the variation of the growth rate, 8 other tests were prepared with NaCl and acetate amounts equally placed around the

central point in the design space: 3 ± 1.5% of NaCl and 1124 ± 562 mg COD/L as acetate. Two control tests were prepared with sludge dewatering liquors, without any addition of acetate or NaCl.

All the tests were inoculated with *B. antiquum* at 1% v/v ratio, stoppered with foam polystyrene plugs and then incubated for 7 days. Samples were taken daily to measure the growth rate of *B. antiquum* using flow cytometry. At the end of the incubation period, bio-struvite produced was filtered, identified and quantified.

4.2.4 Bio-struvite formation under optimal growth conditions

4.2.4.1 One-Stage incubation experiments

Experiments were completed to help understand the impact of NaCl and acetate on bio-struvite production. Four 125 mL bottles with 50 mL of dewatering liquors were supplemented with 562, 1124, 1686, and 2248 mg COD/L as acetate, four other bottles were supplemented with 1.5, 3.0, 4.5, and 6.0% of NaCl. Control tests were also prepared without any addition of acetate or NaCl, or addition of *B. antiquum* to assess spontaneous struvite precipitation and microbial contamination, respectively. All the inoculated tests were added to *B. antiquum* at 1% v/v ratio and all tests were incubated for 6 days. Samples were taken daily to measure the growth rate of *B. antiquum* using flow cytometry. In this experiment the bio-struvite production was quantified by measuring the PO_4-P quantities after isolation of the bio-struvite with differential density centrifugation. This method was considered more accurate as it allowed the quantification of bio-struvite crystals <10 µm.

4.2.4.2 Two-Stage incubation experiments

In the 1-Stage incubation experiments the bio-struvite production was very low. To understand these results, a 2-Stage incubation experiment was completed. Stage 1 aimed at promoting growth of *B. antiquum* by adding acetate and NaCl to the sludge dewatering liquors. Stage 2 aimed at bio-struvite production with no addition of NaCl.

In Stage 1, acetate was added at 0, 281, 562, 1124, and 1686 mg COD/L (as acetate) and NaCl was added at 3.0% w/v. After 3 days of incubation the liquors were centrifuged (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 10 min), the supernatant sludge dewatering liquors were collected, and replaced with sludge dewatering liquors with acetate (equivalent to 281 mg COD/L), without NaCl addition. Control tests were also prepared without acetate or NaCl, or addition of *B. antiquum* to assess spontaneous struvite precipitation and microbial contamination, respectively. Samples were taken daily to measure the growth rate of *B. antiquum* using flow cytometry. At the end of the incubation period, bio-struvite produced was filtered for identification using electron microscopy. The bio-struvite production was quantified by measuring the PO_4-P quantities after isolation of the bio-struvite with differential density centrifugation.

4.2.5 Sample analysis and analytical methods

Total phosphorus (TP), and COD were measured using the Merck cell test kit according to the manufacturer's instructions. Magnesium was analysed using an atomic absorption spectrophotometer (Analyst 800, Perkin Elmer Ltd, Beaconsfield, UK) equipped with an air/acetylene burner system. The pH was measured with a Fisherbrand hydrous 300 pH meter (Fisher Scientific, Loughborough, UK) immediately after sampling. Ammonium and PO_4 were measured using a Smartchem 200 discrete analyser according to the manufacturer's instructions (Labmedics, Abingdon, UK).

The growth rates of the selected bacteria were calculated from intact cell counts measured daily using flow cytometry. Intact cell counts were measured using a SYBR Green I and propidium iodide dye mixture with an incubation period of 11 min at 37°C and following the procedure detailed in Lipphaus *et al.* (2014).

In order to identify and quantify the bio-struvite produced, the sludge dewatering liquor samples were vacuum-filtered through a previously dried (2 hours, 37°C), and weighted, 10 µm aperture nylon mesh sheet (Plastok Associates, Birkenhead, UK). The crystals were washed with a small quantity of washing water (deionised water adjusted to pH 10 with 1M NaOH) and allowed to dry at 37°C for 2 hours before being weighed. The crystals were identified using high

resolution scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDX) (scanning electron microscope XL 30 SFEG, Phillips, The Netherlands).

In order to account for bio-struvite crystals smaller than 10 µm, a differential density centrifugation method was applied (Cromar and Fallowfield, 1992). This method allows the separation of liquids (and particles) according to their density. The density of the sludge dewatering liquors was assumed to be similar to that of water (1 kg/L); bacterial cells and proteins have relative densities of 1.1 kg/L and 1.3, respectively (Milo *et al.*, 2010), and struvite crystals have a relative density of 1.7 kg/L (Le Corre *et al.*, 2009). A sucrose solution (2000 g/L; density of 1.3 kg/L) was layered at the bottom of a centrifuge tube (5 mL) and the sludge dewatering liquors were added on top. After centrifugation for 10 min (Sanyo MSE Falcon 6/300 centrifuge, 2400g), the supernatant, made up of sludge dewatering liquors and bacterial cells, was discarded and removed with 3–4 mL of the sucrose layer. The remainder was dissolved with 10 mL of 0.05M HCl and the phosphate content quantified and used to estimate the amount of bio-struvite produced.

4.3 Results and Discussion

The sludge dewatering liquors used in this study were analysed for typical wastewater quality parameters across the different samples collected (Table 4-3). The COD concentrations were stable at around 455 ± 12 mg/L. The measured nutrient concentrations were 882 ± 31 mg NH_4-N/L (63.0 mM), 33 ± 5 mg PO_4-P/L (1.1 mM) and 55 ± 9 mg Mg^{2+}/L (2.3 mM) with a pH of 7.9 ± 0.1. Existing literature on sludge dewatering liquors originating from biological nutrient removal sites, indicated lower NH_4-N concentrations at 551-610 mg NH_4-N/L (39.3-43.6 mM) and Mg^{2+} at 11.2-29.2 mg Mg^{2+}/L (0.5-1.2 mM) but higher phosphate at 39 mg PO_4-P/L (1.3 mM), up to 167 mg PO_4-P/L (5.4 mM) (Ueno and Fujii, 2001; Pastor, Martí, *et al.*, 2008). Hence the sludge dewatering liquors used in this study had lower PO_4-P concentrations

than typically found in sludge dewatering liquors originating from biological nutrient removal sites.

Table 4-3 Characteristics of the sludge dewatering liquors collected from a full scale site (mean ± standard error).

	pH	**Total COD** (mg/L)	**Dissolved COD** (mg/L)	**NH_4-N** (mg N/L)	**PO_4-P** (mg P/L)	**Mg^{2+}** (mg/L)
Screening experiment	8.1	440 ± 6	n.d.	839 ± 1	14 ± 1	66
PSA experiment	8.0	482 ± 1	n.d.	941 ± 4	32 ± 1	n.d.
CCD experiment	8.0	479 ± 1	n.d.	866 ± 10	38 ± 1	33
Salt/Acetate experiment	7.8	458 ± 1	n.d.	n.d.	40 ± 1	46
Bio-struvite production experiment	7.8	500 ± 3	418 ± 4	n.d.	41 ± 1	74

PSA, path of steepest ascent method; CCD, central composite experimental design; n.d., not determined.

The PB experiment was carried out to screen for the impact of selected factors on the growth rate of *B. antiquum* on sludge dewatering liquors to obtain a first approximation linear model. These factors included Mg^{2+} (low:2.7 mM; high:3.2 mM), NH_4-N (low:36.4 mM; high:59.9 mM), PO_4-P (low:0.4 mM; high:2.9 mM), Ca^{2+} (high: 2.5 mM added), NaCl and two carbon sources (a readily available source – acetate, and a more complex form of carbon – oleic acid). The growth rates observed ranged from 0.50 to 3.44 1/d with the control test (Test 1) having a growth rate of 0.93 1/d (Table 4-4). The significance of each factor was assessed by a multi variance analysis of the fitted model of growth rate response (Table 4-5). The fitted model was found to have a significant correlation of 98% (r^2 – adjusted for the degrees of freedom) with a mean absolute error of 0.06, and a global *p*-value of 0.03%. These values indicate a very low probability that the results obtained could have occurred by random chance, and indicate that the fitted model explains >98% of the variability observed in the growth rate.

Table 4-4 Maximum observed growth rate of *B. antiquum* for each test of the screening experiment.

	Test											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Growth rate (μ, 1/d)	0.93	0.61	0.74	1.36	0.99	1.11	0.99	1.04	0.50	3.32	3.44	1.34

Table 4-5 Multiple regression analysis of the screening experiment towards the response variable growth rate.

Parameter	**Contribution to growth rate** (1/d)	***p*-value** (%)
Acetic acid	0.56	0.01
Ammonium	0.38	0.06
Calcium	–0.41	0.04
Magnesium	0.08	9.55
Oleic acid	0.41	0.04
Phosphate	–0.01	75.85
Sodium chloride	0.26	0.25
Model constant [a]	1.36	–

[a] Parameter-independent component of the regression fitted model.

The highest growth rate was obtained when high levels of Mg^{2+} (3.2 mM), NH_4-N (59.9 mM), PO_4-P (2.9 mM), NaCl, acetate and oleic acid and low levels of Ca^{2+}, were present in the liquors. Both acetate and oleic acid demonstrated a significant and comparable impact on the growth rate of *B. antiquum* with acetate having a better response (μ = 1.92 1/d) than oleic acid (μ = 1.77 1/d). The addition of a carbon source in WWTPs, is a common practice in denitrification and enhanced biological phosphate removal processes, in order to stimulate the proliferation of selected bacteria such as denitrifiers and phosphorus accumulating organisms (Cherchi *et al.*, 2009; Manyumba *et al.*, 2009). A similar approach might also benefit the growth of *B. antiquum*. The addition of NaCl at a rate of 6% w/v showed a positive effect on the growth rate

(μ = 1.62 1/d) and can possibly be used as a selective pressure to create a process environment where *B. antiquum* can outcompete the indigenous bacteria, and thus remain in stable concentrations to allow the use of sludge dewatering liquors without the need for sterilisation.

The first order model, obtained in the screening experiment functioned as an initial approximation of the growth rate of *B. antiquum* as a function of the factors tested. To further screen for the optimal growth rates, the model space was additionally explored following the PSA method. Hence, increased amounts of acetate (+562 mg COD/L) and of NaCl (+1.5% w/v), were tested. The model was not further optimised in terms of Ca^{2+} or NH_4 concentrations given that controlling these factors seemed impractical in a WWTP.

The results for the PSA experiment proved that the conditions that resulted in the highest growth rate, 3.32 1/d, were found with 3.0% w/v NaCl and 1124 mg COD/L as acetate (Table 4-6). Test 1 and Test 3 followed with the 2nd and 3rd highest growth rates of 2.87 and 2.75 1/d, respectively. The remaining observed growth rates were also well within the range observed in the screening experiment of 0.50 to 3.44 1/d. This indicated that the factors required for maximum growth rate were close to the conditions found in Test 2 (3.0% w/v NaCl and 1124 mg COD/L as acetate). A CCD experiment was used to assess the main effects and interactions of acetate and NaCl around a central point with 3.0% w/v NaCl and 1124 mg COD/L as acetate. The growth rates observed were between 2.43-3.20 1/d and statistically similar (mean at the central point was μ = 2.75 ± 0.08 1/d). These results demonstrate that the maximum growth rate measured could be obtained at a range of conditions tested, indicating *B. antiquum's* adaptability and flexibility. This can be seen as an important advantage allowing for flexibility on the design of a bio-struvite production process as high growth rates can be obtained for NaCl and acetate concentrations within 3.0 ± 1.5% and 1124 ± 562 mg COD/L as acetate, respectively.

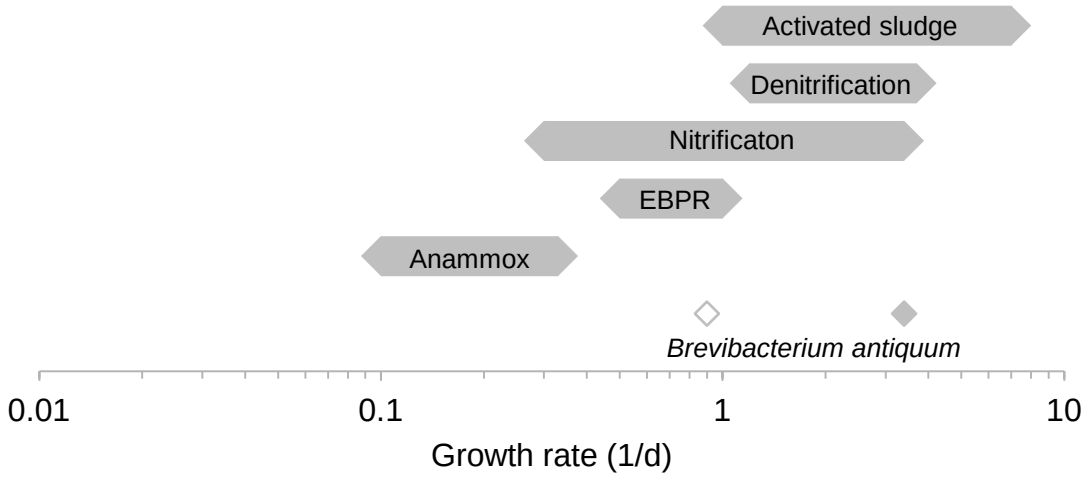
Table 4-6 Maximum observed growth rate of *B. antiquum* for each test on the path of steepest ascent of the linear model identified on the screening experiment. Each step added 1.5% (w/v) of NaCl and 562 mg COD/L as acetate.

	Test										
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Growth rate (μ, 1/d)	1.21 ± 0.25[a]	2.75	3.32	2.87	2.39	2.73	2.09	1.61	1.23	1.21	0.80

[a] standard error of replicate tests.

The *B. antiquum* growth rate of 3.44 1/d was 60 fold higher than previously reported at 0.05 1/d (Soares *et al.*, 2014). Nevertheless, the lower growth rate was estimated using turbidity measurements and no addition of extra carbon source or NaCl, which could potentially explain the difference in results.

The *B. antiquum* growth rates observed herein were obtained by varying selected environmental conditions and substrates at 2 levels (screening and PSA experiments), thus a maximum specific growth rate could not be calculated. Nevertheless, when comparing the growth rates of *B. antiquum* in this study (μ = 3.44 1/d) with other bacteria commonly found in WWTPs, it was observed that *B. antiquum* is a relatively fast growing organism (



). Heterotrophic bacteria growing in activated sludge systems have been reported to have maximum specific growth rate (μ_{MAX}) varying between 1-7 1/d, both in domestic and several industrial wastewaters (Sözen *et al.*, 1998). Heterotrophic denitrification bacteria were reported have maximum specific growth rate of 3.7 1/d when using acetate as a carbon source, and 1.2 1/d with methanol (Mokhayeri *et al.*, 2006). Autotrophic ammonia oxidizing bacteria have been reported to have μ_{MAX} = 0.3-3.4 1/d (Cherchi *et al.*, 2009). Even without the use of acetate and NaCl, *B. antiquum* growth rate in sludge dewatering liquors (μ = 0.93-1.21 1/d) was high relative to reported maximum specific growth rates of phosphorus removal bacteria (μ_{MAX} = 0.5-1.0 1/d) (Isaacs *et al.*, 1995; Whang *et al.*, 2007) and Anammox (μ_{MAX} = 0.09-0.33 1/d) (Laureni *et al.*, 2015; Lotti *et al.*, 2015). All these bacteria which have been successfully applied are a commercially viable biological process for the treatment of wastewater and sludge dewatering liquors. This was accomplished through application of processes and reactors with particular engineered operational conditions that work as a selective pressure, limiting other competing microorganisms and allowing for them to proliferate in open mixed-culture conditions (Tchobanoglous *et al.*, 2003; Manyumba *et al.*, 2009).

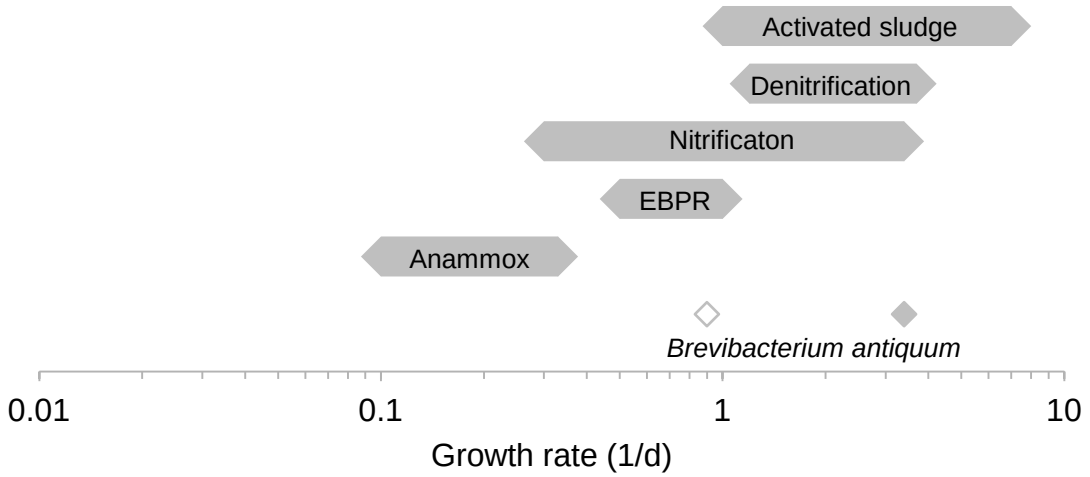


Figure 4-1 **Comparison of the growth rates of *B. antiquum* in sludge dewatering liquors (◇, non-optimised; ◆, optimised with 3.0% w/v NaCl and 1124 mg COD/L as acetate) with the maximum specific growth rate (μ_{MAX}) range of values found in published literature for heterotrophic bacteria in activated sludge systems, heterotrophic denitrifying bacteria, nitrification bacteria, enhanced biological phosphorus removal (EBPR) bacteria, and Anammox bacteria Bio-struvite production (Isaacs *et al.*, 1995; Sözen *et al.*, 1998; Cherchi *et al.*, 2009; Laureni *et al.*, 2015; Lotti *et al.*, 2015; Reino *et al.*, 2016).**

Visual inspection of the tests in the screening and the PSA experiments indicated that the formation of bio-struvite was taking place but was not associated with the growth rates. In the CCD experiment, the quantity of bio-struvite recovered in the inoculated tests dosed with NaCl and acetate was very low at 10 ± 6 mg/L. These results implied that the application of acetate and NaCl prevented the formation of bio-struvite.

The effects of adding separate different quantities of NaCl or acetate to sludge dewatering liquors were tested (one-stage experiment). It was observed that *B. antiquum* had considerably higher cell counts in the tests with added acetate: 11-28x more cells than in the inoculum, compared to 2x more in tests dosed with NaCl alone (Figure 4-2). Bio-struvite was formed in all tests supplemented with only acetate (9.6-21.5 mg P/L) and not in the tests dosed with NaCl (0.8-1.7 mg P/L). When both NaCl and acetate were added, the cell counts were comparable to the growth in the tests with acetate (Figure 4-2) but the bio-struvite production was similar to the tests with NaCl alone (1.2 mg P/L). The results here support the fact that it was the NaCl, and not acetate, that prevented the formation of bio-struvite in sludge dewatering liquors dosed with both NaCl and acetate.

Organic substances as humic and citric acids have been shown to inhibit struvite formation (Zhang *et al.*, 2015; Zhou *et al.*, 2015) and acetate has been reported to delay the precipitation of struvite at more than 5 mM (equiv. 321 mg COD/L), but the effect was not noticeable after 25 min (Song *et al.*,

2014) – a small time interval when compared to the incubation times tested here. The solubility of struvite has been found to not change significantly at NaCl concentrations up to 60mM (equiv. 0.35% w/v) (Bhuiyan *et al.*, 2007). Nevertheless, time required for struvite precipitation was reported to increase at sodium concentrations above 50 mM (Kabdaşli *et al.*, 2006). In the current study, NaCl was used at 1.5 to 4.5% w/v, hence 5 fold higher than the concentration of NaCl that was reported to affect chemical struvite precipitation. In a study investigating the occurrence of bio-mineralisation in artificial marine-salts media by halophilic bacteria, increasing salt concentrations (from 2.5 to 20% w/v) was reported to lead to minerals of smaller size, and to increase the time required for precipitation from 3 to 15 days (Rivadeneyra, Delgado, *et al.*, 2006). Although biomineralization mechanisms may be able to selectively change specific micro-environments independently of the macroscopic environmental conditions (Mann, 2001), results here demonstrated that NaCl prevented bio-struvite formation in sludge dewatering liquors.

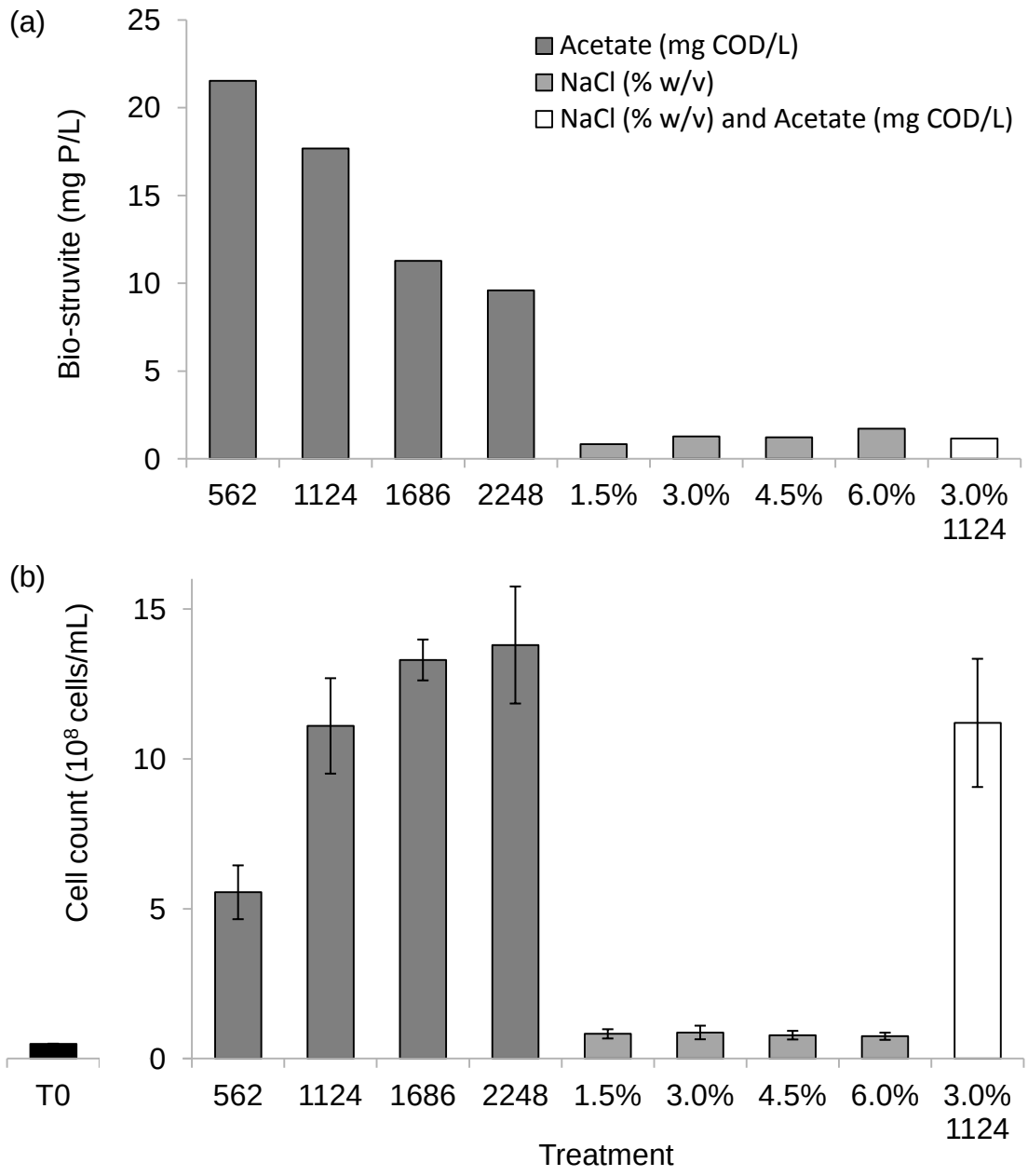


Figure 4-2 One-Stage experiment - (a) bio-struvite production (only one replica measured) and (b) cell counts after incubating *B. antiquum* for 6 days in sludge dewatering liquors supplemented with 1.5, 3.0, 4.5, and 6.0% w/v of NaCl, and 562, 1124, 1686, and 2248 mg COD/L of acetate. Error bars in (b) detail the standard error of replicate tests.

Henceforth, an experiment was carried out to assess the production of bio-struvite in two Stages. A Stage 1 of 3 days was used to promote growth of the selected bacteria with the addition of acetate and NaCl. This was followed by a Stage 2 of 5 days without added NaCl. Acetate was dosed in Stage 1

experiments at different initial concentrations and then added to various tests in Stage 2 (281 mg COD/L). In Stage 2, bio-struvite was formed in all tests, except for those without the addition of acetate in either of the stages, as bacterial growth was limited (Treatment A, Figure 4-4).

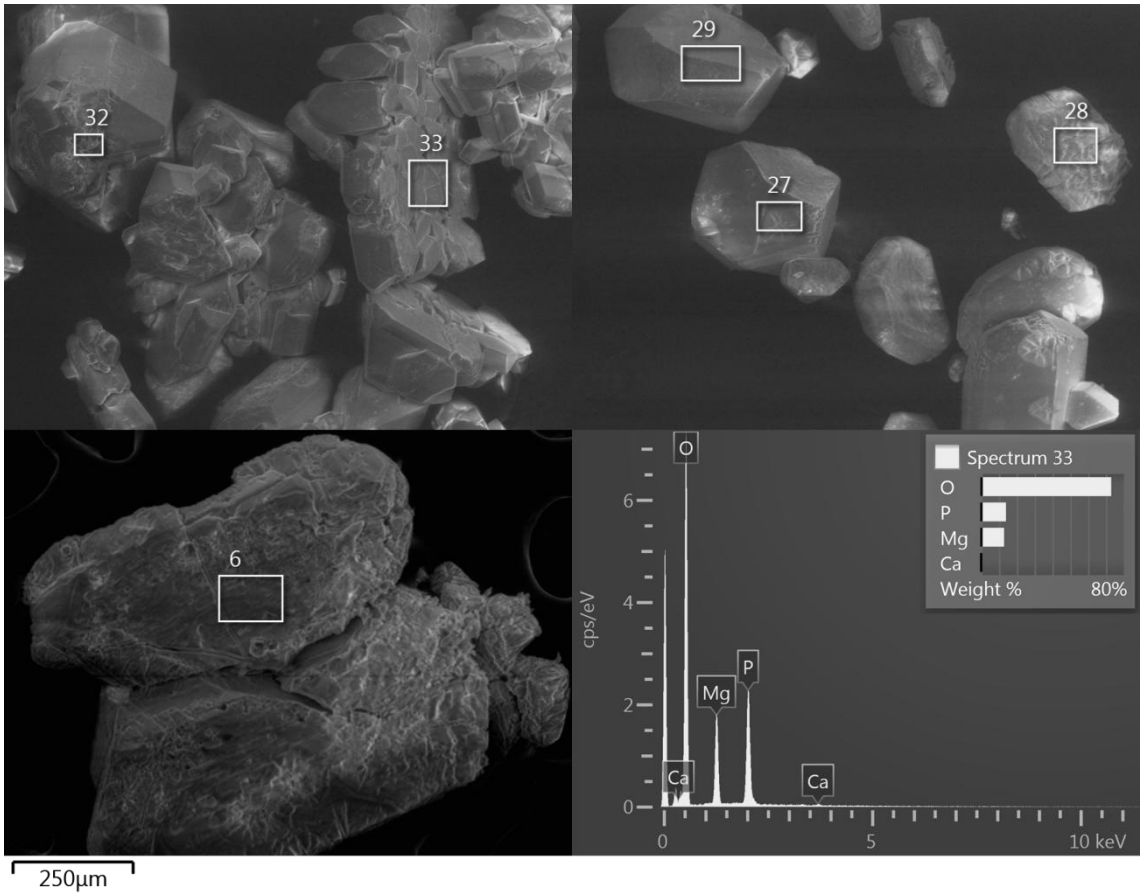


Figure 4-3 Electron scanning microscope photos of the bio-struvite collected after the Two-Stage experiment: (a) no acetate in Stage 1, followed by 281 mg COD/L of acetate in Stage 2; (b) 1124 mg COD/L in Stage 1, followed by 281 mg COD/L of acetate in Stage 2; (c) 1686 mg COD/L of acetate in Stage 1, followed by no acetate in Stage 2. Energy dispersive X-ray micro-analysis spectrum of site 33 highlighted in photo (a), characteristic struvite crystals.

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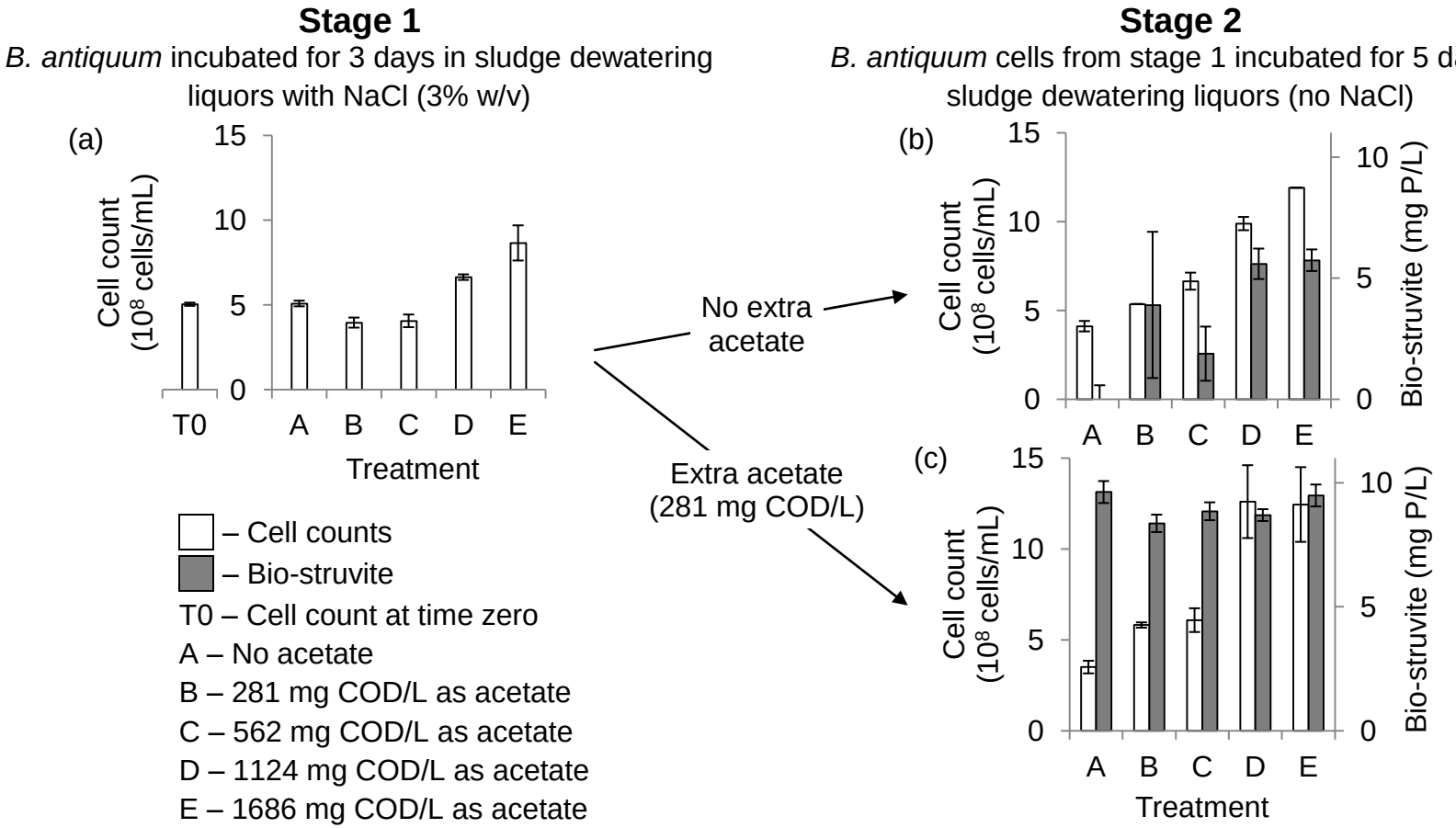

Figure 4-4 Maximum cell counts during incubation and bio-struvite production above control tests in the 2-Stage incubation experiment: (a) Stage 1 cell counts when incubating *B. antiquum* in sludge dewatering liquors with 3.0% w/v NaCl and different concentrations of acetate (Treatment A: 0, B: 281, C: 562, D: 1124, and E: 1686 mg COD/L); (b) Stage 2 cell counts and bio-struvite production after incubating *B. antiquum* cells collected from Stage 1, in sludge dewatering liquors with no NaCl and no acetate; and (c) Stage 2 cell counts and bio-struvite production after incubating *B. antiquum* cells collected from Stage 1, in sludge dewatering liquors with no NaCl and with 281 mg COD/L of acetate. T0 is the cell count at time zero, after inoculating *B. antiquum* from starter cultures. Error bars detail the standard error of replicate tests.

The presence of bio-struvite was confirmed with energy-dispersive X-ray spectroscopy. The spectra obtained matched the spectra expected for struvite crystals (Figure 4-3d), and the morphology of the bio-struvite crystals, observed in the electron microscope, was found to follow characteristic prismatic, tabular, and twinned-crystal habit (Figure 4) similar to the morphologies found in other published reports on struvite (Sánchez-Román *et al.*, 2007; Song *et al.*, 2014; Zhang *et al.*, 2015; Zhou *et al.*, 2015)

These results clearly demonstrated that NaCl at 3.0% w/v impacted the biomineralization of the bio-struvite. Both growth and bio-struvite production could be promoted by establishing a 2 Stage process. In the tests where acetate was added to Stage 2, bio-struvite production was higher reaching a maximum value of 9.0 ± 1.4 mg P/L (Figure 4-4c). The addition of acetate in Stage 2 contributed towards bio-struvite production whilst the addition of acetate in Stage 2 was not essential for bio-struvite production.

Cell counts in Stage 2 (where no extra acetate was added) reached 4.1×10^{8} to 1.2×10^{9} cells/mL (Figure 4-4b), similar to the cell counts obtained in the experiment in Figure 4-2b (single Stage) (from 5.6×10^{8} to 1.4×10^{9} cells/mL, Figure 4-2b). However, the bio-struvite produced did not follow the same trend. In the latter, increasing the acetate doses (from 562 to 2248 mg COD/L) led to lower rates of bio-struvite production (from 21.5 to 9.6 mg P/L, respectively, Figure 4-2a). It is possible that bio-struvite formation was associated with the end of the exponential growth phase. In the experiment in Figure 4-2, a dose of 2248 mg COD/L acetate was likely to be the result in a longer growth phase. In the case of the 2-Stage incubation experiment (Figure 4-4) the exponential growth phase was interrupted, potentially causing stress to the bacterial cells. The production of secondary metabolites, as antibiotics, is associated with the end of the exponential growth phase (Ruiz *et al.*, 2010). Classical methods to increase the production of secondary metabolites involves the manipulation of the culture conditions and biological stress responses (Craney *et al.*, 2013). Hence, it is possible that the differences observed could be due to the

production of bio-struvite as secondary metabolism, although further tests are required to verify this statement.

4.4 Conclusions

Biological processes used in conventional wastewater treatment are typically performed in open mixed-culture conditions. Ideally, a bio-struvite production process should be carried out in open mixed-culture conditions. To allow the stable growth of the bio-struvite bacteria *B. antiquum* in open mixed-culture conditions, a process needs to be designed in a way that allows *B. antiquum* to grow at a faster pace than other microorganisms present in the sludge dewatering liquors. The goal of this work was to identify the optimal growth rate of *B. antiquum* in sludge dewatering liquors as a function of factors such as Mg^{2+}, NH_4-N, PO_4-P, Ca^{2+}, NaCl and two carbon sources: acetate (ready available) and oleic acid (complex form of carbon). Moreover, it aimed to assess the impact of the factors responsible for the observed optimal growth on bio-struvite production.

Acetic acid, oleic acid, NaCl, and NH_4 were shown to have a positive impact on the growth rate of *B. antiquum* with average growth rates of 1.92, 1.77, 1.57, 1.69 1/d, respectively, in the tests with high levels. Additionally, Ca^{2+} was found to have a negative impact (0.95 1/d). Controlling the content of NH_4 and Ca^{2+} in sludge dewatering liquors, in a full-scale WWTP, to favour the growth rate of *B. antiquum*, was considered unpractical but insight was gained on their impact on the *B. antiquum* growth rate.

The growth rate (μ) of *B. antiquum* in sludge dewatering liquors was 0.93-1.21 1/d but this could be increased by 3 fold to 3.44 1/d when supplementing the sludge dewatering liquors with acetate (1124 mg COD/L) and NaCl (3.0% w/v).

However NaCl at 3.0% w/v was found to hinder the biomineralization of the bio-struvite. Both growth and bio-struvite production could be promoted by establishing a 2 Stage process. The growth stage would combine the sludge

dewatering liquors with acetate and NaCl, followed by a second stage for bio-struvite production with just acetate dosing.

The fact that the growth rate of *B. antiquum* in sludge dewatering liquors can be made to reach 3.44 1/d, broadens the probability of finding conditions in sludge dewatering liquors, or other wastewater streams, where *B. antiquum* can be used to produce bio-struvite and recover phosphorus from wastewater streams in open mixed-culture conditions.

4.5 Acknowledgements

The authors are grateful for the financial support and contribution from Severn Trent Water Ltd (Coventry, UK) and for the financial support from POPH/FSE as a Ph.D. grant (SFRH/BD/84116/2012) awarded by the Fundação para a Ciência e Tecnologia (FCT, Portugal).

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Chapter 5 Understanding the impact of the source of phosphorus on biological struvite formation

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Abstract

This work investigates which forms of phosphorus were used by *Brevibacterium antiquum* to form bio-struvite in wastewater sludge dewatering liquors. After 72 h of incubation, 25.6 mg P/L were recovered as bio-struvite from 12.4 mg P/L of the initial organic and condensed phosphorus and 13.2 mg P/L of the initial phosphate. This study demonstrated that *B. antiquum* can convert of organic and condensed phosphorus into bio-struvite, and this opens up a completely new way to recover forms of phosphorus that are not typically available for phosphorus recovery in a single process.

Ten types of sludge liquors (including primary, secondary, digester feed and digestate from 3 types of wastewater treatment plants) were characterized to assess their potential to be used for phosphorus recovery in the form of bio-struvite. Sludge liquors were characterized for their phosphorus fractions and other parameters relevant for bacteria growth. Results indicated that liquors obtained from digested, primary, and digester feed were the most suitable to produce bio-struvite, thus expanding the range of sludge liquors that can be used phosphorus recovery in a wastewater treatment plant.

Keywords

Biomineralization; centrate; sidestream; P-recovery; acid-hydrolysable phosphorus.

5.1 Introduction

Phosphorus is a non-replaceable nutrient essential for all living organisms. Application of phosphorus fertilizers has enabled the increase of agricultural crop yields making phosphorus necessary to secure food production (Cordell *et al.*, 2012). However, usable sources of phosphorus are limited and have a widely asymmetric world distribution. Five countries are responsible for 80% of the world production of phosphate rock, and three countries own >80% of the reserves (Jasinski, 2016).

Phosphorus recovery from wastewater is expected to contribute to fill this gap in phosphorus supply (Cordell *et al.*, 2009). Liquors from dewatering sludge have been pointed as the wastewater stream with better potential for phosphorus recovery (Jaffer *et al.*, 2002). Sludge dewatering liquors can have high concentrations of phosphate (1-167 mg P /L) while making up a small fraction of the main stream of treatment flow (1-2% v/v) (Fux *et al.*, 2002; Pastor, Martí, *et al.*, 2008). When returned to the main stream of treatment, sludge dewatering liquors can account for 40% of the phosphorus load in the secondary treatment (Jardin and Pöpel, 1994; Battistoni *et al.*, 1997). The removal of phosphorus from sludge dewatering liquors has the potential to be more economical than removing phosphorus in the main stream of treatment, and thus can lower operational costs and extend the usable lifetime of the wastewater treatment plant (WWTP).

Phosphorus recovery from sludge dewatering liquors can be accomplished through precipitation of phosphate minerals such as hydroxyapatite or, more common, struvite, a salt of magnesium, ammonium, and phosphate ($MgNH_4PO_4.6H_2O$). Conventional chemical struvite requires magnesium dosing and pHs >8.5 that are often accomplished with chemicals, or CO_2 stripping (Le

Corre *et al.*, 2009). Commercially available processes need a source of liquors with phosphorus above 100 mg P /L to remain economically viable (Dockhorn, 2009).

Selected bacteria as *B. antiquum* were shown to mediate the biological formation of struvite (bio-struvite) when incubated in sterile sludge dewatering liquors with 31 mg P /L, without addition of other chemicals (Soares *et al.*, 2014). This suggested the possibility of a process to recover phosphorus from low phosphorus wastewater streams without the requirement for chemical additives. However, the underlying bio-struvite formation mechanisms, and which forms of phosphorus are used by the bacteria to produce the bio-struvite are not yet known.

Phosphorus in wastewater exists in a complex variety of forms that can be difficult to separate and quantify (Majed *et al.*, 2012). The most accepted definitions separate phosphorus in: readily reactive, organic and condensed phosphorus, according to the reactivity with ammonium molybdate and susceptibility to acid hydrolysis (APHA, 2005; Gu *et al.*, 2011). Readily reactive phosphorus includes the dissolved inorganic phosphate-ion forms, and can include loosely adsorbed phosphate ions and phosphate ions associated in molecular complexes. This form of phosphorus is the only directly available for the precipitation of chemical struvite. The organic phosphorus fraction in wastewater includes substances as DNA, adenosine triphosphate (ATP), phospholipids, and other organic substances containing phosphorus. This fraction has been reported to make up 14 to 21% of the total phosphorus in sludge collected from enhanced biological phosphorus removal (EBPR) processes treating domestic wastewater (Xie *et al.*, 2011). Substances in the organic phosphorus fraction are resistant to hydrolysis when subjected to acid conditions. A strong acid oxidant, such as perchloric acid, is required to free the phosphate ions from organic phosphorus substances in order to quantify the total phosphorus in wastewater samples (APHA, 2005). On the other hand, substances in the condensed phosphorus fraction can be hydrolysed in acid conditions under mild to high temperature conditions (100-121°C) (Gu *et al.*,

2011). This fraction includes condensed inorganic phosphates as the polyphosphates (poly-P) accumulated by bacteria in EBPR, and biological nutrients removal (BNR) processes (Myung *et al.*, 2014). Given the acid conditions used, a fraction of adsorbed phosphates can also solubilise and add to the amount of phosphorus quantified as condensed phosphorus (APHA, 2005).

Sludge dewatering liquors can be sourced from a wide variety of sludges, with expected differences in their phosphorus fractions, particularly if different phosphorus removal technologies are used. The sludge from secondary treatment processes such as EBPR / BNR processes can have 300 mg poly-P/g VSS, well above 30 mg P/g VSS in conventional activated sludge (Acevedo *et al.*, 2012). However, there is very limited information regarding the amounts of different forms of phosphorus in the various types sludge dewatering liquors.

This work aims to identify what different fractions of phosphorus were used by *B. antiquum* to promote bio-struvite formation in sludge dewatering liquors, and what proportion of the different fractions of phosphorus can be found in the various type sludge dewatering liquors (including primary, secondary, digester feed and digestate from various types of wastewater treatment plants), in order to assess what is the potential of the bio-struvite formation process for phosphorus recovery.

5.2 Materials and Methods

5.2.1 Bacteria source

Brevibacterium antiquum, strain DSM 21545, was obtained from a commercial culture collection (German Resource Centre for Biological Material, Brunswick, Germany). Pure bacteria starter cultures were prepared by growing *B. antiquum* in sterile synthetic media (4 g/L of yeast extract, 2 g/L of magnesium sulphate heptahydrate, and 2 g/L of di-potassium hydrogen phosphate) at room temperature (20-22°C), under agitation at 150 rpm (Stuart SSL1, Fisher Scientific, Loughborough, UK) for 4 days (i.e. 96 hours).

5.2.2 Sludge dewatering liquors source

Different types of sludge (including primary, secondary, digester feed and digestate) were collected from three full-scale WWTP with distinct processes regarding phosphorus removal, including: a WWTP with no phosphorus removal processes (NPR), a WWTP with chemical phosphorus removal (CPR), and a WWTP with biological nutrients removal (BNR).

The BNR WWTP had a capacity of 500,000 population equivalent (PE) with biological nutrient removal as secondary treatment. The CPR site had a capacity of 700,000 PE with activated sludge as secondary treatment and ferric chloride addition to the primary effluent for phosphorus removal. Both BNR and CPR, primary and secondary sludges were pre-thickened separately to 7-8% solids content, and stabilized in anaerobic digesters with imported sludge from nearby municipal WWTP (approx. 40% v/v, in both sites). The digested sludge was stored in holding tanks, from 10 to 27 days before dewatering from typical 7% solids to 22% solids in horizontal centrifuge-decanters. Cationic polymer, anti-scaling and antifoam additives were used to aid the centrifugation process. For this study the primary sludge was collected in the feed to the pre-digestion thickening centrifuges, and the secondary sludge was collected from the feed line to the pre-digestion belt thickeners. Digester feed sludge was collected from the anaerobic digesters feed line. Digested sludge was collected from the outlet line of an anaerobic digester in the BNR site, and after the post-digestion holding tanks in the CPR site.

The NPR site (200,000 PE) had no specific processes for phosphorus removal. The primary settlers were de-sludged manually and the primary effluent was split between activated sludge process and trickling filters (50% v/v). The primary and the secondary sludges were mixed in a pre-thickening weir tank. From the pre-thickening weir tank, the sludge was stored in holding tanks until fed to the anaerobic digester. Samples were collected in the sludge line of the primary settler tanks and both from the feed and outlet lines of the anaerobic digester.

The same dewatering procedure was applied to all the collected sludges, in order to obtain the respective dewatering liquors. The collected sludges were centrifuged in 50 mL disposable centrifuge tubes (Sanyo MSE Falcon 6/300 centrifuge, 300g, 5 min). The supernatant was collected and stored under refrigerated conditions (<4°C) for further analysis and testing, while the caked pellet was discarded.

5.2.3 Bacteria cultivation in sludge dewatering liquors

B. antiquum was added to autoclaved (121°C, 20 min) sludge dewatering liquors from the starter culture and incubated at room temperature (20-22°C), under agitation at 150 rpm (Stuart SSL1, Fisher Scientific, Loughborough, UK) for 4 days (*i.e.* 96 hours). Control tests, not inoculated with *B. antiquum*, were also prepared.

The pH of the sludge liquors used was adjusted before autoclaving to ensure that the sterile sludge liquors keep a pH between 7.3 and 7.7. The pH of some sludge liquors was as been found to increase to pH values >9 during autoclaving, which is not representative of the pH observed for sludge liquors (Simoes *et al.*, 2017b). To minimize the addition of PO_4, NH_4, and Mg^{2+} from the starter culture to the sludge dewatering liquors, the bacterial cells were separated from the synthetic media by centrifugation (2400g, 5 min, Sanyo MSE Falcon 6/300 centrifuge), and then resuspended to the original volume with sterile 0.9% NaCl solution. Inoculation of sludge liquors was done with 1 volume of resuspended bacteria to 10 volumes of sludge liquors.

5.2.4 Investigation of the sources of phosphorus for biological struvite formation

To follow the fate of the various phosphorus fractions during the growth of *B. antiquum* in sludge liquors, an experiment was setup with 40 sacrificial bottles. Each bottle was prepared with 50 mL of sludge liquors obtained from digested sludge of the BNR site. Five test bottles were prepared for each of eight sampling points. In total 24 bottles were inoculated with the selected bacteria

and 16 bottles were prepared without bacteria but with the same volume of sterile 0.9% NaCl solution.

To maximize the potential for struvite production, the sludge liquors were supplemented with magnesium making phosphate the limiting ion for struvite formation. Magnesium sulphate was added to a final concentration of 52 mg Mg^{2+}/L, using a 290 g/L solution of magnesium sulphate heptahydrate (Fischer BioReagents, Loughborough, UK).

During sampling, each test bottle was divided into aliquots: 10 mL of sample were saved to quantify pH and total phosphorus (TP). The remaining volume was mixed with 5 mL of 2000 g/L sucrose solution in a 50 mL centrifuge tube and centrifuged (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 10 min). This procedure was completed to separate the bio-struvite (density 1.7 g/L) from the liquid and bacterial cells using density (Cromar and Fallowfield, 1992). Only precipitates with higher density than the sucrose solution (1.3 g/L) settled to the bottom of the sucrose layer (Simoes *et al.*, 2017a). The pellet was saved with a small quantity of sucrose solution. The bio-struvite content was quantified by measuring the phosphate content of the pellet after solubilisation with 0.05M HCl. The supernatant was collected together with 3.5 ± 1.0 mL of the sucrose layer, placed in clean 50 mL centrifuge tube and centrifuged again (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 10 min). The supernatant was collected to quantify the condensed phosphorus (Cond-P) and phosphate (PO_4-P). Total dissolved phosphorus was quantified from this supernatant at the beginning and at the end of 72 hours of incubation. The biomass pelletized in this second centrifugation step was saved and kept frozen until analysed for total cellular phosphorus .

5.2.5 Quantification of condensed and organic phosphorus

The condensed phosphorus were converted to phosphate using a modified APHA 4500-P.B.2 method to quantify acid-hydrolysable phosphorus (APHA, 2005). Each sample (250 µL) was added to 4.65 mL of de-ionized water, mixed, and then acidified with 100 µL of sulphuric and nitric acid solution (75 mL 96% sulphuric acid mixed with 150 mL of de-ionized water and then supplemented

with 1 mL of conc. 68% nitric acid). The cell tube was then closed, mixed and allowed to digest for 30 min at 120°C in a preheated thermoreactor (Spectroquant TR620, Merck-Millipore, Watford, UK). After digestion the cell test kit tubes were allowed to cool down to room temperature (20-22°C) for 2 h, and then neutralized with 150 µL of 6 M aqueous sodium hydroxide solution. This modified method was validated and calibrated against set nine filtered sludge liquors samples spiked with 5 levels of sodium hexametaphosphate (Fisher Scientific, Loughborough, UK). Organic phosphorus was calculated by subtracting the condensed phosphorus from the total phosphorus.

5.2.6 Analytical methods

Bacterial counts were taken with flow cytometry using a live/dead cells staining method that provides the number of cells with intact membrane (Simoes *et al.*, 2017b).

The concentrations of total phosphorus (TP) fractions, chemical oxygen demand (COD), chloride and sulphate ions, were measured using cell test kits according to the manufacturer instructions (Merck-Millipore, Watford, UK). Total suspended solids (TSS), and alkalinity to pH 4.5 were measured following the standard methods. The pH was measured with a Fisherbrand hydrous 300 pH meter (Fisher Scientific, Loughborough, UK) immediately after sampling. Magnesium was analysed using an atomic absorption spectrophotometer equipped with an air/acetylene burner system (AAnalyst 800, Perkin Elmer Ltd, Beaconsfield, UK). Ammonium (NH_4), nitrite (NO_2), oxidised nitrogen, and phosphate (PO_4) were measured using a SmartChem 200 automated discrete analyser and the recommended reagents that adapt colorimetric standard methods (Labmedics, Abingdon, UK).

Aluminium, arsenic, boron, cadmium, calcium, chromium, copper, iron, lead, mercury, nickel, potassium, selenium, and zinc content of the sludge liquors was assessed by an external lab (Scientific Laboratories Limited, Manchester, UK) using inductively coupled plasma optical emission spectrometry (ICP-OES).

Values presented detail the mean and standard error of duplicate or triplicate tests unless stated otherwise.

5.3 Results and Discussion

5.3.1 Validation of the modified method for measuring condensed and organic phosphorus

The Standard Method, APHA 4500-P.B.2 (APHA, 2005) used to quantify acid-hydrolysable phosphorus was modified to reduce health and safety risks. The new method was validated and calibrated against nine filtered sludge liquors samples spiked with 5 levels of sodium hexametaphosphate. The results obtained clearly showed a good correlation between the amounts of PO_4 measured in function of hexametaphosphate (HMP) (21.6-127.3 mg P/L) with r^2 that varied between 0.987 and 0.998 (Table 5-1). The average slope and standard deviation was 0.302 ± 0.023 (± 7.5%) (mg P/mg HMP). This represents a recovery of 94.6% of the hexametaphosphate.

Table 5-1 Summary of the linear regressions for the set of nine filtered sludge liquors samples spiked with 5 levels of sodium hexametaphosphate.

Sample ID	Slope (mg P/mg HMP)	r^2	Phosphate after acid-hydrolysis without addition of HMP (mg P/L)
1	0.312	0.987	60.1
2	0.293	0.998	129.1
3	0.285	0.999	44.1
4	0.278	0.986	78.3
5	0.338	0.998	56.3
6	0.318	0.992	40.7
7	0.307	0.992	43.1
8	0.267	0.998	20.6
9	0.317	0.998	47.3

HMP, hexametaphosphate.

5.3.2 Phosphorus fractions used by *B. antiquum* to produce bio-struvite in sludge dewatering liquors

Liquors collected at the sludge dewatering plant of the BNR site were used to study which fractions of phosphorus were used by *B. antiquum* to produce bio-struvite. The sludge dewatering liquors used were analysed for typical wastewater quality parameters (Table 5-2). Total phosphorus (65.5 mg P/L), magnesium (18.6 mg/L) and COD (308 mg/L) were below published values of 79, 24, and 308 mg/L, respectively. Phosphate (49.5 mg/L) was within the range of published values but still in the lower range (43 mg/L). Ammonium content (889 mg N/L) and pH (7.7) were well within the published values of 355-1170 mg N/L and 7.2-7.9 pH. Hence, the used liquors can be considered low strength for BNR sludge dewatering liquors.

Table 5-2 Characteristics of the sludge dewatering liquors collected from the dewatering plant of a full-scale biological nutrients removal (BNR) site, and comparison with a values found in published literature for sludge dewatering liquors from BNR sites.

Parameter	BNR sludge dewatering liquors	Range in literature	References
pH	7.7	7.2-7.9	(Battistoni *et al.*, 2001; Martí *et al.*, 2010)
Total phosphorus (mg P/L)	65.5 ± 2.1	79-169	(Pastor *et al.*, 2010)
Phosphate (mg P/L)	49.5 ± 7.7	43-169	(Battistoni *et al.*, 2001; Pastor *et al.*, 2010)
Magnesium (mg/L)	18.6 ± 0.4	24-110	(David, 2007; Pastor *et al.*, 2010)
Ammonium (mg N/L)	888.6 ± 33.8	355-1170	(Battistoni *et al.*, 2001; Pastor *et al.*, 2010)
Total chemical oxygen demand (mg/L)	302.0 ± 1.4	308-1762	(Ahn and Choi, 2006; David, 2007)

When incubating *B. antiquum* in the BNR sludge dewatering liquors, the cell counts increased from 1.7×10^8 to 2.7×10^8 cells/mL after 72 h of incubation (Figure 5-1a). This indicates the *B. antiquum* growth was limited. Likely caused by low amount of available carbon source in the sludge dewatering liquors (302 mg COD /L, Table 5-2). Despite the increase in the number of cells, the amount of phosphorus associated with cellular material decreased slightly from 6.1 to 5.3 mg P /L (Figure 5-1b). When assessing the change in concentration of phosphorus per cell it is possible to observe that the cellular phosphorus decreased from 35 to 20 fg P /cell (Figure 5-1c). Microorganisms isolated from the aerobic section of EBPR process have been reported to contained 1 to 60 fg P/cell, and cells with >11 fg P/cell have been categorised as high P accumulators (Sidat *et al.*, 1999). Hence, *B. antiquum* can be classified as a high P accumulation organism. Furthermore, *B. antiquum* has been reported to accumulate intracellular inorganic phosphate when grown in synthetic media with 71-356 mg PO_4-P/L and high availability of carbon source (5 g/L glucose, 3 g/L yeast extract, and 5 g/L peptone) (Smirnov *et al.*, 2005). The results

obtained in this study show similar capabilities of *B. antiquum* to accumulate cellular phosphorus in real sludge dewatering liquors even with a low availability of carbon source (302 mg COD/L) and initial phosphate of 28.6 mg PO_4-P/L.

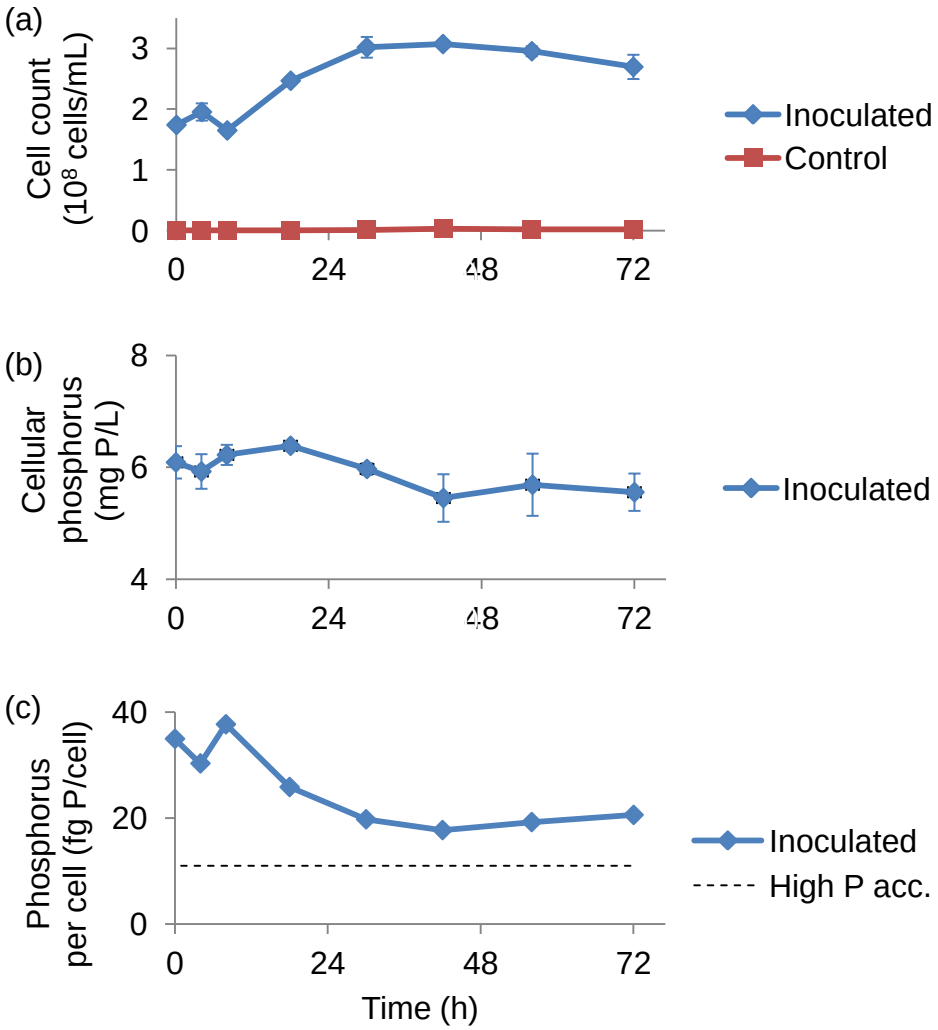

Figure 5-1 *B. antiquum* cell count (a) and variation of the cellular phosphorus (b), and phosphorus content per cell (c) when incubated in sludge dewatering liquors at room temperature for 72h. High P acc., reference value of phosphorus per cell for EBPR microorganisms with high phosphorus accumulation (11 fg P/cell) (dashed line) (Sidat *et al.*, 1999).

Phosphorus fractions were measured during the incubation of *B. antiquum* in the sludge dewatering liquors for 72 h using sacrificial bottles. Phosphate decreased from 28.6 mg PO_4-P/L to 15.4 mg PO_4-P/L after 72 h (Figure 5-2a).

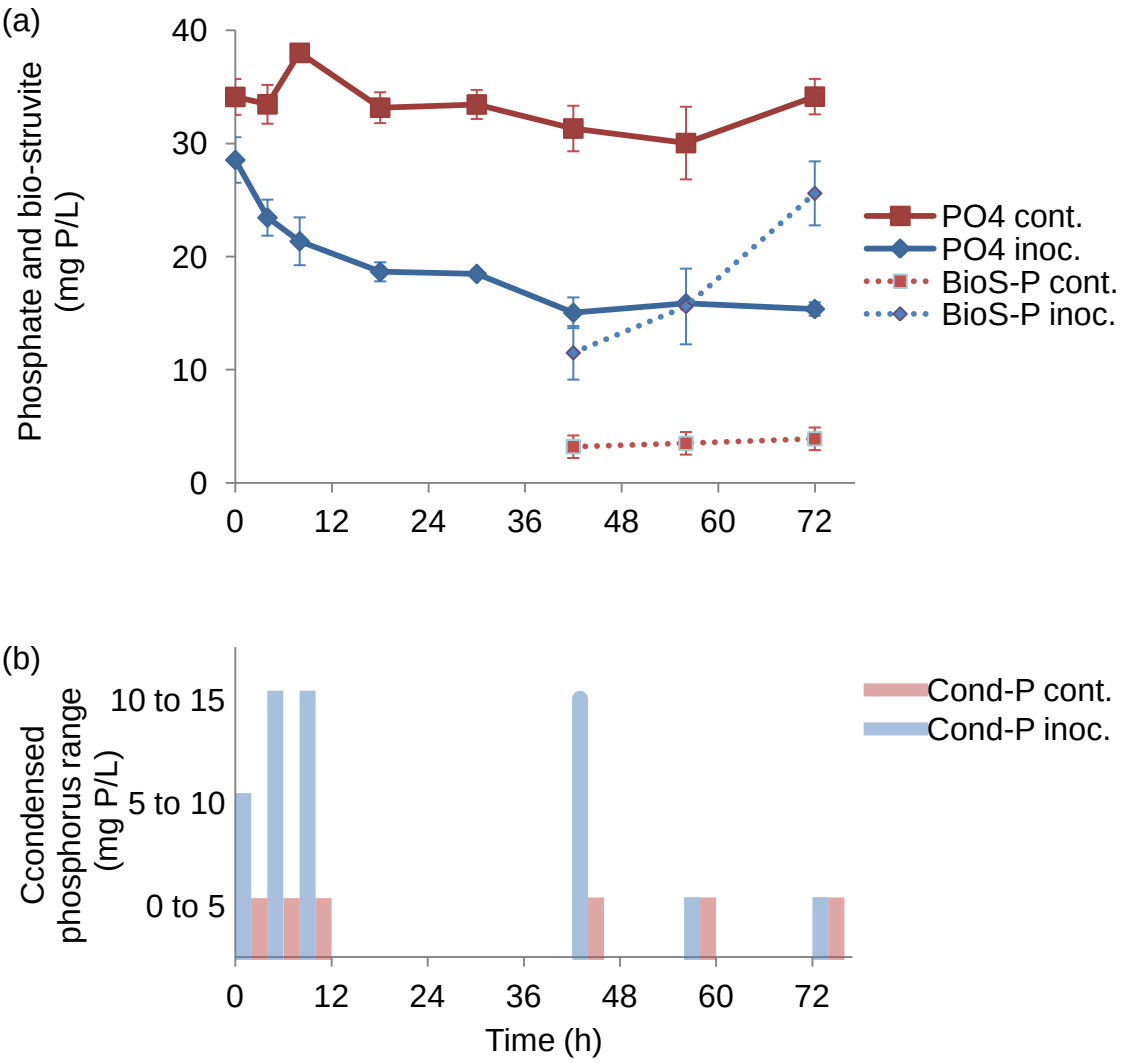

Figure 5-2 Evolution of bio-struvite, phosphate and condensed phosphorus when growing *B. antiquum* in sludge liquors from a BNR site, at room temperature for 72h. (a) concentration of: phosphate in inoculated tests (PO4 inoc.), phosphate in un-inoculated control tests (PO4 cont.), bio-struvite in inoculated tests (BioS-P inoc.), and bio-struvite in un-inoculated control tests (BioS-P cont.). (b) semi-quantitative assessment of the amount of condensed phosphorus: in inoculated tests (Cond-P inoc.), and in un-inoculated control tests (Cond-P cont.).

Bio-struvite production increased from 0 to 11.5 to 25.6 mg P/L, from 0, 42 and 72 h incubation, respectively, well above the un-inoculated control tests (Figure 5-2a). Bio-struvite production was also seen previously to increase between days 2 to 4 of incubation, from not detected to 6.8 mg P/L, in BNR sludge dewatering liquors (Simoes *et al.*, 2017b).

Measurements of the condensed phosphorus fraction based on the modified acid-hydrolysis procedure, presented a high variation and did not allowed for the complete closure of the phosphorus mass balance, particularly for the un-inoculated controls. As such, the condensed phosphorus fraction was presented as semi-quantitative measurement (Figure 5-2b). Condensed phosphorus concentration increased from 5-10 mg P/L at time 0, to 10-15 mg P/L at 42 h, and then it decreased to 0-5 mg P/L at 56 and 72 h (Figure 5-1b). These results suggest that condensed phosphorus was released for the first 42 h of incubation and then used for bio-struvite production, as this later increased (Figure 5-2).

Taking into account the bio-struvite and the phosphate concentrations between 42 and 72 h, against the initial total phosphorus (59.5 mg P/L) it can be asserted that the combined organic and condensed phosphorus fraction decreased by 14.4 mg P/L (from 33.0 mg P/L to 18.5 mg P/L). This matched an increase of 14.1 mg P/L in bio-struvite for the same period (from 11.5 to 25.6 mg P/L).

A mass balance to the fractions of phosphorus highlights the decrease of condensed phosphorus and organic phosphorus throughout the incubation period and the contribution of these fractions to the formation of bio-struvite (Figure 5-3). The fractions of organic phosphorus and condensed phosphorus contributed with close to half the phosphorus recovered in bio-struvite (12.4 mg P /L) (Figure 5-3). The remaining phosphorus originated from phosphate (13.2 mg P /L). As such, sludge liquors with high content in organic and condensed phosphorus are expected to contribute to formation of bio-struvite. However, there is a lack of information about the organic and condensed

phosphorus in sludge liquors, including different approaches to phosphorus removal.

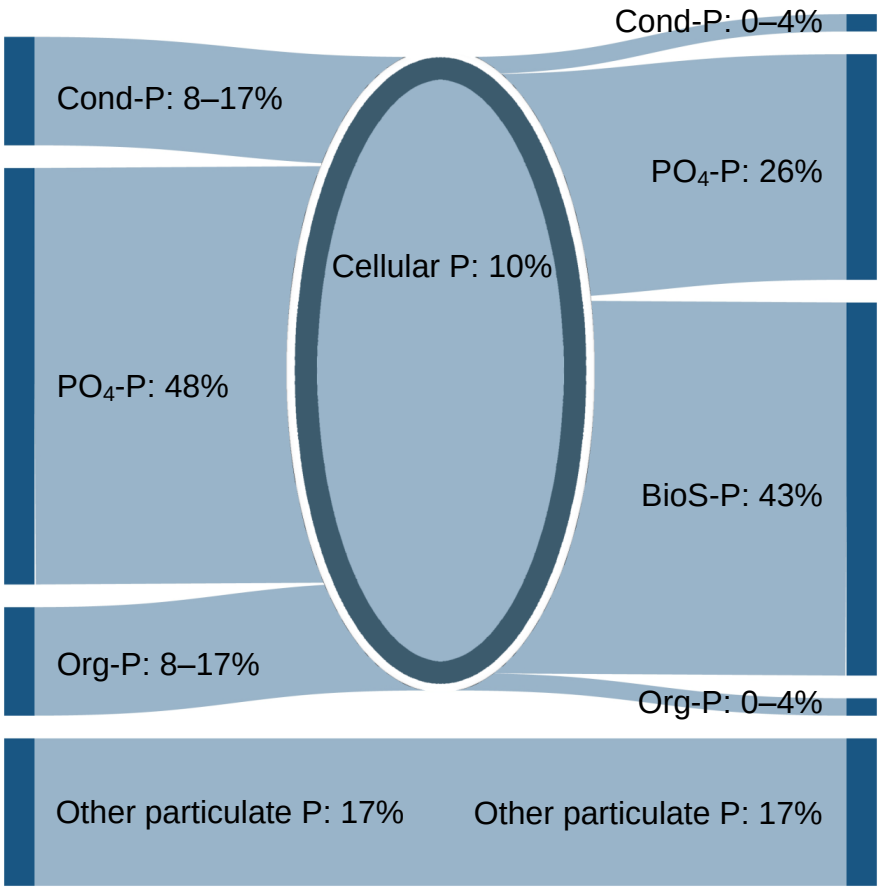

Figure 5-3 Variation in the proportion (%) of each form of phosphorus as a result of incubating *B. antiquum* in sludge dewatering liquors at room temperature for 72 h. Cond-P, condensed phosphorus; Org-P, organic phosphorus; BioS-P, bio-struvite phosphorus.

5.3.3 Characterisation of liquors prepared from different types of wastewater sludge

Sludge dewatering liquors from primary, secondary, digester feed and digestate sludge, collected from 3 full-scale WWTP (with different P-removal technologies) were characterized for the phosphorus fractions and other wastewater quality parameters in order to assess the potential of each sludge liquors type for the application of a bio-struvite process (Table 5-3).

Phosphate content varied from <0.3 mg PO_4-P/L in liquors produced from CPR digester feed sludge, to a maximum of 112.5 mg PO_4-P/L, in liquors obtained from NPR digester feed sludge. These match values reported for digested sludge liquors, which ranged from 0.6 to 167 mg PO_4-P/L (Fux *et al.*, 2002; Pastor, Martí, *et al.*, 2008). Only one of the liquors had a content of phosphate above 100 mg PO_4-P/L, which is the minimum amount of phosphate necessary for the economical recovery of struvite using conventional chemical precipitation process (Dockhorn, 2009). Hence, based on phosphate content, none of the sludge liquors tested were a good candidate for phosphorus recovery using conventional chemical struvite precipitation. On the other side, results from this study clearly indicate that *B. antiquum* can use various fractions of phosphorus (soluble, organic and condensed fractions) to produce bio-struvite. Hence, the total phosphorus content of the sludge liquors was one of the key parameters here evaluated to understand and potentially predict the potential of particular sludge dewatering liquors to apply biological recovery of phosphorus through bio-struvite.

Total phosphorus in the liquors varied by as much as 2 orders of magnitude, from 1.1 mg P/L in the CPR secondary sludge, up to 767.5 mg P/L in the BNR digested sludge. These values widen the range found in literature (4-200 mg P/L) (Parsons *et al.*, 2001; Ahn, 2006). In all sites, digested sludge liquors had >200 mg P/L total phosphorus. This was three times more than the total phosphorus in the liquors obtained from the full-scale site with 65.5 mg P/L (Table 5-2), through dewatering by centrifugation. This difference is thought to be related with the use polymers in the full-scale dewatering processes to increase sludge dewaterability (Kurade *et al.*, 2014).

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Table 5-3 Characterisation of sludge liquors produced from primary sludge, secondary sludge, digester feed sludge, and digested sludge collected from three full-scale WWTPs with different phosphorus removal technologies.

Site	Liquors sludge source	Total phosphorus (mg P/L)	Organic phosphorus (mg P/L)	Condensed phosphorus (mg P/L)	Phosphate (mg P/L)	Ammonium (mg N/L)	Dissolved Chemical Oxygen Demand (mg/L)	Total suspended solids (mg/L)	Dissolved oxidised nitrogen (mg N/L)	Dissolved nitrite (mg N/L)	Alkalinity (as mg $CaCO_3$/L)	pH	Magnesium (mg/L)	Calcium (mg/L)	Chloride (mg/L)	Sulphate (mg/L)	Aluminium (mg/L)	Arsenic (mg/L)	Boron (mg/L)	Chromium (mg/L)	Copper (mg/L)	Iron (mg/L)	Lead (mg/L)	Nickel (mg/L)	Potassium (mg/L)	Zinc (mg/L)
BNR	Primary	30.2 ± 1.6	10 to 30	<10	2.5 ± 0.0	64.4	770 ± 23	858 ± 25	<0.1	<0.1	610 ± 14	6.9	33	130	126	84	<0.02	<0.02	0.32	<0.01	0.01	16	<0.03	<0.01	46	<0.01
		19.6 ± 0.6	<10	<10	9.8 ± 0.2	28.3 ± 2.0	228 ± 13	383 ± 3	<0.1	<0.1	377 ± 15	6.7	27	110	113	73	<0.02	<0.02	0.26	<0.01	<0.01	3.4	<0.03	<0.01	30	<0.01
		92.0 ± 2.8	10 to 30	10 to 30	65.7 ± 2.2	45.5 ± 5.0	420	1409 ± 97	<0.1	<0.1	583 ± 10	6.6	40	150	150	47	0.94	<0.02	0.41	<0.01	<0.01	2.7	<0.03	0.02	63	0.18
	Secondary	46.0 ± 1.0	<10	<10	35.2 ± 0.0	<4.0	105 ± 3	117 ± 6	2.1	<0.6	248 ± 8	7	32	81	150	144	<0.02	<0.02	0.26	<0.01	<0.01	0.1	<0.03	<0.01	50	<0.01
		19.0 ± 0.0	<10	<10	11.1 ± 1.2	<4.0	<25	52 ± 23	1.2 ± 0.1	<0.1	213 ± 6	7.2	26	88	120	122	<0.02	<0.02	0.22	<0.01	<0.01	<0.01	<0.03	<0.01	36	<0.01
		39.9 ± 0.1	<10	<10	33.8 ± 0.3	2.4 ± 0.7	<25	213 ± 13	0.6 ± 0.3	<0.6	224 ± 1	7.1	28	90	264	138	<0.02	<0.02	0.37	<0.01	<0.01	0.1	<0.03	<0.01	50	0.01
	Digested	70.8 ± 2.0	10 to 30	30 to 100	8.5 ± 0.1	861.6	639 ± 119	2787 ± 71	<0.1	<0.1	4250 ± 0	7.9	33	130	168	90	2.00	0.03	0.15	<0.01	0.08	8.9	0.07	0.06	170	0.52
		87.2	30 to 100	<10	55.3 ± 1.5	713.2 ± 19.5	629 ± 15	1208 ± 106	<0.1	<0.1	3403 ± 6	8	32	120	132	43	0.71	<0.02	0.08	<0.01	0.07	8.8	<0.03	0.04	87	0.12
		767.5 ± 60.1	>100	>100	35.0 ± 2.0	713.4 ± 33.5	555	21617 ± 424	<0.1	<0.1	5883 ± 64	7.8	15	68	188	28	0.21	0.04	0.15	<0.01	<0.01	0.76	<0.03	0.04	170	0.09
NPR	Primary	134.0	<10	30 to 100	63.0 ± 0.1	193.6	4296 ± 356	2073 ± 35	<0.1	<0.1	913 ± 38	5.2	38	370	132	78	0.49	<0.02	0.15	<0.01	0.06	16	<0.03	0.02	73	0.15
		11.7 ± 1.4	<10	<10	12.8 ± 0.2	31.0 ± 0.7	32 ± 1	243 ± 3	<0.1	<0.1	377 ± 6	7.2	18	95	80	45	<0.02	<0.02	0.07	<0.01	0.02	0.44	<0.03	<0.01	31	<0.01
		21.8 ± 0.3	<10	<10	15.4 ± 0.5	17.3 ± 2.4	230	344 ± 15	<0.1	<0.1	422 ± 6	7.2	15	100	316	34	0.04	<0.02	0.10	<0.01	0.02	0.32	<0.03	<0.01	35	<0.01
	Digester feed	96.3 ± 1.3	10 to 30	10 to 30	50.2 ± 0.1	167.2	3335 ± 155	1773 ± 72	<0.1	<0.1	820 ± 10	5.4	34	320	144	78	1.20	<0.02	0.18	<0.01	0.15	24	0.04	0.03	66	3.3
		87.6 ± 1.7	30 to 100	<10	51.4 ± 2.2	189.9 ± 11.1	7710	4783 ± 370	<0.1	<0.1	1090 ± 10	5	47	580	158	95	0.14	<0.02	0.18	<0.01	0.03	110	<0.03	0.05	80	0.23
		216.0 ± 65.0	30 to 100	30 to 100	112.5 ± 4.3	287.1 ± 63.4	5397	3757 ± 261	<0.1	<0.1	1113 ± 33	5.2	56	450	368	15	0.13	<0.02	0.20	<0.01	0.03	4.9	<0.03	0.04	96	0.24
	Digested	59.7 ± 2.9	10 to 30	10 to 30	27.5 ± 1.2	551	189 ± 93	1297 ± 18	1.02	<0.6	2700 ± 10	7.7	28	150	216	78	0.14	<0.02	0.08	<0.01	0.05	0.58	<0.03	0.02	78	0.06
		18.1 ± 1.3	10 to 30	<10	<0.3	612.1 ± 12.9	290 ± 40	1557 ± 95	<0.1	<0.1	3377 ± 15	7.8	34	110	166	22	1.10	<0.02	0.08	<0.01	0.11	9.5	<0.03	0.04	85	0.13
		360.5 ± 7.8	>100	>100	3.4 ± 0.4	554.5 ± 14.9	508	7717 ± 317	<0.1	<0.1	4160 ± 10	7.6	33	150	352	25	0.31	0.03	0.06	<0.01	0.03	3.3	<0.03	0.04	100	0.07
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CPR	Primary	73.5 ± 2.3	30 to 100	<10	11.3 ± 2.3	211.2 ± 6.4	3752 ± 62	3763 ± 156	<0.1	<0.1	1323 ± 29	5.9	36	300	172	39	<0.02	<0.02	0.66	<0.01	<0.01	21	<0.03	0.02	110	<0.01
		186.0 ± 22.6	10 to 30	>100	46.6 ± 6.3	113.9 ± 6.9	6668	3583 ± 148	<0.1	<0.1	1037 ± 15	5.1	48	460	476	17	0.12	<0.02	0.76	<0.01	0.02	29	<0.03	0.05	100	0.20
	Secondary	1.7 ± 0.1	<10	<10	0.5 ± 0.0	<4.0	82 ± 1	60 ± 3	2.6	1.2	233 ± 6	7.3	18	87	204	114	<0.02	<0.02	0.50	<0.01	<0.01	0.06	<0.03	<0.01	40	<0.01
		1.1 ± 0.1	<10	<10	0.4 ± 0.1	4.3 ± 0.1	<25	46 ± 8	7.7 ± 0.4	0.2	247 ± 64	7.1	17	85	144	72	<0.02	<0.02	0.33	<0.01	0.01	0.01	<0.03	<0.01	36	<0.01
		2.2 ± 0.1	<10	<10	0.1 ± 0.0	2.3 ± 0.4	36	147 ± 20	2.8 ± 0.4	<0.6	223 ± 8	7.3	20	94	332	97	<0.02	<0.02	0.43	<0.01	0.02	0.05	<0.03	<0.01	55	<0.01
	Digester feed	78.9 ± 1.2	10 to 30	30 to 100	0.7 ± 0.0	264.3	5238 ± 153	5185 ± 191	<0.1	<0.1	1410 ± 113	5.3	51	340	276	132	<0.02	0.03	0.40	<0.01	0.02	250	<0.03	0.21	130	1.00
		59.5 ± 0.5	30 to 100	10 to 30	<0.3	510.6 ± 24.7	5325 ± 149	6773 ± 32	<0.1	<0.1	2487 ± 35	6.6	56	290	184	66	<0.02	0.03	0.48	<0.01	<0.01	120	<0.03	0.10	170	<0.01
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Digested	54.4 ± 1.1	10 to 30	10 to 30	21.8 ± 0.4	750.4	680 ± 265	1495 ± 156	<0.1	<0.1	3840 ± 0	7.8	30	140	402	126	0.29	0.04	0.59	<0.01	0.04	2.1	<0.03	0.06	160	0.09
		36.0 ± 2.4	30 to 100	<10	<0.3	829.6 ± 42.3	2442 ± 38	7717 ± 74	<0.1	<0.1	4483 ± 61	7.4	46	150	400	24	1.90	0.05	1.20	0.01	0.11	61	0.12	0.11	310	0.50
		276.5 ± 101.1	<10	>100	0.4 ± 0.1	832.0 ± 43.6	523	16993 ± 203	<0.1	<0.1	6820 ± 35	7.7	46	82	870	29	0.10	0.10	1.50	<0.01	0.01	2.2	<0.03	0.10	460	0.08

BNR, biological nutrients removal site; NPR, no phosphorus removal site; CPR, chemical phosphorus removal site.

Cadmium (<0.01 mg/L), mercury (<0.01 mg/L) and selenium (<0.04 mg/L) were below the limit of detection for all samples.

Values detail the mean ± standard deviation when replicate measurements were taken.

On the other side, the concentration of phosphate was similar in both prepared liquors in this study (35.0 mg P/L) and liquors collected at full-scale site (49.5 mg PO_4-P/L). These results suggest that the dewatering process has a relevant impact on the content and speciation of phosphorus as more organic and condensed phosphorus was present in the liquors prepared without polymers. Consequently, the type of dewatering process as well as the chemicals added are expected to impact the fractions of phosphorus available for bio-struvite formation.

Organic phosphorus in the digested sludge liquors from the NPR and BNR sites ranged from 10-30, up to >100 mg P/L. Hence, these liquors have a considerable potential for bio-struvite formation through metabolisation of the organic phosphorus. To a lesser extent, liquors from the primary sludge of all sites, from the NPR digester feed sludge, and from the CPR digested sludge, also presented an opportunity for the recovery of organic phosphorus. In these liquors, 5 of 14 samples were classed as having 10 to 30 mg P/L of organic phosphorus. In contrast, low amounts of organic phosphorus (<10 mg P/L) were found in the primary sludge liquors from the NPR site, and in all secondary sludge liquors tested. Thus secondary sludge liquors presented the lower potential for recovery bio-struvite via conversion of organic phosphorus.

The concentrations of condensed phosphorus also varied considerably for all sludge liquors, except liquors from secondary sludge. In digested sludge liquors, the condensed phosphorus varied 2 orders of magnitude, from <10 mg P/L to >100 mg P/L, in all sites. As in the case of the organic phosphorus, the digested sludge liquors had a considerable potential for bio-struvite production through metabolisation of condensed phosphorus fraction, not typically recovered in existing processes. In digester feed sludge liquors, 4 of 5 samples had condensed phosphorus between 10 and 100 mg P/L. The content of condensed phosphorus in secondary sludge liquors was <10 mg P/L for all samples.

Because of the presence of phosphorus accumulating organisms in the BNR sites (Acevedo *et al.*, 2012), more condensed phosphorus was expected to be

found in these liquors, in particular, for secondary and digested sludge liquors. The lack of evidence for a higher content in condensed phosphorus in liquors from the BNR site, relative to liquors from the other sites, might be the result of the BNR condensed phosphates remaining with solid fraction, in the dewatering process where the liquors are made. Another potential explanation is that the acid hydrolysis method, used for the quantification of condensed phosphorus, solubilised loosely adsorbed inorganic phosphates in the liquors from non-BNR sites, thus over representing the amount of condensed phosphorus in those liquors. The solubilisation of loosely adsorbed inorganic phosphates is noted as possible in the acid hydrolysis method description of the Standard Methods (APHA, 2005). Hence, both the partition of the BNR condensed phosphorus between solid and liquors, and the release of adsorbed phosphate in non-BNR liquors can justify the lack of difference between the condensed phosphorus content of the liquors from BNR and non-BNR sites.

Beyond phosphorus, struvite formation requires magnesium and ammonium at the same molar amounts as phosphate. Hence, both magnesium and ammonium can also limit the struvite production potential of the liquors. The NPR, and CPR digester feed sludge liquors had highest average magnesium concentrations at 45.8, and 53.5 mg Mg^{2+}/L, respectively. Accounting for all liquors, the content of magnesium varied between 15 and 56 mg Mg^{2+}/L. Published values show that magnesium in sludge liquors can range from 3 to 170 mg/L (David, 2007). Magnesium was the limiting ion for struvite production in 7 out of the 28 samples of sludge liquors tested. Magnesium limitation was observed more often in BNR digested sludge liquors, and in NPR digester feed sludge liquors. Moreover a $Mg:PO_4$ molar ratio above 1.3:1 is frequently recommended as necessary to trigger the precipitation, or to achieve sufficient rates of phosphorus recovery (Jaffer *et al.*, 2002; Bhuiyan *et al.*, 2008; Geerts *et al.*, 2015). Magnesium source can represent up to 75% of the costs in chemical struvite precipitation processes (Dockhorn, 2009).

The need for a molar ratio of Mg:P higher than 1:1 has not been addressed yet in the bio-struvite process. Nevertheless, because the *B. antiquum* can make

use fractions of phosphorus beyond the soluble phosphate used in conventional chemical precipitation, magnesium use can be maximized and limit the recoverability of the organic and condensed phosphorus in a bio-struvite process. Of the sludge liquors tested, 7 samples had enough magnesium to combine with all the phosphate and part of the organic and condensed phosphorus in a bio-struvite process. In these 7 samples, conventional struvite precipitation could potentially recover 0.4-46.6 mg PO_4-P/L, while a bio-struvite process could potentially recover 35.7-61.2 mg P/L. On other 11 samples, magnesium was present in enough quantity to combine with all the phosphate and all of the organic and condensed phosphorus. In these 11 samples, conventional struvite precipitation could potentially recover 0.8-407.1 mg PO_4-P/L, while a bio-struvite process could potentially recover 8.7-474.8 mg P/L.

Ammonium was present in excess concentration relative to phosphate and magnesium, in liquors from primary sludge, digester feed sludge, and digested sludge (17.1 to 888.6 mg NH_4-N/L). Ammonium was the limiting ion in liquors obtained from secondary sludge, for both the CPR and BNR sites (<4.3 mg NH_4-N/L). This further reinforces the secondary sludge liquors as having the least potential for struvite formation.

Additionally, to produce bio-struvite it is also necessary that the liquors have conditions to sustain the viability and growth of the bio-struvite forming bacteria.

Previous research has shown that a carbon source is required to promote the growth of *B. antiquum* in sludge liquors. The growth rate was increased 3 fold, from 0.9 to 3.4 1/d, when the COD of digested sludge liquors (<500 mg COD/L) was supplemented with 562-1686 mg COD/L as acetate (Simoes *et al.*, 2017a). The amount of COD in the liquors here investigated varied with the type of sludge. The liquors with the highest COD were digester feed sludge (3334-7710 mg COD/L), followed by primary sludge (32-6667 mg COD/L), digested sludge (189-2442 mg COD/L), and secondary sludge (<25.0-105 mg COD/L). As such, the liquors from digester feed sludge and primary sludge have sufficient COD to sustain the growth of *B. antiquum,* whilst the liquors from digested sludge and secondary sludge were likely to limit its growth rate. An assessment of readily

biodegradable COD in the liquors should provide a better account of the observed effect of acetate in the growth rate of *B. antiquum*.

Previous studies, have also highlighted the importance of managing the pH. The production of bio-struvite, and *B. antiquum* cell growth were reduced at pH values <7.3, and >8.3. In liquors from digested sludge, the degassing of CO_2 was seen to increase the pH to values unsuitable for *B. antiquum* (Simoes *et al.*, 2017b). For the liquors tested here, the pH decreased along the series: digested sludge liquors (7.4-8.0), secondary sludge liquors (7.0-7.3), primary sludge liquors (5.1-7.2), and digester feed sludge liquors (5.0-6.6). A CO_2 degassing case-by-case assessment might be necessary to show that *B. antiquum* remains viable and grows.

An increase in calcium content of 100 mg Ca^{2+}/L has been associated with a decrease in the growth rate of *B. antiquum* (Simoes *et al.*, 2017a). The content of calcium in digested sludge liquors were similar for all sites and varied between 68.0 and 150.0 mg/L. The lowest amounts were found in secondary sludge liquors, from 81.0 to 94.0 mg Ca^{2+}/L. Primary sludge liquors from the BNR site had a content of calcium close to what was found in digested sludge liquors 110.0 – 150.0 mg Ca^{2+}/L. The digester feed sludge liquors from the CPR and NPR sites had the highest values found going from 290.0 to 580.0 mg Ca^{2+}/L. The CPR primary sludge liquors were also high with 300.0 and 450.0 mg Ca^{2+}/L, whilst in the NPR site the calcium content varied from 95.0 to 370.0 mg Ca^{2+}/L.

Parameters as chloride, sulphate, boron, and aluminium, cadmium, chromium, copper, lead, mercury, selenium, and zinc did not show a relationship with the type of sludge, or P-removal technology used in the main stream of treatment. Moreover, the impacts of these parameters on the formation of bio-struvite, and on the growth of *B. antiquum*, are not yet known. Thus, the results here indicate that choosing the type of liquors is not likely to be the solution to manage the presence of these substances for a bio-struvite process.

5.3.4 Sludge liquors potential for bio-struvite production

A qualitative assessment of potential for use with *B. antiquum* to produce bio-struvite was made of each type of sludge liquors characterized (Table 5-4).

Table 5-4 Qualitative assessment of the potential application of the bio-struvite process for different types of sludge liquors. The potential for struvite, and for bio-struvite, accounts for the percentage of the maximum points possible (struvite:10, bio-struvite:20).

Sludge source	Site	Phosphate	Magnesium	Ammonium	pH	Struvite potential (%)	Phosphorus	Magnesium	Ammonium	COD	pH	Alkalinity	Calcium	Bio-struvite potential (%)
Primary	BNR	–	–	+	–	30	+	+	+	+	–	+	+	80
	NPR	–	–	+	–	30	+	+	+	–	–	+	–	60
	CPR	– –	+	+	– –	30	+ +	+ +	+	+ +	–	+	–	85
Secondary	BNR	–	+	–	–	30	+	+	–	– –	+	+	+	65
	CPR	– –	–	+	–	20	– –	–	+	– –	+	+	+	50
Digester feed	NPR	–	–	+	– –	20	+ +	–	+	+ +	–	–	–	60
	CPR	– –	+	+	– –	30	+ +	+ +	+	+ +	–	–	–	75
Digested	BNR	–	–	+	–	30	+ +	–	+	–	+	–	+	65
	NPR	– –	+	+	–	40	+	–	+	– –	+	–	+	55
	CPR	– –	+	+	–	40	+ +	+	+	–	+	–	+	75

+ + Always suitable
+ Variable and not always suitable
– Variable and mostly not suitable
– – Not suitable

Primary sludge liquors stand out as the liquors with more potential for growing *B. antiquum* and recover phosphorus as bio-struvite. Secondary and digester feed sludge liquors showed specific challenges. Secondary sludge liquors were not suitable due to the low carbon source available and phosphorus content. Digester feed sludge liquors are at risk for reaching too high pH values. The

combination of the liquors from digested sludge and primary sludge has the potential to address magnesium, pH, and carbon source limitations of the digested sludge liquors alone. The application of external carbon sources as the fermentation liquid produced from the organic fraction of municipal solid waste, or the drainage liquid produced from the organic fraction of municipal solid waste, have been successfully applied for via-nitrite removal of nutrients from anaerobically digested sludge liquors (Katsou *et al.*, 2014). These alternative external carbon sources can supply a readily biodegradable carbon source required to improve the growth rate of *B. antiquum* in sludge liquors.

5.4 Conclusions

B. antiquum can use >50% of the organic and condensed phosphorus fractions to produce bio-struvite. This opens the opportunity to recover two phosphorus fractions that were not available for recovery before.

Different types of sludge liquors were found to frequently have a high content of organic and condensed phosphorus. Up to 276.1, 357.1, and 732.5 mg P/L in liquors from digested sludges.

Liquors, from all the investigated sites, presented higher potential for bio-struvite production and phosphorus recovery, than with conventional struvite precipitation. But, none the liquors were found to have all the conditions suitable for bio-struvite formation. Combining primary sludge with digested sludge was suggested to mitigate carbon source limitation without increasing the calcium content of the combined liquors.

Bio-struvite produced with *B. antiquum* enables the recovery of phosphorus that was not available for recovery using current processes. This widens range of sludge dewatering liquors that can potentially be used for phosphorus recovery.

5.5 Acknowledgements

The authors are grateful for the financial support and contribution from Severn Trent Water Ltd (Coventry, UK) and for the financial support from POPH/FSE as a PhD grant (SFRH/BD/84116/2012) awarded by the Fundação para a Ciência e Tecnologia (FCT, Portugal).

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Chapter 6 Enrichment *Brevibacterium antiquum* in sludge dewatering liquors in open mixed-culture conditions to produce bio-struvite

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Abstract

Brevibacterium antiquum has been shown to mediate the formation of struvite from wastewater sludge dewatering liquors in sterile conditions. This work investigated the application of *B. antiquum* in sludge dewatering liquors under open mixed-culture conditions. A sequencing batch reactor (SBR) was run with sludge dewatering liquors collected from a full-scale wastewater treatment plant. Sodium chloride (3% w/v) was used as selective pressure agent aimed to favour the growth of *B. antiquum*. Flow cytometry intact cell counts, and bacterial community profiling using 16S rRNA gene barcoded pyrosequencing, were done to measure changes in microbial community and assess the enrichment of *B. antiquum*. The results showed that *B. antiquum* represented only 2.8%, and 0.9% of the bacteria found after 13, and 25 days, respectively, and hence the enrichment strategy was not successful. Furthermore, no struvite was collected at the end of the tests. Other strategies were proposed for enriching *B. antiquum* such as improving the retention of *B. antiquum* cells within the SBR, and increasing the selective pressure to limit the growth the competing indigenous microorganisms and favour *B. antiquum* growth.

Keywords

Biomineralization; phosphorus recovery; wastewater; sequencing batch reactor; selective agent; growth rate; bioaugmentation.

6.1 Introduction

Brevibacterium antiquum has been shown to achieve phosphorus recovery from wastewater sludge dewatering liquors via biological formation of struvite crystals (bio-struvite) (Soares *et al.*, 2014). However, this was accomplished in sterile conditions with single-culture incubation. Conventional wastewater treatment processes are completed in open mixed-culture conditions. Existing biological wastewater treatment processes are designed to enrich specific microbial communities, with the goal of removing particular pollutants from the wastewater. The enrichment is accomplished by a combination of specific bio-reactor design and operational conditions that results in the selective pressure that favours the growth rate of the target microorganisms.

The development of a bio-struvite process using bacteria such as *B. antiquum,* to recover phosphorus from wastewater, should ideally be done in open mixed-culture conditions, removing the need for sterilization of the wastewater, which is energy intensive resulting in increased costs. Sequencing batch reactors (SBRs) have been pointed to be a convenient reactor design for the enrichment of microorganisms. SBRs have been used to enrich Anammox bacteria and implementation of the process (Strous *et al.*, 1998, Sánchez Guillén *et al.*, 2016). Recently, the bio-plastic accumulating bacteria *Plasticicumulans acidovorans* was enriched to a relative abundance of >70% in a pilot-scale SBR using fermented wastewater (Tamis *et al.*, 2014). The simplicity of the SBR allows independent control of parameters such as residence time, solids retention, and the tailoring of alternating aerobic, anoxic and anaerobic stages. Furthermore, SBR can be combined with different physical processes such as sedimentation or membranes (Du *et al.*, 2014) which can be advantageous for the recovery of bio-struvite.

In batch incubation, in single-culture conditions, *B. antiquum* was able to grow at a rate of 0.9 1/d in sludge dewatering liquors, and at 3.4 1/d when supplementing acetate (562-1686 mg COD/L) and sodium chloride (NaCl, 1.5 to 4.5% w/v) (Simoes *et al.*, 2017a). However, bio-struvite formation was shown to be hindered by NaCl, and a two-stage approach was necessary. *B. antiquum* growth was favoured with acetate and NaCl (1st stage), and bio-struvite formation was promoted in a stage without NaCl (2nd stage) (Simoes *et al.*, 2017a). Previously, bio-struvite production in sludge dewatering liquors with 45 mg P/L reached 77 mg/L after 4 days of incubation. Hence, in this study, an SBR process was designed to cycle a stage promoting the growth in sludge dewatering liquors supplemented with acetate and NaCl, followed by a stage with addition of acetate but no NaCl to allow for bio-struvite formation.

Solids retention in SBR is typically achieved with a sedimentation stage. The length of the settling time depends on both the characteristics of the particles, and the characteristics of the liquid matrix. The choice of settling conditions is recommended to be done based on experimental testing (Tchobanoglous *et al.*, 2003). However, there are no reference values for the settling of *B. antiquum* in sludge dewatering liquors. Settling times for biomass on other wastewater biological processes using sludge dewatering liquors include: 15 min for Anammox in synthetic mineral medium (Strous *et al.*, 1998), and 40 min for both Anammox and nitrate denitrification bacteria (Du *et al.*, 2014).

The aim of this study was to enrich *B. antiquum* in wastewater sludge dewatering liquors under open mixed-culture conditions, using a SBR supplemented with acetate and NaCl to favour *B. antiquum* growth (1st stage), followed by a 3-days stage with acetate and no NaCl, to allow for bio-struvite formation (2nd stage). The sludge in the mixed culture together with *B. antiquum* were settled for 30 min at the end of each stage, before removing 90% of the supernatant, that was replaced with fresh sludge dewatering liquors.

6.2 Materials and Methods

6.2.1 Biological material

Brevibacterium antiquum (VKM Ac-2118) was acquired from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). Starter cultures were grown for 4 days in autoclaved (121°C, 20 min) synthetic media (4 g/L of yeast extract, 2 g/L of magnesium sulphate heptahydrate, and 2 g/L of di-potassium hydrogen phosphate). The bacteria was incubated at room temperature (20-25°C), in an orbital shaker at 150 rpm (Stuart SSL1, Fisher Scientific, Loughborough, UK).

6.2.2 Source of sludge dewatering liquors

Sludge dewatering liquors were collected from a full-scale municipal wastewater treatment plant (WWTP) with an average inlet flow of 1 m^3/s and biological nutrient removal activated sludge as secondary treatment. Onsite primary and secondary sludge, and import sludge from neighbouring WWTP (40% v/v) were digested in a standard mesophilic anaerobic digesters, and then hold for 10 to 27 days before dewatering. Sludge dewatering liquors were obtained from the centrate from a horizontal centrifuge-decanter used to separate sludge cake (22% solids) from the digested sludge (7% solids), with the help of cationic polymer flocculant, anti-scale, and anti-foam agents.

6.2.3 Operation and set-up of the SBR

The SBR were set-up with four 500 mL borosilicate-glass Duran bottles, inside a water bath kept at temperature between 20-25°C. Magnetic stirrers were used in each SBR to keep the media in suspension and ensure aeration. Dissolved oxygen was measured at the end of stage 1 of cycle 2, with concentrations of 6.2 mg O_2/L.

Two SBR were inoculated with *B. antiquum* (replicates Ba and Bb) and compared with two control non-inoculated SBR (replicates Sa and Sb). All 4 SBRs were run for 25 days, for a total of 6 cycles. In order to emulate a process where freshly produced sludge liquors, with a microbial population adapted to

anaerobic conditions, are added to a reactor enriched with *B. antiquum*, initial inoculation was done with a one-time addition (10% v/v) of B. antiquum cells resuspended in sterile 0.9% w/v NaCl aqueous solution, after being centrifuged (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 5 min) from the starter culture. Stabilizing the reactor first was not done to prevent having *B. antiquum* competing with a microbial population adapted to the conditions of the reactor. Sterile 0.9% w/v NaCl solution was added to the control un-inoculated reactors at the same ratio. The expected solids retention time of the SBRs enriched with *B. antiquum* was 21 days, based on a yield coeficient of 9.9×10^{8} cells/mg COD, and the maximum number of *B. antiquum* cells observed in sludge liquors 13.6×10^{8} cells/mL (Simoes *et al.*, 2017a) and a using endogenous decay coeficient of 0.1 1/d and fraction of biomass that remains as cell debris of 10% (Tchobanoglous *et al.*, 2003). Each SBR cycle was made of 3 stages: 1 day supplement with 3% w/v NaCl and 562 mg COD/L as acetate (stage 1); 3 days supplement with 562 mg COD/L as acetate (without added NaCl) (stage 2) and settling (30 minutes before decanting out approximately 90% of the volume, leaving 40 mL of spent sludge dewatering liquors in the SBR). Each SBR cycle was started by adding 360 mL of fresh sludge dewatering liquors to the reactor. On-off pH controllers (Wenbo PH-2010, Pivotal Systems, Leeds, UK) were used to ensure the pH was kept below 8.0. Each of the controllers activated a peristaltic pump (Watson Marlow 120S, Falmouth Cornwall, UK) that supplied 5M HCl acid solution to the reactors at a rate of 10 rpm via a 0.6 mm bore-size Marprene tubing (Fischer Scientific, Loughborough, UK). During the settling periods, the pH controller probes were immersed for 10 min in a 2% Decon 90 alkaline solution for washing, and calibrated with pH 7, and pH 10 standard solutions (Fischer Scientific, Loughborough, UK).

6.2.4 Bacterial community profiling with barcoded pyrosequencing of 16S rRNA gene fragments

Sludge dewatering liquors samples were mixed with an equal volume of 100% ethanol (Acros Organics, Fisher Scientific, Loughborough, UK) and kept frozen at -80°C until analysed. After thawing, the samples were placed in a lysing matrix tube and the DNA was extracted 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 (Quince *et al.*, 2011). Sample multiplexing was accomplished with the use of error correcting Golay barcodes (Hamady and Knight, 2009). Polymerase chain reaction (PCR) amplified products (amplicons) were purified using HighPrep magnetic beads (Magbio, Gaithersburg USA) and quantified using QuantiFluor ONE (Promega, Madison USA). An equimolar pool of amplicons was sequenced using Illumina MiSeq with 2x300 v2 chemistry (Illumina, San Diego USA). The number of reads per sample varied from 3.4×10^5 to 1.6×10^6. The sequences were analysed in QIIME 1.9 software (Caporaso *et al.*, 2010) and the SILVA 16S rRNA gene database v123.1 (Quast *et al.*, 2013).

6.2.5 Analytical methods

Intact cell counts were measured at the end of cycles 3 and 6, using flow cytometry. SYBR Green I and propidium iodide dye mixture was allowed to incubate with the samples for 11 min at 37°C, following the procedure detailed previously (Simoes *et al.*, 2017b).

The presence of bio-struvite was monitored at the end of all cycles. One drop of settled sludge was collected with a glass Pasteur pipette from the bottom of the SBR after having removed the supernatant. The collected sample was then laid on a slide with a drop of glycerol and checked in an optical transmission microscope.

Phosphate (PO_4-P) was quantified in a Smartchem 200 spectrophotometric discrete chemical analyser using the recommended reagents (Labmedics, Abingdon, UK). Chemical oxygen demand (COD), total phosphorus, and dissolved phosphorus were measured with Merck cell test kits, according to instructions. Dissolved magnesium (Mg^{2+}) was quantified using atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer Ltd, Beaconsfield, UK) equipped with an air/acetylene burner system. The pH was measured with a Fisherbrand hydrous 300 pH meter (Fisher Scientific, Loughborough, UK).

6.3 Results and Discussion

The sludge dewatering liquors collected the full-scale site treating mostly BNR sludge had a dissolved PO_4-P content of 40 ± 1 mg P/L, dissolved Mg^{2+} content of 73 ± 1 mg/L, total COD of 500 ± 3 mg/L and a pH of 7.8 (Table 6-1). These concentrations compare with values of 37-167 mg PO_4-P/L, 11-51 mg Mg^{2+}/L, 70-308 mg/L for total COD, and 7.6-8.2 for pH, reported for sludge dewatering liquors from BNR sites (Ueno and Fujii, 2001; Battistoni *et al.*, 2006; David, 2007; Pastor, Martí, *et al.*, 2008). Hence, the liquors used here can be considered standard in terms of dissolved PO_4-P and pH but had higher Mg^{2+} and total COD.

Table 6-1 Characteristics of the sludge dewatering liquors collected from a full scale BNR municipal WWTP (mean ± standard error).

Total P (mg P/L)	**Dissolved P** (mg P/L)	**Dissolved PO_4-P** (mg P/L)	**pH**	**Mg^{2+}** (mg/L)	**Dissolved COD** (mg/L)	**Total COD** (mg/L)
50 ± 1	42 ± 1	41 ± 1	7.8	74 ± 1	418 ± 4	500 ± 3

Bio-struvite formation throughout the experiment was monitored through visual observation of the settled biomass collected at the end of each cycle using an optical microscope. Nevertheless, very low amounts of crystals with morphology typical of struvite, were found although higher abundance was observed in the inoculated tests relative to the non-inoculated tests (Figure 6-1,Table 6-2). Additionally, the sludge dewatering liquors used had a theoretical potential for struvite production of 316 mg/L (1370 mg at the end of the 6 cycles). These amounts of struvite should have been evident when the samples were observed in a microscope. Hence, these results indicated that bio-struvite was not accumulating in the SBRs.

Phosphate and Mg^{2+} concentrations were measured over 5 cycles in the 4 SBRs (Figure 6-2). Differences between the inoculated and non-inoculated SBR were only noted during cycle 2. Comparing the inoculated and non-inoculated

SBR, the PO_4-P was 2.8 mg P/L lower at end of cycle 2, and magnesium was 1.5 mg/L lower.

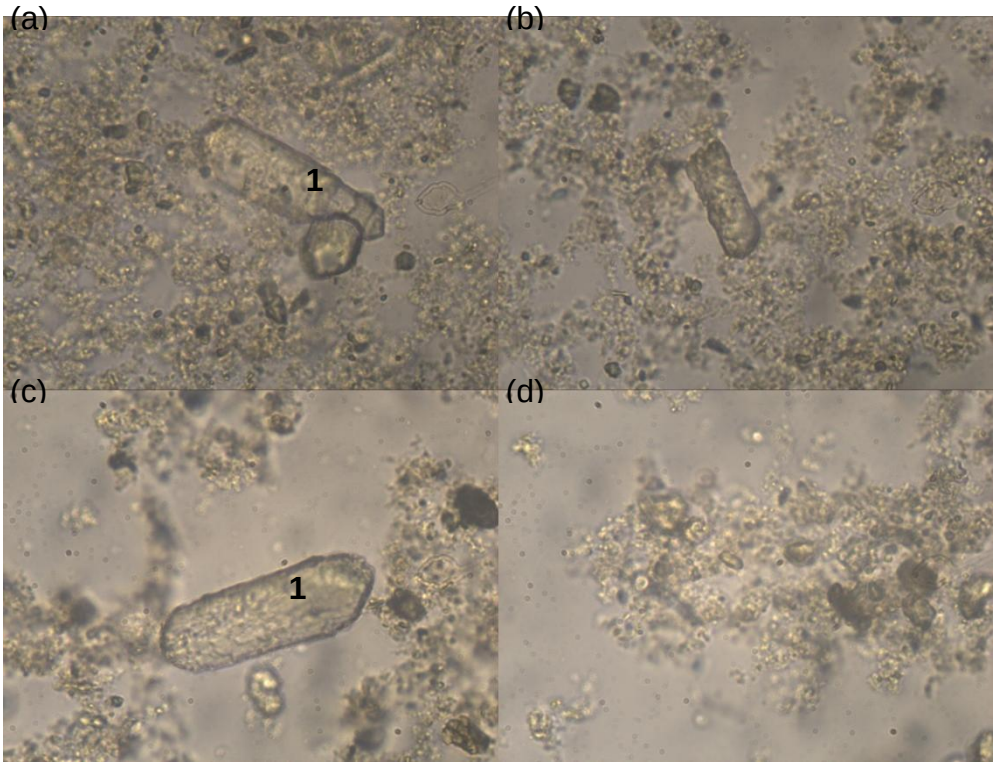

Figure 6-1 Optical microscope 500x magnification photographs of crystals with morphology typical of struvite (1), found in settled sludge collected from the bottom of the SBR at the end of the cycle. *B. antiquum* inoculated at cycle 2 (a), and cycle 3 (c). Non-inoculated control at cycle 2 (b), and cycle 3 (d).

Table 6-2 Qualitative assessment of crystals with morphology typical of struvite monitored through visual observation of the settled biomass collected at the end of each cycle using an optical microscope: *B. antiquum* inoculated (Ba, Bb), and non-inoculated control (Sa, Sb).

	Inoculated SBR		Non-inoculated SBR	
Cycle Nr.	**Ba**	**Bb**	**Sa**	**Sb**
1	+	+	+	−
2	•	•	−	+
3	+	+	−	•
4	+	+	−	•
5	NC	NC	NC	NC
6	−	−	−	•

+ crystals with morphology typical of struvite; •, ambiguous form; − no crystals with morphology typical of struvite. NC, sample not collected.

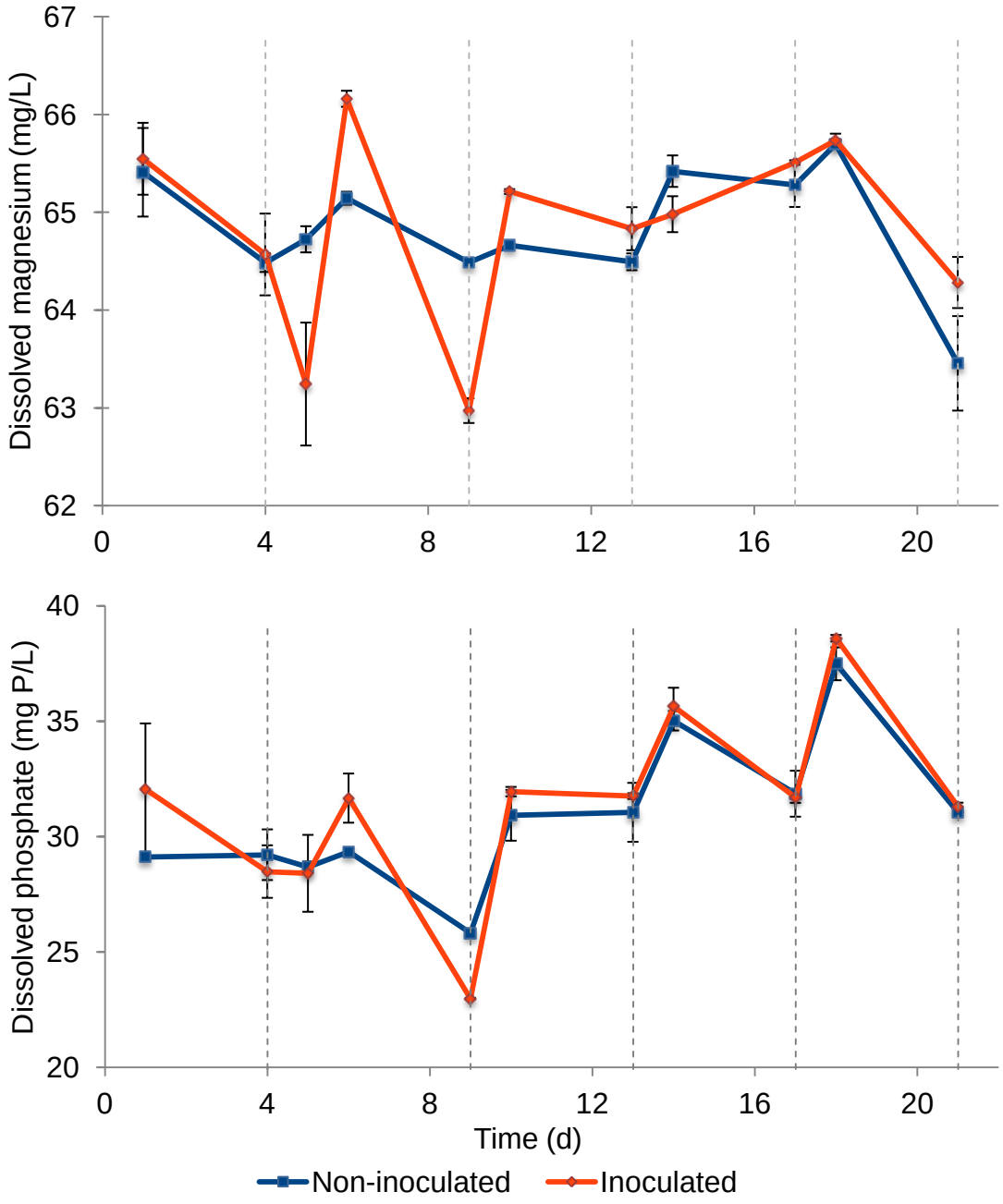

Figure 6-2 Concentrations of dissolved Mg^{2+} and dissolved PO_4-P at the end of each stage, for 5 cycles. SBR inoculated with *B. antiquum* (inoculated) and non-inoculated control SBR (non-inoculated). Dashed vertical lines mark the end of each cycle. Error bars detail the standard error of measurements from replica SBR.

Cell counts were measured at the end of cycles 3 and 6 (Figure 6-3). The results show that there was microbial growth in all SBRs, with higher cell counts

increasing to >6.7×10^7 cells/mL, in relation to time zero at 4.1×10^7 cells/mL. However, the cell counts in *B. antiquum* inoculated SBRs were <2.1×10^8 cells/mL, always below the initial cell counts at time zero counts (5.5×10^8 cells/mL). Additionally, differences were observed between the two inoculated SBRs as the cell counts decreased exponentially in the replicate Ba (Figure 3), and cell counts in the replicate Bb were always below the counts in non-inoculated SBR. This suggests that in SBR Bb the inoculum cells were reduced at a steeper rate than in SBR Ba. The difference in cell count trends observed for the inoculated SBR indicate that the addition of *B. antiquum* to sludge dewatering liquors did not behave in a monotonous, repeatable way.

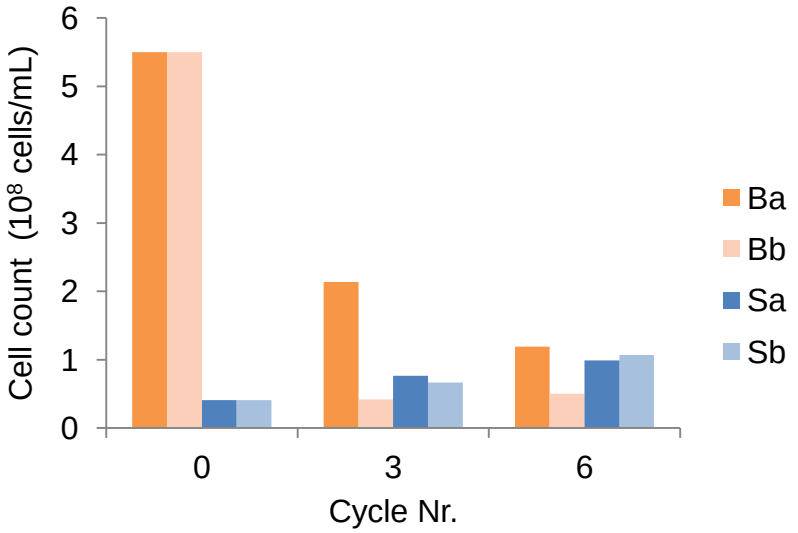

Figure 6-3 Cell counts in samples collected from SBRs: inoculated with *B. antiquum* (Ba, Bb), and non-inoculated control (Sa, Sb). Samples were collected at time zero and at the end of cycles 3 and 6.

Bacterial community profiling indicated that the relative abundance of *B. antiquum* in the inoculated SBR Ba was 96.4% at time zero (Figure 6-4). Nevertheless, the relative abundance of *B. antiquum* was 2.8% at the end of cycle 3, and 0.9% at the end of cycle 6. In second replicate inoculated SBR, Bb,

the relative abundance of *B. antiquum* was 1.5% at end of cycle 6. These results point to a steep decrease in *B. antiquum* relative abundance. Hence, the SBR operational conditions tested did not led to the enrichment of *B. antiquum*. On the other side, the *Corynebacterium* genera were enriched to 56.1, 61.9, and 50.5% relative abundance, in the SBR Ba, Bb, and Sa, respectively, after 6 cycles.

Of interest was the fact that *Brevibacterium* bacteria relative abundance in the non-inoculated SBR (Sa) was less than 0.01% for the time zero sample, which was made up of sludge dewatering liquors without inoculation, and for the samples after cycles 3 and 6 (Figure 6-4). As such, techniques based on amplification of the 16S rRNA gene can be used to show the enrichment of *Brevibacterium* species in sludge dewatering liquors.

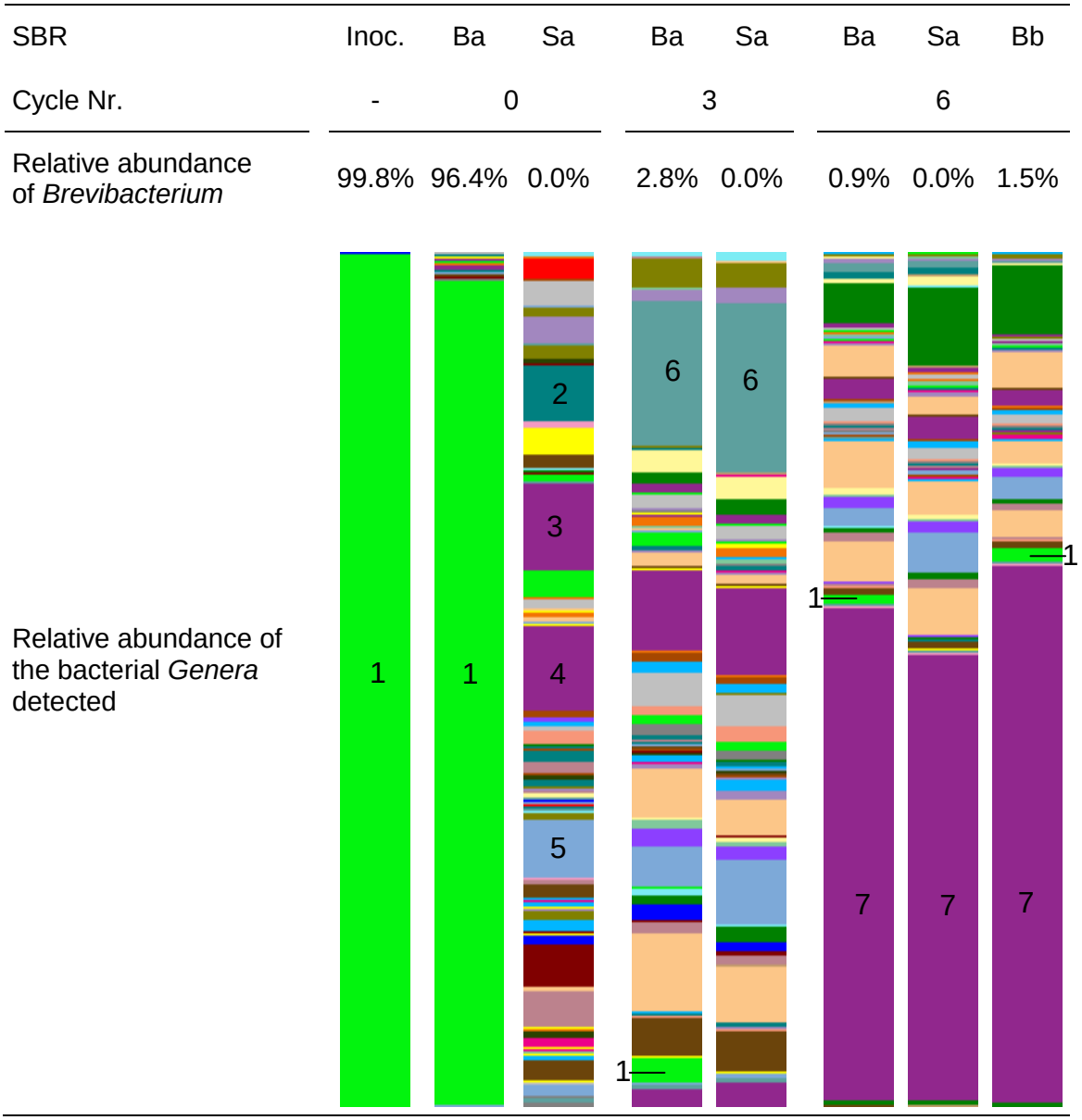

Figure 6-4 Bacterial community diversity and relative abundance of *Brevibacterium* relative to other taxonomic genera on samples collected from three SBR and a starter culture (Inoc.). SBR Ba, and Bb, were inoculated with *B. antiquum* (10% v/v from starter culture.). SBR Sa was a non-inoculated control, with only the indigenous microbial population from the sludge dewatering liquors. Colours in the charts refer to different bacterial Genera: *Brevibacterium* (1), *Smithella* (2), *Simplicispira* (3), uncultured bacterium of the Order *Rhizobiales* (4), *Proteiniclasticum* (5), *Acinetobacter* (6), *Corynebacterium* (7).

In order to understand what were the potential causes for the poor success of the enrichment experiment Equation 6-1 was developed from classic Monod kinetics. The theoretical cell counts for *B. antiquum* ($ThCC_{B.\ ant.}$) were calculated from the relative abundance (Figure 6-4) and the total cell counts (Figure 6-3). The theoretical cell counts for *B. antiquum* were then used to calculate the settled fraction (f_s) of *B. antiquum* cells, at the end of each stage, as a function of the growth rate of *B. antiquum* (Equation 6-1). The Lag stage time was assumed as zero and both the settling fraction and growth rate were considered equal for the 2 stages of the cycle.

$$ThCC_{B.\ ant.,n} = ThCC_{B.\ ant.,n-1} \cdot f_s \cdot 2^{tn.\mu} \qquad ECC_n = ECC_{n-1} \cdot f_S \cdot 2^{t_n \cdot \mu}$$

$$ECC_n = CC_{n-1} \cdot f_S \cdot 2^{t_n \cdot \mu}$$

Equation 6-1 Estimation of *B. antiquum* cell count in an SBR as a function of growth rate and settled fraction.

Where,

$ThCC_{B.\ ant.,n}$, is the theoretical cell count of *B. antiquum* at the end of stage n (calculated from relative abundance and total cell counts) (cell/mL)

$f_{s,}$ is the fraction of settled *B. antiquum* cells (%)

tn, is the time until the end of stage n (days)

n, is the number of the stage

μ, is the *B. antiquum* growth rate (1/d)

From Equation 6-1 it can be observed that the fraction of settled *B. antiquum* cells (f_s) varies inversely to the growth rate, if we fix $ThCC_{B.ant.,n}$. Hence, the maximum value for the settled fraction occurs when the growth rate is at its minimum value, and *vice versa*.

By investigating possible values for the settled fraction, or for the growth rate, it is possible to understand in more detail why the enrichment was not successful. The impact of conservative values for the settling fraction (10%, corresponding

to no settling of *B. antiquum*) and growth rate (μ = 0) were investigated. summarizes the values used, the assumptions made as well as the results obtained from Equation 6-1.

Table 6-3 Summary of values and assumptions used to estimate the settled fraction and the growth rate of *B. antiquum*.

SBR	Cycle	Time[a] (d)	Stage[a]	RA (%)	CC_{Total} (cell/mL)	$ThCC_{B.\ ant.}$ (cell/mL)	Assumption (f_s in %, and μ in 1/d)	Result[b]
Ba	0	0	0	96.4	5.5×10^8	5.3×10^8	-	-
Ba	3	13	7	2.8	2.1×10^8	6.0×10^6	$f_s = 10$	μ = 1.3
							μ = 0	$f_s = 53$
Ba	6	25	13	0.9	1.2×10^8	1.1×10^6	$f_s = 10$	μ = 1.4
							μ = 0	$f_s = 62$
Bb	6	25	13	1.5	5.0×10^7	7.3×10^5	$f_s = 10$	μ = 1.4
							μ = 0	$f_s = 60$

RA, relative abundance of *Brevibacterium*; CC_{total}, total cell count; $ThCC_{B.\ ant.}$, theoretical cell count of *B. antiquum* (RA x CC_{total}).

[a] Refers to the total since time zero.

[b] Lag stage time was assumed as zero, and both the settling fraction (f_s) and growth rate (μ) were assumed equal for the 2 stages cycled.

The results in indicate that the value of the settled fraction is likely within the range of 10 to 62%, and the growth rate of *B. antiquum* between 0 and 1.4 1/d.

A growth rate <1.4 1/d was less than half of what was observed previously (3.4 1/d) in single-culture sludge dewatering liquors supplemented with acetate and sodium chloride (562-1686 mg COD/L and 1.5-4.5% of NaCl) (Simoes *et al.*, 2017a). Likely, the competing microorganisms had a higher affinity to uptake nutrients essential to sustain the growth rate of *B. antiquum*. On the other hand, <62% of the *B. antiquum* cells settled to the volume kept between stages. It follows that the settling fraction of *B. antiquum* has margin to be improved.

Two broad strategies are recommended to achieve higher abundance and enrich *B. antiquum* in the inoculated SBR:

1. improving the retention of *B. antiquum* cells in between stages, so that more cells can remain in the SBR.

2. increasing the selective pressure to limit the growth the competing indigenous microorganisms, and, possibly, select a carbon source for which *B. antiquum* has a higher affinity.

The enrichment of ammonium oxidizing bacteria (AOB) relative to nitrite oxidizing bacteria (NOB) has been shown to be possible in the CANON process, by making use of differences in microbial affinity for oxygen, often described as the substrate half saturation constant in the Monod kinetics – *Ks*. Even though NOB have higher maximum specific growth rate than AOB (Arnaldos *et al.*, 2015), limiting the oxygen supply created the conditions where the AOB were enriched as they have a lower *Ks* value for oxygen (0.99 g O_2/m^3) compared with NOBs (1.4 g O_2/m^3) (Ciudad *et al.*, 2006).

The enrichment of selected bacteria from a mixed culture requires tight control of several parameters and a good understanding of the biochemical pathways of the target bacteria. In the CANON Anammox process, Anammox and AOB were enriched from activated sludge (AS) mixed liquors by doing full biomass retention (biomass carrier material was recommended, if there was no formation of >2mm flocs), maintaining excess ammonium (>30 mg/L) and doing online control of oxygen (0.1 mg O_2/L) and nitrite (<15 mg/L), at pH 7.8 and 30°C (Third *et al.*, 2005).

On another example, the enrichment of phosphate accumulating organisms (PAOs) in the EBPR processes was accomplished from a starting inoculum of AS mixed liquors. By sequencing of anaerobic-aerobic stages as a selective pressure, the growth of bacteria that accumulate a source of energy in the form of polyphosphates such as PAOs was favoured, resulting in the bacteria enrichment (Hui *et al.*, 2014). Similarly, the cycling of feast-famine stages was reported to lead to the enrichment of bacteria able to accumulate polyhydroxyalkanoate (PHA) bio-plastics, which are a form of accumulating

energy and a carbon source (Frison *et al.*, 2015; Valentino *et al.*, 2015). In all these cases, the enrichment target was a community of functionally related microorganisms and not a specific bacterium species such as with *B. antiquum*.

A single species of *Plasticicumulans acidivorans* has been reported to have been enriched to an abundance >70%, in a pilot-scale process designed to recover PHA using industrial wastewater on a feast-famine cycle (Tamis *et al.*, 2014). *P. acidivorans* was enriched from AS sludge, from where it had initially been identified (Jiang *et al.*, 2011), and was not inoculated from a pure culture, as in this work with *B. antiquum*.

In other cases, single bacterial cultures were added to wastewater treatment, in open mixed-culture conditions, but the chosen strains had been previously isolated from a wastewater treatment source (Chen and Ni, 2011; Shi *et al.*, 2015; Chen *et al.*, 2016). For bacteria not originally isolated from wastewater, as is the case of *B. antiquum*, there is no successful case of enrichment reported to date. As an alternative, repeated additions of *B. antiquum*, into the sludge dewatering liquors is a possible route moving forward. This repeated addition of selected microorganisms into a treatment process is often described as bioaugmentation. However, inoculum survival after bioaugmentation in mixed cultures is a recurrent issue. Insufficient inoculum size, predation, competition by indigenous populations, presence of inhibiting substances and availability of alternative substrates are pointed as causes for the poor success rate of bioaugmentation (Raper *et al.*, 2017).

The retention of the inoculated bacteria within the reactor, by minimizing the wastage, is also a common feature of successful cases. The bacteria *Serratia sp.* BDG-2 was retained inside the reactor after adding the coagulant polyaluminium chloride to enhance its settleability (Chen *et al.*, 2016). Similarly, the enrichment of *Arthrobacter* sp. W1 into zeolite-biological aerated filters, treating coking wastewater, was achieved after immobilizing the bacteria in alginate (Shi *et al.*, 2015). The relative abundance of *Arthrobacter sp.* W1 was 14.3% after 100 days on operation, while it was only 3.9% when it was added as a free suspension. Bouchez *et al.* (2000) reported the relative abundance of

Microvirgula aerodenitrificans one week after inoculation. When the bacteria was immobilized in alginate before inoculation, the relative abundance was 7%, which was higher than using coagulant and flocculant agents (2%), which was still higher than adding the bacteria alone (0%). Moreover, the alginate beads were reported to have protected the inoculated bacteria from protozoa grazing. Although the beads broke down within 3 days, the fragments were incorporated into the indigenous flocs.

Besides immobilization of *B. antiquum* in carrier beads to increase the retention and settled fraction when transitioning stages, settleability can also be increased by promoting granulation of the biomass in the SBR. Reducing the settling times has been reported to be a key factor to inducing granulation. Granulation was induced in a mixed culture SBR of *Rhizobium* sp. NJUST18, by allowing settling for 12 min plus 3 min for effluent withdrawal, in a reactor with 1 m height (Tay *et al.*, 2002; Liu *et al.*, 2015). The granulation of nitrifying microorganisms was achieved with 30 min settling plus 4 min effluent withdrawal in a 1.2 m reactor (Tay *et al.*, 2002).

Another way of achieving enrichment of a specific bacteria, or bacterial community, is by decreasing the number of competing microorganisms. The pre-treatment of sludge for enrichment of bio-hydrogen producing bacteria has been researched as a way of eliminating the hydrogen consumers while preserving the hydrogen producers (Wang and Yin, 2017).

Currently proposed regulations for the commercialization of fertilizers by the European Commission, require the pasteurization (at 70°C for 1 h) of components of fertilizers originating from wastewater digestate (European Commission, 2016). Struvite is aimed to be used as phosphorus rich fertilizer. If this specific pasteurization process becomes a required part of recovering phosphorus from wastewater digestate, the decrease in the microbial load in the digestate, and its liquors, might favour the applicability of *B. antiquum*. The same principle can be thought of for liquors from digestate previously submitted to thermal hydrolysis.

In any case, the addition of a bacterial community or a bacteria species to an established microbial community, has been pointed to be more susceptible to uncertainty and variability in lab-scale systems when compared with pilot- and full-scale processes (Stephenson and Stephenson, 1992).

6.4 Conclusions

- A onetime inoculation of *B. antiquum* into a SBR with sludge dewatering liquors with acetate and NaCl, (30 min settling, and 90% volume replacement) did not conduct to the establishment of a stable bacterial community composed mostly of *B. antiquum* bacteria.

- Bacterial community profiling showed that *Brevibacterium* species were not present in the sludge dewatering liquors indigenous microbial population (<0.01% of sequenced amplicons). This confirmed that techniques based on amplification of the 16S rRNA gene can be used to show the enrichment of Brevibacterium species in sludge dewatering liquors.

This work represents early stages in the development of a stable bio-struvite process and further trials should explore the enrichment of *B. antiquum* in sludge dewatering liquors via improving the retention of *B. an*tiquum cells in between stages, and by increasing the selective pressure to limit the growth the competing indigenous microorganisms.

6.5 Acknowledgements

The authors would like to thank the financial contribution and help from Severn Trent Water Ltd (Coventry, UK), and the financial support awarded by the Portuguese foundation for science and technology (Fundação para a Ciência e Tecnologia, FCT, Portugal) in the form of a PhD grant (SFRH/BD/84116/2012) covered by the POPH/FSE program.

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Chapter 7 Fertilizer value of bio-struvite produced from sludge dewatering liquors

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Abstract

The aim of this study was to compare biomineralized and chemical struvite produced from sludge dewatering liquors as a fertiliser, including phosphate content, impact on plant germination and presence of micropollutants. Bio-struvite was found to have higher purity than chemical struvite, 91%, and 50%, respectively. Impact on plant growth was assessed by measuring the growth of mustard seedlings (*Brassica nigra*) germinated in a controlled environment. No differences were found between using bio-struvite and chemical struvite. Micropollutants content was assessed by measuring the content of 11 heavy metals and 16 poly-aromatic hydrocarbons. The presence of 198 organic polar substances was screened with a liquid chromatograph quadrupole time-of-flight mass spectrometer (QTOF). However, only small differences were found in terms of micropollutants content with chemical struvite having 1.0×10^3 µg Hg/kg of mercury, and 7.5×10^3 µg As/kg of arsenic, compared with 7.4×10^2 µg Hg/kg and 1.2×10^4 µg As/kg in bio-struvite. These results indicate that bio-struvite displays similar or better characteristics and hence it has the potential to reach the same market value as chemical struvite. This highlights the potential of the bio-struvite process for phosphorus recovery from wastewater streams.

Keywords

Biomineralization; phosphorus recovery; wastewater; heavy metals; micropollutants.

7.1 Introduction

Phosphorus is a non-replaceable essential nutrient widely used in agriculture for food production and essential for food security (Cordell *et al.*, 2009). Up to 90% of the phosphorus available worldwide is used in agriculture as fertilizer (Desmidt *et al.*, 2015). This comes from the abstraction of mineral rock phosphate, but there are limited reserves with widely asymmetric world distribution, making phosphorus a strategic, essential resource of limited availability (Elser, 2012).

Up to 15% of the phosphorus needs can be potentially recovered from wastewater (van Dijk *et al.*, 2016). Struvite, a phosphate, ammonium and magnesium salt, is seen as the most promising end product of phosphorus recovery from wastewater streams due to the value of struvite as a marketable fertilizer. The recovery of phosphorus as struvite has been reported to be particularly interesting when done from sludge dewatering liquors, a side-stream rich in nutrients that is typically returned to the main stream of treatment within the wastewater treatment plant (WWTP) (Jaffer *et al.*, 2002). Although the precipitation of struvite from sludge dewatering liquors is a process already implemented at full-scale in several sites in Europe, Canada, and Japan (Desmidt *et al.*, 2015) it is a process that requires the addition of chemicals as a source of magnesium, and requires a pH increase to trigger the precipitation and to maximize the recovery of phosphorus. Hence, chemical struvite precipitation has been reported to only be economically viable in cases where the sludge dewatering liquors stream has a phosphate content above 100-200 mg PO_4-P/L (Dockhorn, 2009; Le Corre *et al.*, 2009). Although struvite could be a new revenue stream for the water industry, products obtained from wastewater are often stigmatized raising concerns in terms of safety. In

particular, when destined to be used in food production, the concerns range from the presence of pathogenic microbial contamination, heavy metal content and other organic micropollutants (Weidelener *et al.*, 2008).

An alternative process for struvite production from wastewater that can be used in streams as sludge dewatering liquors or activated sludge liquors, uses bacteria to mediate struvite formation (bio-struvite) without the addition of chemicals (Soares *et al.*, 2014). Selected bacteria as *Brevibacterium antiquum* incubated in sludge dewatering liquors with 45 mg PO_4-P/L, were shown to lead to the formation of up to 138 mg/L of bio-struvite (Simoes *et al.*, 2017b). This biological process has yet to be optimized and the bio-struvite formed has not assessed for the presence of hazardous micropollutants relative to struvite obtained using conventional chemical process.

Conventional chemical struvite (chemical struvite) has been shown to be an effective fertilizer in field tests with several different cultivars, covering multiple crop seasons, with results comparable to triple superphosphate and di-ammonium phosphate (Latifian *et al.*, 2012; Kataki *et al.*, 2016). In terms of microbial safety, struvite produced the effluent of an upflow anaerobic sludge blanket reactor digesting black water, was found to not have detectable levels of *Enterobacteriaceae* and to be safe according to the European Union Animal By-products Regulation, although the upflow anaerobic sludge blanket (UASB) sludge contained 100 colony forming units per gram (Gell *et al.*, 2011). Furthermore, chemical struvite has been repeatedly shown to have heavy metal content below traditional fertilizers (Antonini *et al.*, 2012; Latifian *et al.*, 2012; Xu *et al.*, 2012; Kratz *et al.*, 2016). In terms of organic micropollutants, studies have pointed the association of tetracyclines (3.0×10^4 µg/kg), sulfonamindes (1.9×10^3-1.4×10^4 µg/kg), and fluoroquinolones (5.1×10^3-5.9×10^4 µg/kg) with struvite (Kemacheevakul *et al.*, 2014, 2015; Lou *et al.*, 2015). In addition the list of potential hazardous micropollutants keeps growing as new analytical methods show the effects of these chemicals at concentrations that were previously not detected (Petrie *et al.*, 2014).

In this paper commercially available soil bacteria *Brevibacterium antiquum* was used to produce bio-struvite from sludge dewatering liquors collected at a full-scale WWTP. The bio-struvite was compared with struvite obtained from the same sample of sludge dewatering liquors using a conventional chemical process, in terms of phosphate content, plant growth, and presence of micropollutants.

7.2 Materials and Methods

7.2.1 Biological material

Brevibacterium antiquum, strain DSM 21545 (German Resource Centre and Biological Material, Germany) was used to produce bio-struvite from sludge dewatering liquors. Starter cultures were grown for 4 days in autoclaved (121°C, 20 min) synthetic media (4 g/L of yeast extract, 2 g/L of magnesium sulphate heptahydrate, and 2 g/L of di-potassium hydrogen phosphate). Incubation of the cultures was done at room temperature (20-25°C), in an orbital shaker at 150 rpm (Stuart SSL1, Fisher Scientific, Loughborough, UK).

To assess the fertiliser value of the bio-struvite, seeds of two plants were germinated in phosphorus-poor soils under controlled lab conditions: sweet corn seeds (*Zea mays*, White Pearl Popping Corn, Map Trading Ltd, UK), and mustard seeds (*Brassica nigra*, Thompson & Morgan, UK).

7.2.2 Sludge dewatering liquors

Sludge dewatering liquors were collected from a full-scale biological nutrient removal (BNR) WWTP with an average inlet flow of 1 m^3/s. Primary and secondary sludge produced onsite and import sludge from nearby municipal WWTP (40% v/v) were anaerobically digested, and then stored in holding tanks for 10 to 27 days, before dewatering. A horizontal centrifuge-decanter was used to dewater the digested sludge from about 7% solids to 22% solids content with the help of cationic polymer, anti-scaling and antifoaming additives.

7.2.3 Production of the different types of struvite

Bio-struvite was produced by incubating sludge dewatering liquors with *B. antiquum* for 7 days (Simoes *et al.*, 2017b). Inoculation was done with *B. antiquum* cells centrifuged (Sanyo MSE Falcon 6/300 centrifuge, 2400g, 5 min) from starter cultures, and resuspended to the original volume with autoclaved 0.9% w/v NaCl aqueous solution, in order to remove the phosphate, ammonium and magnesium present in the starter cultures media. Sludge dewatering liquors were pH corrected to ensure a post-autoclave pH between 7.0 and 7.8. To ensure enough carbon source was available for the bacteria, 562 mg/L of chemical oxygen demand (COD) was added from a stock of 357 g/L of sodium acetate tri-hydrate aqueous solution. Phosphate concentration in the sludge dewatering liquors was increased by 40 mg P/L using a 25 g P/L stock of solution of di-potassium hydrogen phosphate and potassium di-hydrogen phosphate with pH 7.8. The magnesium concentration was increased to 110% of the total molar ratio of phosphate with 226 g/L magnesium chloride solution, taking into consideration the original concentration of magnesium in the sludge dewatering liquors.

Sludge dewatering liquors supplemented with the same amounts of phosphate and magnesium were used to produce struvite using a conventional chemical process (chemical struvite), this process was completed by increasing the pH to 8.7 using 40 g/L sodium hydroxide solution. The solutions was left under agitation in the same conditions as used for the incubation of *B. antiquum* in sludge dewatering liquors for 4 hours before separating the precipitated struvite from the liquid.

To prepare struvite from pure salts in de-ionized water (here referred to as synthetic struvite), a 10.2 g/L solution of magnesium chloride hexahydrate (Fisher Scientific, Loughborough, UK) and a 23.2 g/L solution of ammonium dihydrogen phosphate (Fisher Scientific, Loughborough, UK) were prepared. The pH of each solution was adjusted to pH 9 with a 240 g/L sodium hydroxide solution. Then 500 mL of magnesium chloride was mixed with 250 mL of the

ammonium phosphate solution in a 2 L Duran placed on a magnetic stirrer for 4 hours until separation of the precipitated struvite crystals (Le Corre *et al.*, 2005).

7.2.4 Plant growth with different types of struvite

To compare the fertilizer value of the bio-struvite with the chemical and synthetic struvite, seeds were germinated in a phosphorus-poor soil collected nearby (52.068432N, 0.594859W, Cranfield, UK). Fenland peat soil (Ely, Cambridge, UK) was used to compare the application rate of struvite for the germination and growth of the seedlings. Before use both soils were air-dried at 35°C in a drier cabinet for at least 24 hours, then grinded and sieved at 4 mm to break clumps and remove stones. The growth of germinated seedlings was compared after 5 days of incubation at 28°C with a 12 hour light cycle in an environmental growth chamber (Sanyo MLR-351H).

The struvite application rate was first studied for changes in the extractable phosphate on both Fenland peat soil and Cranfield soil, using synthetic struvite at 0.00, 0.05, 0.10, 0.30, and 1.00% w/w of struvite in soil. The struvite-supplemented soils were placed in plastic Petri plates watered and incubated as described before. Extractable phosphate in the soil was measured at beginning and at the end of 5-7 days. Application rates were also tested for plant growth with sweet corn and mustard seeds in both Fenland peat, and Cranfield soils supplemented with synthetic struvite using the procedure and application rates detailed. Tests with sweet corn seeds were done with 5 plate replicas, and tests with mustard seeds were done with 4 plate replicas.

Each type of struvite was tested at an application rate of 0.3% w/w in Cranfield soil. Six seeds were evenly placed over 25 g of struvite-supplemented soil in 9 mm diameter plastic Petri plates (Fisher Scientific, Loughborough, UK). The plates were then watered with 20 mL of de-ionized water, and placed the environmental growth chamber. Each plate was then watered daily with 15 to 20 mL of de-ionized water, to compensate for evaporation. Plant growth was measured as total of roots length and stem length. Eight plate replicas were done for each of the 3 types of struvite. Extractable phosphate was assessed at the end of incubation.

7.2.5 Analytical methods

All types of struvite were separated from the liquid media where they were produced by vacuum filtration with a 10 μm aperture nylon mesh filter (Plastok Associates, Birkenhead, UK) and then air dried for at least 2 hours at 37°C.

The bio-struvite and chemical struvite obtained from the sludge dewatering liquors were photographed in an environmental scanning electron microscope (ESEM; XL 30 SFEG, Phillips, The Netherlands). ESEM energy dispersive X-ray spectroscopy (EDX) was used to verify the elemental composition of the exposed surface of selected regions.

Samples of bio-struvite and chemical struvite were dissolved in de-ionized water, and acidified to a final pH of 3, in acid washed borosilicate glassware with PTFE lined caps, to assess the presence of a suite of dissolved metals: arsenic, cadmium, chromium, copper, lead, mercury, nickel, zinc, aluminium, iron, and potassium; and a suite of poly-aromatic hydrocarbons (PAH): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b/k)fluoranthene, benzo(a)pyrene, indeno(123-cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene, and total PAH; in an external laboratory using UKAS accredited methods (UK Accreditation Services). Dissolved chemical struvite and bio-struvite were also used to measure their content of magnesium and phosphate, and to check for the presence of 198 polar organic hazardous micropollutants (listed in Appendix B). The polar organic hazardous micropollutants separation was conducted by a 1290 series liquid chromatography from Agilent (Santa Ana, USA) equipped with an Agilent Zorbax Eclipse Plus C18 rapid resolution column (2.1×50 mm, 1.8 μm) kept at 21°C. The mobile phase consisted of water (A) and acetonitrile (B) with both eluents containing 0.1% formic acid. An elution gradient of 0.3 mL/min was applied with the initial concentration (5% B) hold for 1 min, increased to 75% B for 9 min and thereafter hold for 5 min. A 3-min post run was required for column equilibration. The injection volume was 20 μL. 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 250 °C; nebulizer 45 psi; gas flow 8 L/min; gas temperature 250°C; skimmer 65 V; fragmentor 175 V; nozzle 0 V; octopole RF 750 V; and capillary 3.5 kV. Accurate mass spectra were acquire in scan mode (50 – 1200 *m/z*). Reference masses were 121.0509 and 922.0098 *m/z* with a resolution of 19546 at 922.0106 *m/z*. Data were acquired and analysed with MassHunter software.

To measure plant growth the plant seedlings were carefully separated from the soil, and flattened over a paper after rinsing the roots with a trickle of water. The plants were then photographed with a reference scale, and image analysis software, ImageJ (version 3.2.0), was used to measure the length of the roots and the stem.

Soil pH measurement was taken from a 20% v/v suspension of soil in 1 M potassium chloride solution according to the British Standard BS ISO 10390:2005. The extractable phosphate in the soil was extracted with 0.5 M sodium hydrogen carbonate solution at pH 8.5 according to the method described on the British Standard BS 7755-3.6:1995. Phosphate and ammonium were measured with a Smartchem 200 discrete analyser and recommended reagents (Labmedics, Abingdon, UK). Dissolved magnesium was quantified by atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer Ltd, Beaconsfield, UK) equipped with an air/acetylene burner system. Chemical oxygen demand was quantified with Merck cell kit (VWR, Lutterworth, UK). The pH was measured with a Fisherbrand hydrous 300 pH meter (Fisher Scientific, Loughborough, UK). Values presented in this work detail the mean and standard error of the tested replicas.

7.3 Results

Both bio-struvite and chemical struvite were produced from the same sludge dewatering liquors (Table 7-1) that presented 594.5 mg COD/L) and a pH of 7.8. These values were similar to the 308-564 mg COD/L and pH of 7.8- 8.0

reported in sludge liquors of municipal wastewater sludge (David, 2007). However, the content of total phosphate (1.4 mg P/L) was low in comparison with 44-136 mg P/L to the values reported (David, 2007). As consequence, the sludge dewatering liquors were supplemented with phosphate so struvite formation could be obtained.

Table 7-1 Characteristics of the sludge dewatering liquors collected from a full scale site and used to produce both bio-struvite and chemical struvite. Values detail the mean ± standard error of replicate measurements

pH	**Total phosphorus** (mg P/L)	**PO_4** (mg P/L)	**Mg^{2+}** (mg/L)	**Total COD** (mg/L)	**Dissolved COD** (mg/L)
7.8	11.7 ± 0.4	1.4 ± 0.0	74.5 ± 2.1	594.5 ± 4.9	391.5 ± 4.9

The phosphate and magnesium content as measured in the bio-struvite, chemical struvite and synthetic struvite, to estimate their purity (Table 7-2). The contents of magnesium and phosphate matched well the 1:1 molar stoichiometric ratio of Mg:PO_4 in struvite. The mass percentages indicated that up to 50% of the weight of the chemical struvite was made of struvite, whilst the remaining fraction was composed of other substances without phosphate or magnesium. On the other side, up to 91% of the weight was struvite in the bio-struvite produced. Hence, these results demonstrate that bio-struvite had a higher purity than chemical struvite produced from the same sludge dewatering liquors. CrystalGreen, a commercially available chemical struvite obtained from other sources of sludge dewatering liquors is reported to have 10% w/w of Magnesium and 12% of phosphorus which points to a purity of 97% (Ostara USA, 2017).

Table 7-2 Struvite purity and mass percentage of phosphorus, and magnesium in synthetic struvite, bio-struvite, and chemical struvite. Values detail the mean ± standard error

	Source	Phosphorus (%)	Magnesium (%)	Purity (%)
Struvite	Empirical chemical formula	12.6	9.9	-
Synthetic struvite	$NH_4H_2PO_4$, $MgCl_2.6H_2O$, NaOH	12.9 ± 0.4	9.9 ± 0.3	100
Bio-struvite	Sludge dewatering liquors	11.9 ± 0.3	9.0 ± 0.2	91
Chemical struvite	Sludge dewatering liquors	6.7 ± 0.1	4.9 ± 0.1	50

Further confirmation of the purity of the recovered products was assessed using electron microscope observation and EDX microanalysis where the spectrograms of bio-struvite and chemical struvite were typical of struvite (Table 7-1). The spectrogram for chemical struvite indicated significant levels of calcium, which was estimated at approximately 20% w/w (Figure 1c). Phosphorus and magnesium were present at less than half the expected proportion for pure struvite crystals, matching the struvite purity analysis of 50% w/w. For both bio-struvite and chemical struvite, the EDX spectrograms indicated the presence of iron. In chemical struvite, the iron content was 2 fold the weight proportion of the bio-struvite (Table 7-1a and c).

Both types of struvite showed crystals with typical trapezoidal shape with lengths up to 200 µm (Table 7-1b and c). Chemical struvite showed the presence of amorphous particles with a high content of calcium (Table 7-1d).

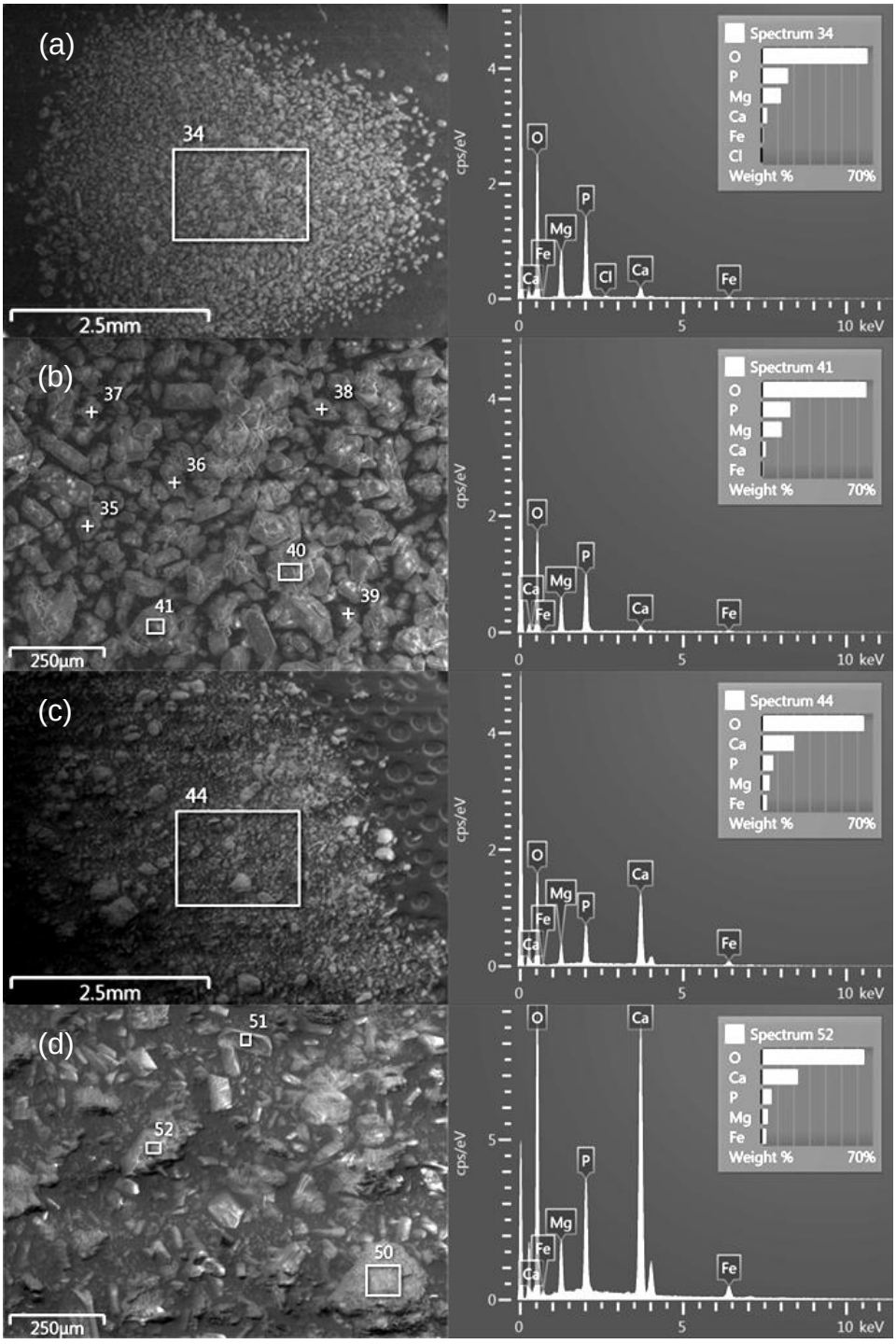


Figure 7-1 Electron scanning microscope photos, and energy dispersive X-ray (EDX) spectra of selected areas highlighted in the photos, of bio-struvite: (a) 18x magnification, and (b) 100x magnification. And of chemical struvite: (c) 18x magnification, and (d) 100x magnification.

The presence of organic polar hazardous micropollutants was evaluated with QTOF mass spectrometry in synthetic, chemical and bio-struvite. No differences were found between the different types of struvite. Of all of the 198 substances screened, dibutyl phthalate, diethyl phthalate, diisobutyl phthalate, and nonylphenol triethoxylate were found in all 3 types of struvite, including the synthetic struvite produced from pure salts. Hence, the presence of these substances was attributed to the method used. No other substances were quantified.

No significant differences were found between the bio-struvite and the chemical struvite in relation to poly-aromatic hydrocarbons (Table 7-3), with $3.0\times10^{1} \pm 1.0\times10^{1}$ µg/kg in the bio-struvite, and $3.7\times10^{1} \pm 1.2\times10^{1}$ µg/kg in the chemical struvite. Also, no differences were found for 9 of the 11 metals measured. However, bio-struvite contained $1.2\times10^{4} \pm 5.0\times10^{2}$ µg/kg of arsenic, which was higher than $7.5\times10^{3} \pm 2.6\times10^{2}$ µg/kg found on the chemical struvite. On the other side, bio-struvite had less mercury ($7.4\times10^{2} \pm 9.9$ µg/kg) than the chemical struvite with $1.0\times10^{3} \pm 1.3\times10^{1}$ µg/kg (Table 7-3).

Table 7-3 Quantification of metals, and poly-aromatic hydrocarbons (PAH) released by chemical struvite and by bio-struvite obtained from the same sample of sludge dewatering liquors, when dissolved at 0.5 g/L in pH 3 de-ionized water. Values detail the mean ± standard error of replicate measurements.

Micropollutant	LOD (µg/kg)	Chemical struvite (µg/kg)	Bio-struvite (µg/kg)
Arsenic (dissolved)	5.0×10^{2}	$7.5\times10^{3} \pm 2.6\times10^{2}$ [a]	$1.2\times10^{4} \pm 5.0\times10^{2}$ [a]
Cadmium (dissolved)	5.0×10^{1}	7.5×10^{1} [b]	<LOD
Chromium (dissolved)	2.5×10^{3}	<LOD	<LOD
Copper (dissolved)	1.2×10^{3}	$1.5\times10^{4} \pm 4.4\times10^{3}$	$8.0\times10^{3} \pm 1.0\times10^{3}$
Lead (dissolved)	7.5×10^{2}	<LOD	$1.1\times10^{3} \pm 1.0\times10^{2}$
Mercury (dissolved)	1.2×10^{2}	$1.0\times10^{3} \pm 1.3\times10^{1}$ [a]	$7.4\times10^{2} \pm 9.9\times10^{0}$ [a]
Nickel (dissolved)	2.5×10^{3}	$8.7\times10^{3} \pm 1.3\times10^{3}$	8.0×10^{3} [b]
Zinc (dissolved)	5.0×10^{3}	$3.2\times10^{4} \pm 5.0\times10^{3}$	$2.1\times10^{4} \pm 1.0\times10^{3}$
Aluminium (dissolved)	2.5×10^{5}	$6.2\times10^{5} \pm 2.0\times10^{5}$	$5.5\times10^{5} \pm 9.9\times10^{3}$
Iron (dissolved)	1.2×10^{4}	$6.5\times10^{5} \pm 1.3\times10^{5}$	$3.6\times10^{5} \pm 9.7\times10^{3}$
Potassium	2.5×10^{5}	$2.5\times10^{6} \pm 1.2\times10^{6}$	2.6×10^{6} [b]
Naphthalene	2.5×10^{1}	<LOD	<LOD
Acenaphthylene	2.5×10^{1}	<LOD	<LOD
Acenaphthene	2.5×10^{1}	$2.5\times10^{1} \pm 1.2\times10^{1}$	$2.5\times10^{1} \pm 1.2\times10^{1}$
Fluorene	2.5×10^{1}	<LOD	<LOD
Phenanthrene	2.5×10^{1}	2.5×10^{1} [b]	2.5×10^{1} [b]
Anthracene	2.5×10^{1}	<LOD	<LOD
Fluoranthene	2.5×10^{1}	<LOD	<LOD
Pyrene	2.5×10^{1}	<LOD	<LOD
Benzo(a)anthracene	2.5×10^{1}	<LOD	<LOD
Chrysene	2.5×10^{1}	<LOD	<LOD
Benzo(b/k)fluoranthene	2.5×10^{1}	<LOD	<LOD
Benzo(a)pyrene	2.5×10^{1}	<LOD	<LOD
Indeno(123-cd)pyrene	2.5×10^{1}	<LOD	<LOD
Dibenzo(ah)anthracene	2.5×10^{1}	<LOD	<LOD
Benzo(ghi)perylene	2.5×10^{1}	<LOD	<LOD
PAH (total)	2.5×10^{1}	$3.7\times10^{1} \pm 1.2\times10^{1}$	$3.0\times10^{1} \pm 1.0\times10^{1}$

LOD, limit of detection; PAH, poly-aromatic hydrocarbons.

a Significant difference between bio-struvite and chemical struvite with a p-value below 5%.

b Single measurement.

Iron quantified from the dissolved samples was $3.6\times10^5 \pm 9.7\times10^3$ µg/kg for bio-struvite, and $6.5\times10^5 \pm 1.3\times10^5$ µg/kg for chemical struvite (Table 3). Up to 1.8 fold more iron was found in the chemical struvite relative to the bio-struvite, which matched the electron microscope energy-dispersive X-ray microanalysis results (Figure 7-1).

The fertilizer value of the bio-struvite was also assessed by measuring the growth of plant seedlings. Two different sources of soil were used to assess the impact of different application rates of synthetic struvite. The parameters measured included extractable phosphate and the growth of roots and stems of sweet corn and mustard seedlings. Both soils had a pH of 7.4, hence struvite was not expected to be easily dissolved. The soils had distinct amounts of extractable phosphate, 118.2 ± 0.7 mg/kg for the Fenland peat soil and 6.2 ± 0.2 mg/kg in the Cranfield soil (Table 7-4).

Table 7-4 Extractable phosphate and pH of Fenland peat soil, and Cranfield soil. Values detail the mean ± standard error of replicate measurements.

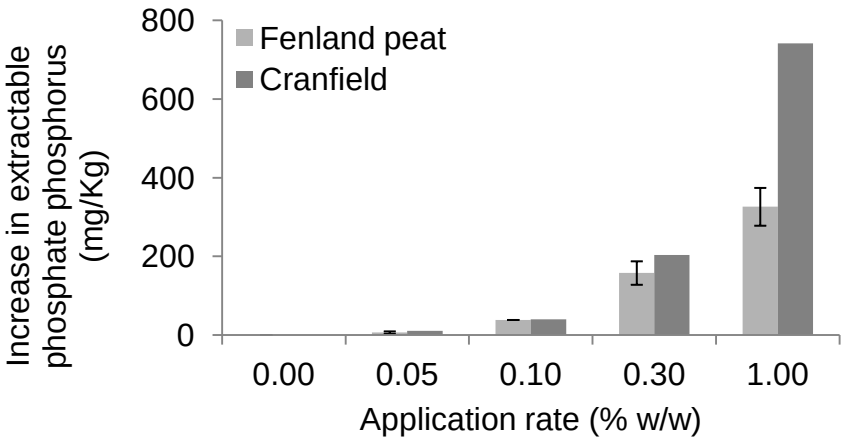
Soil	$pH_{(KCl)}$	Extractable phosphate (mg P/kg)
Fenland peat	7.38 ± 0.01	118.2 ± 0.7
Cranfield	7.40 ± 0.01	6.2 ± 0.2

The proportion of extractable phosphate released from synthetic struvite in un-cultivated soil increased when using higher application rates (Figure 7-2a). At 0.05% w/w application rate the release of phosphate was 10.8 mg/kg in Cranfield soil, and 6.4 mg/kg in Fenland peat. At 0.10% w/w application rate the release of phosphate was 40.3 mg/kg in the Cranfield soil, and 38.6 mg/kg in Fenland peat. The amount of phosphate released for the application rates of 0.30, and 1.00% w/w was higher in the Cranfield soil (203.2, and 741.2 mg/kg, respectively) than in the Fenland peat soil (157.9, and 326.2 mg/kg, respectively).

In Fenland peat soil, 10% mol/mol of phosphate was released at the lowest application rate (0.05% w/w), and 42% mol/mol was released at 0.3% w/w

application rate (Figure 7-2b). In the Cranfield soil, 17% mol/mol was released at 0.05% w/w, and 60% mol/mol was released at 1% w/w application rate. These results suggest that a form of saturation was taking place where less initial phosphate in the soil, as the Cranfield soil, leads to more release of phosphate from the added struvite.

(a)



(b)

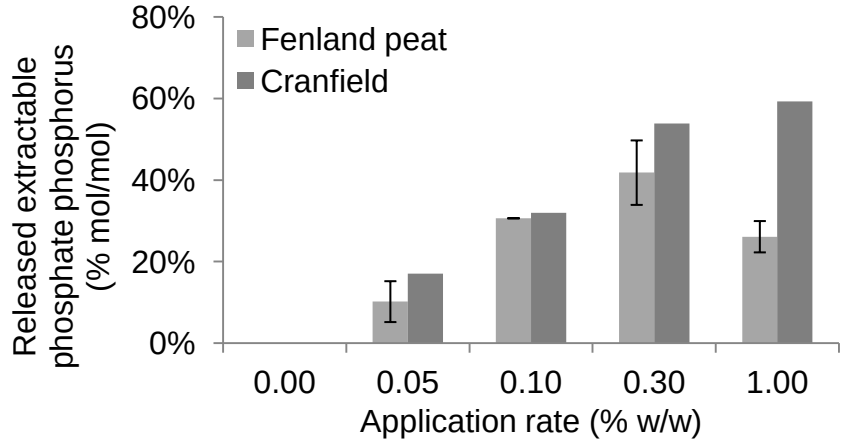


Figure 7-2 Increase of extractable phosphate (a) and fraction of phosphorus added with struvite accounted in extractable phosphate (b) in Fenland peat soil and Cranfield soil at different application rates of synthetic struvite after incubating the soil in an environmental growth chamber for 5 days at 28°C and 12 h day-light cycles. Error bars represent the standard error of the replicate tests.

The length of sweet corn seedlings in Fenland peat soil reached a maximum of 2.0 ± 0.6 cm in the tests with 0.30% w/w of struvite. In the tests with 0.10, and 1.00% w/w of synthetic struvite, the seedlings grew as much as in the 0.00% w/w control tests (1.2 ± 0.4 cm) (Figure 7-3). With 0.05% w/w the seedlings grew 0.3 ± 0.1 cm, which was less than in the 0.00% w/w control tests. The limited growth at 0.05% w/w application rate was also observed for the mustard seedlings in Cranfield soil (1.1 ± 0.2 cm, at 0.05% w/w) which was lower than the growth in 0.00% w/w in control tests (3.0 ± 0.4 cm). It is not clear what the cause for this difference was. The results suggest two concurrent phenomena whose cumulative effect resulted in a minimum at application rate of 0.05% w/w.

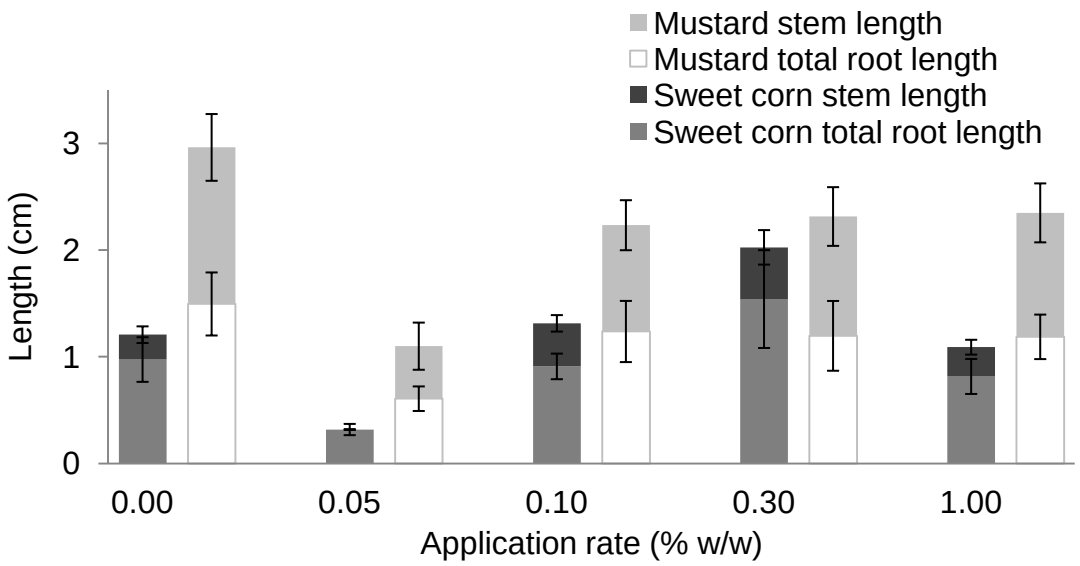


Figure 7-3 Length of roots and stem of sweet corn seedlings sown in Fenland peat soil, and of mustard seedlings sown in Cranfield soil, with different application rates of synthetic struvite, after incubating in an environmental growth chamber for 5 days at 28°C and 12 h day-light cycles. Error bars represent the standard error of the replicate tests.

The total root length of the mustard seedlings in Cranfield soil was similar for the application rates above 0.10% w/w and the control tests with no struvite added (1.2 ± 0.5 cm). The stem length for all application rates did not reach the

length observed in the tests with no struvite. The minimum stem length observed occurred at 0.05% w/w application rate.

An application rate of 0.30% w/w of struvite in soil was selected to test for differences in the growth of stems and roots of mustard seedling in Cranfield soil, and for the release of extractable phosphate. The application rate of 0.30% w/w of struvite in soil allowed to maximize the release of phosphorus from dissolved struvite. At the same time, higher dissolution of struvite was expected to release more hazardous micropollutants which can have a phytotoxic impact on the seedling growth (Chouychai *et al.*, 2007).

At an 0.30% w/w application rate, the extractable phosphate in Cranfield soil at the end of growing mustard seedlings was similar for tests with bio-struvite, and synthetic struvite (202 ± 26 mg/kg), and almost 2 fold higher than in the chemical struvite tests (115 ± 10 mg/kg) (Figure 7-4). Hence, extractable phosphate seemed directly related with the purity of the struvite used. No significant differences were found for the total root length of the mustard seedlings grown with different types of struvite (Figure 7-5). For the stem length, 1.7 ± 0.0 and 1.8 ± 0.2 cm were observed for bio-struvite and synthetic struvite, respectively, and 1.2 ± 0.5 cm in tests with chemical struvite. These results confirm that there were no differences between synthetic struvite, bio-struvite, and chemical struvite.

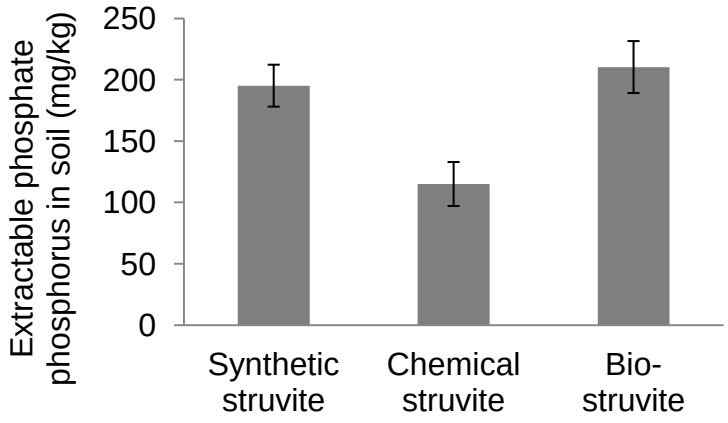


Figure 7-4 Comparison between the soil extractable phosphate at the end of 7 days of incubation of mustard seedlings in Cranfield soil treated with 0.30% w/w of synthetic, chemical or bio struvite, in an environmental growth chamber at 28°C and 12 h day-light cycles. Error bars represent the standard error of the replicate tests.

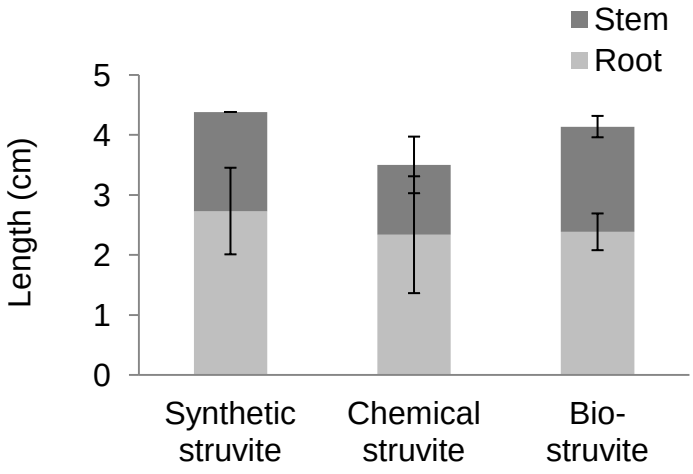


Figure 7-5 Comparison between the soil extractable phosphate levels at the end of 7 days of incubation of mustard seedlings in Cranfield soil, in an environmental growth chamber at 28°C and 12 h day-light cycles, when treating the soil with 0.30% w/w of synthetic, chemical or bio-struvite. Error bars represent the standard error of the replicate tests.

7.4 Discussion

Electron microscope observation and EDX microanalysis confirmed the purity analysis of both bio-struvite and chemical struvite. Bio-struvite purity was comparable to reported values of 98-99% for struvite obtained from sludge centrate with 37-107 mg PO_4-P/L through the addition of magnesium chloride and sodium hydroxide (Britton *et al.*, 2005; Bhuiyan *et al.*, 2008). The purity of chemical struvite (50%, Table 7-2) was in line with published values of 41% in struvite produced from non-digested sludge dewatering liquors with 48 mg PO_4-P/L (Ping *et al.*, 2016), and 48-52% obtained for struvite produced from centrate of digested pig slurry with 28.6 mg P/L (Cerrillo *et al.*, 2015). This difference in purity makes bio-struvite more valuable for its higher phosphate content, and for its lower amount of impurities. Compounds with higher purity can be more easily transformed into other forms too, allowing for the use of additives, as binding agents. (Latifian *et al.*, 2012), have reported the need produce a granular struvite fertilizer that can be used in normal farm fertilizer spreader equipment and for that the use binding agents is often required.

Chemical and bio-struvite were shown to have different content of mercury and arsenic. For arsenic, the ratio between the amounts found seem related with the purity of the struvite tested (7.5×10^3 : 1.2×10^4 µg/kg *versus* $50\%_{\text{chemical struvite}}$: $91\%_{\text{bio-struvite}}$) suggesting that arsenic was associated with the struvite fraction. Arsenic (V) has been said to co-precipitate with struvite, and arsenic (III) to adsorb to the surface of struvite crystals (Ma and Rouff, 2012; Lin *et al.*, 2014; Rouff and Juarez, 2014). The differences found for mercury (1.0×10^3, and 7.4×10^2 µg/kg for chemical struvite, and bio-struvite, respectively) suggest that mercury associated preferentially with the impurities of the chemical struvite.

The chemical struvite had a mercury content equal to the limit of 1 mg/kg for German and Austrian fertilizers (Table 7-5). When comparing the metals content of both the chemical and bio-struvite with known regulation limits for fertilizers, none was found above legal limits reported for Germany, Austria, Sweden, China, Turkey, and new proposed European Union (EU) limits (Table 7-5) (Latifian *et al.*, 2012; Xu *et al.*, 2012; European Commission, 2016; Kratz *et*

al., 2016). This falls in line with published works that have found that struvite produced from wastewater streams complies with regulations for fertilizers and has considerable fewer amounts of heavy metals than found on traditional phosphorus fertilizers (Ronteltap *et al.*, 2007).

Table 7-5 Legal limits on the presence of micropollutants in fertilizers, from several countries, in µg/kg.

Micro-pollutant	Austria	China	European Union (proposed 2016)[a]	Germany	Sweden	Switzer-land	Turkey
Arsenic			6.0×10^4	4.0×10^4			
Cadmium	1.5×10^4	1.0×10^4	2.0×10^4 [c]	5.0×10^4 [ab]	2.2×10^4 [b]	3.0×10^3	
Chromium	6.7×10^5	5.0×10^5	2.0×10^3 [d]	2.0×10^3 [d]		2.0×10^5	2.7×10^5
Copper	7.8×10^5					4.0×10^5	
Lead	1.0×10^5	1.5×10^5	1.5×10^5	1.5×10^5		2.0×10^5	
Mercury	1.0×10^3	5.0×10^3	2.0×10^3	1.0×10^3			
Nickel			1.2×10^5	8.0×10^4		5.0×10^4	1.2×10^5
Titanium				1.0×10^3			
Zinc	3.3×10^6					1.3×10^6	1.1×10^6
PAH[e]			6.0×10^3				

PAH, Poly-aromatic hydrocarbons.

[a] For fertilizers with phosphorus >5% P_2O_5.

[b] In µg/kg P_2O_5.

[c] Starting with 6.0×10^4 µg/kg for 3 yrs, and 4.0×10^4 µg/kg for the following 9 yrs.

[d] For Cr (VI).

[e] For components of fertilizers. Sum of the 16 PAH analysed in this work.

The presence of polar organic hazardous micropollutants showed no difference between types of struvite and this matched the results found for PAH and metals. Results here did not detect the presence of substances as tetracyclines, sulphonamides, and fluoroquinolones reported to associate with struvite produced from human urine and from swine wastewater (Kemacheevakul *et al.*, 2014, 2015; Lou *et al.*, 2015). Furthermore, the growth of mustard seedlings also did not show differences when using either of the struvite types.

Given that no differences were found between bio-struvite and the other types struvite, and these were found to comply with regulations for fertilizers, it can be anticipated that bio-struvite has the same or higher market value than conventional struvite. This further highlights the potential value of the bio-struvite process for the recovery of phosphorus from wastewater. The economic potential of a bio-struvite process will be limited by the bio-struvite production costs relative to conventional chemical struvite processes.

7.5 Conclusions

The presence of heavy metals and organic non-polar and polar micropollutants was found to be similar in both types of struvite produced from sludge dewatering liquors collected in a full scale WWTP. No polar organic micropollutants were found re-dissolved struvite, and poly-aromatic hydrocarbons were present at $<3.7\times10^{1} \pm 1.2\times10^{1}$ µg/kg, which is below existing regulations.

No difference was found between synthetic struvite, bio-struvite and chemical struvite in terms of their impact on mustard seedlings growth.

Bio-struvite can be expected to at least reach the same market value as conventional struvite. This highlights the potential value of the bio-struvite process for the recovery of phosphorus from wastewater.

7.6 Acknowledgements

The authors are grateful for the financial support and contribution from Severn Trent Water Ltd (Coventry, UK), and for the financial support awarded by the Fundação para a Ciência e Tecnologia (FCT, Portugal) as a PhD grant (SFRH/BD/84116/2012) under the POPH/FSE program.

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Chapter 8 Discussion

The aim of the PhD was to understand the key environmental conditions and factors responsible for biological struvite (bio-struvite) production and growth of selected bacteria in sludge dewatering liquors towards delivering a bio-struvite production process in WWTPs using sustainable and economical systems. The value of the bio-struvite was also assessed to understand the process economical feasibility.

The underlying hypothesis of this work was that selected bacteria could produce bio-struvite from sludge dewatering liquors under open mixed culture conditions. This hypothesis stemmed from the report that demonstrated the production of bio-struvite by pure cultures of bacteria (*Brevibacterium antiquum*, and *Bacillus pumilus*) in sludge dewatering liquors and settled wastewater (Soares *et al.*, 2014).

8.1 Selected bacteria can produce struvite in sterile sludge dewatering liquors

Results in Chapter 3 showed that all bacteria investigated, *B. antiquum*, *B. pumilus* and *Halobacterium salinarum*, were able to produce bio-struvite from sludge dewatering liquors under sterile conditions (Figures 3-4, and 3-5). Furthermore, results in Chapters 4, and 7 also showed that *B. antiquum* produced bio-struvite from sludge dewatering liquors under sterile conditions (Figures 4-3, 7-1).

The identity of the bio-struvite produced in experiments of Chapters 3, 4, and 7 was confirmed in an environmental scanning electron microscope (ESEM) and with energy dispersive X-ray spectroscopy (EDX). The surface elemental composition obtained with EDX was compared with the composition expected for reference struvite crystals. And the morphology of the crystals, observed in the ESEM, was compared with the morphology of struvite crystals found in published literature (Sánchez-Román *et al.*, 2007; Zhang *et al.*, 2015). All bio-struvite samples were confirmed to have surface composition matching

reference struvite crystals (Figures 3-3, 4-3, 7-1). Similarly, all bio-struvite samples had crystal morphology typical of struvite crystals (Figures 3-3, 4-3, 7-1).

Furthermore, in all experiments, the biological origin of the formed bio-struvite was confirmed by verifying that no struvite crystals were precipitated from the sludge dewatering liquors in un-inoculated tests (Chapters 3, 4, 5, 6, and 7) with the exception of the experiment in Chapter 3 that assessed the effect of different pHs on bio-struvite production. In this experiment struvite was observed in un-inoculated control tests with pH >8.3 (Figure 3-4).

Overall, these results added confirmation that the production of bio-struvite resulted from the action of the selected bacteria. Thus, confirming the possibility of an alternative process for phosphorus recovery from wastewater streams.

8.2 Selection of *B. antiquum* for further studies of bio-struvite formation in sludge dewatering liquors

Results in Chapter 3 showed that *B. antiquum* was the only bacterium able to increase the cell numbers in sludge dewatering liquors from initial 4.7×10^8 cells/mL, to 5.4×10^8 cells/mL at pH 7.3, and to 4.8×10^8 cells/mL at pH 7.8 (Figure 3-2). *B. pumilus* and *H. salinarum* were only able to remain viable at the pHs between 7.3-9.1, but their cell numbers declined. These results suggested that *B. antiquum* was better adapted to grow in sludge dewatering liquors. Consequently, *B. antiquum* offered a better chance for growth in sludge dewatering liquors under open-mixed culture conditions.

Later results in Chapter 4 indicated that the sludge dewatering liquors have limited availability of carbon source (Figure 4-2, and 4-4) even though sludge dewatering liquors typically have >300 mg COD/L (Table 2-1). This was the likely reason for the limited growth observed Chapter 3.

The production rate of bio-struvite with *B. antiquum* was different than observed the other two bacteria (Figure 3-5). *B. pumilus*, and *H. salinarum* produced

>90% of the final amount of bio-struvite within 24 h of incubation (Figure 3-5). On the other side, *B. antiquum* produced bio-struvite in close correlation with the microbial growth (Figure 3-5). This suggested that the mechanism of biomineralization of *B. antiquum* was more dependent on biology than in the case of *B. pumilus*, or *H. salinarum*. The biomineralization mechanisms were not explored in these studies, but others have indicated that *B. antiquum* bio-struvite production is regulated by biologically controlled mineralisation (BCM) mechanism whilst *B. pumilus* and *H. salinarum* follow a biologically induced mineralisation mechanism (Leng *et al.*, 2017). Biologically controlled mineralisation indicates the microorganism exerts active control over the biomineralization process and environmental conditions are expected to have a smaller influence on the output minerals (Mann, 2001).

In the case of bio-struvite formation from sludge dewatering liquors, the use of *B. antiquum* has the potential to be less susceptible to the varying environmental conditions of the sludge dewatering liquors because *B. antiquum* seems to follow a biologically controlled mechanism of biomineralization.

8.3 Impact of the composition of sludge dewatering liquors on the growth of the selected bacteria

The composition of the sludge dewatering liquors defines almost all physicochemical parameters important for microbial growth (Henze *et al.*, 2008) with the exception of oxygen, temperature and light. The review of published studies in Chapter 2 confirmed that sludge dewatering liquors are a variable complex media with substantial amounts of nutrients (Table 2-1). Results in Chapter 3 showed that the pH of the sludge dewatering liquors can vary widely from typical pH of 7.8 (Table 2-1), up to 9.8 as a consequence of CO_2 degassing. The pH of 9.8 observed in Chapter 3, impaired the growth of selected bacteria and no differences were observed between inoculated tests and un-inoculated control tests at the end of 9 days of incubation (Figure 3-1). Published work on *H. salinarum*, *B. antiquum* and *B. pumilus* have reported optimum growth at pHs 6-7, 5-9, and 5-11, respectively (Parvathi *et al.*, 2009;

Wende *et al.*, 2009; Shivaji *et al.*, 2011). Hence, Chapter 3 was focused on understanding the ability of the selected bacteria to grow and produce bio-struvite at different pH values in sludge dewatering liquors.

In Chapter 3, the selected bacteria were incubated in sludge dewatering liquors treated with non-phosphate buffers at pHs between 5.7 and 9.1. The results have shown that *B. pumilus* and *H. salinarum* remained viable at pHs between 7.3-9.1 and that *B. antiquum* was able to grow at pHs between 7.3-7.8. At the same time, bio-struvite production was observed for all bacteria at pHs between 7.3 to 7.8, with bio-struvite productions between 135-198 mg/L above non-inoculated controls. At pHs >8.3, it was not possible to differentiate between chemical and biological struvite production. It followed that bio-struvite production processes are likely applicable to sludge dewatering liquors with neutral pH.

This study demonstrated for the first time that biological struvite production can occur at a wide range of pHs, hence significantly different from chemical struvite precipitation that occurs at pH >8.3, making it a potentially viable process for phosphorus recovery in the form of struvite from wastewater streams and sludge liquors without strict pH.

Chapter 4 was focused in understanding how the sludge dewatering liquors can be optimised to improve the growth rate of *B. antiquum*. Seven factors were tested at 2 levels in sludge dewatering liquors, for their effect on the growth rate of *B. antiquum* (Tables 4-1 and 4-2). The highest growth rate was observed for the sludge dewatering liquors supplemented with 1000 mg COD/L as acetic acid, 1000 mg COD/L as oleic acid, 6% (w/v) of NaCl, no added calcium (opposite to 100 mg Ca^{2+}/L added), and ammonium at 839 mg NH_4-N/L (opposite to 510 mg NH_4-N/L) (Table 4-4). Phosphate and magnesium did not impact the growth rate of the *B. antiquum* at the concentrations tested. These results indicated that sludge dewatering liquors with more COD, more ammonium, and less calcium can be expected to be better suited for the growth of *B. antiquum*. These results provided the first indication of how the

composition of sludge dewatering liquors impacted the growth rate of *B. antiquum*.

The remaining results of Chapter 4, highlighted that the sludge dewatering liquors COD content, although typically below 300 mg COD/L, was limiting to the growth of *B. antiquum*.

The composition of different types of sludge dewatering liquors was characterised in Chapter 5 (Table 5-3). Three samples of four different types of sludge (primary, secondary, digester feed and digested) were collected in three WWTP with diverse P-removal technologies. A range of parameters were analysed, including: total phosphorus, phosphate, estimation of condensed and organic phosphorus, ammonium, magnesium, COD, total suspended solids, alkalinity, oxidisable nitrogen, nitrite, sulphate, chloride, calcium, iron, aluminium, potassium, boron and a suite of nine heavy metals (arsenic, cadmium, chromium, copper, lead, nickel, mercury, selenium and zinc). Overall, ten different types of sludge dewatering liquors were obtained. Such extensive characterisation of sludge dewatering liquors has not been found in literature, hence these results can provide a reference to frame the characteristics of sludge dewatering liquors in future studies.

The characterisation of the sludge dewatering liquors was used together with information gathered in the review of Chapter 2, and with the results obtained in the Chapters 3, 4 and 5, to make a qualitative assessment of the potential application of the sludge dewatering liquors for bio-struvite production. This assessment considered the knowledge gained previously on the impact of the composition of sludge dewatering liquors on the growth of the bacteria, in particular regarding COD, calcium, and pH. Liquors obtained from digester feed sludge, and from primary sludge in 2 sites were the only liquors with a quantity of COD to provide for carbon source needs of *B. antiquum*. The pH of digested sludge liquors and secondary sludge liquors were the only ones within or close above the optimum range for *B. antiquum* (7.3-7.8), found in Chapter 3. Furthermore, the calcium content of digester feed liquors was above what was found to hamper the growth rate of *B. antiquum*. Together with an assessment

of the phosphorus, magnesium and ammonium contents (which provide the building blocks for bio-struvite formation) it was recommended that blending liquors from primary sludge, and digested sludge should mitigate the carbon source limitation of digested liquors without increasing the calcium content in the combined liquors.

8.4 Impact of the composition of sludge dewatering liquors on the potential amount of bio-struvite

The sludge dewatering liquors used in experimental work throughout this thesis were collected frequently from a full-scale site. The liquors presented significant variability especially for phosphorus and magnesium content. Table 8-1 aggregates the characteristics of the sludge dewatering liquors used in each Chapter, together with information on the amount of bio-struvite produced and bio-struvite productivity based on the experiment incubation time.

Table 8-1 highlights that *B. antiquum* was chosen for further studies after experiments in Chapter 3 when the amount of produced bio-struvite and productivity (99-138 mg/L produced at 9.9-15.3 mg/(L.d)) were lower than those of *H. salinarum* (134-198 mg/L at 14.9-19.8 mg/(L.d)) and *B. pumilus* (131-196 mg/L at 14.8-19.6 mg/(L.d)). *B. antiquum* increased its cell numbers when grown in sludge dewatering liquors whilst both *H. salinarum* and *B. pumilus* only keep viable at a limited range of pHs (7.3-9.1) (Figure 3-2). Additionally, the production rate of *B. antiquum* suggested that bio-struvite was being formed in close correlation with the microbial growth (Figure 3-5). This suggested a biologically controlled mineralization mechanism where the biomineral forming microorganism exerts a high level of control over the biomineralization and where the environmental conditions are expected to impact less on the mineral formation (Mann, 2001). Hence, *B. antiquum* offered a better chance to grow in sludge dewatering liquors under open mixed-culture conditions and a better chance to keep producing bio-struvite under the varying conditions of sludge dewatering liquors.

Table 8-1 Characteristics of the sludge dewatering liquors used and production, and productivity based on incubation time, of bio-struvite obtained in experiments throughout this study.

Chapter	Total phosphorus (mg P/L)	Phosphate (mg P/L)	Ammonium (mg N/L)	Magnesium (mg/L)	COD (mg/L)	pH	Bacteria	Bio-struvite production (mg/L)	Incubation time (d)	Bio-struvite productivity[h] (mg/(L.d))
3	n.d.	45	825	15[d]	455	7.8	*H.s.*	134-198[e]	9-10	14.9-19.8
							B.p.	131-196[e]	9-10	14.6-19.6
							B.a.	99-138[e]	9-10	9.9-15.3
4.1[a]	n.d.	14	839	66	440	8.1	*B.a.*	n.d.[f]	8	n.d.
4.2[a]	n.d.	32	941	n.d.	482	8.0	*B.a.*	n.d.[f]	7	n.d.
4.3[a]	n.d.	38	866	33[d]	479	8.0	*B.a.*	n.d.[f]	7	n.d.
4.4[a]	n.d.	41	n.d.	46	458[d]	7.8	*B.a.*	76-170[g]	6	12.7-28.3
4.5[a], 6[b]	50	40	n.d.	74	500[d]	7.8	*B.a.*	31-76[e]	3+5	3.9-9.5
5	66	50	889	19[d]	308	7.7	*B.a.*	172[g]	3	57.3
7[b]	12	1[c]	n.d.	75	595[d]	7.8	*B.a.*	n.d. [e]	6	n.d.

[a] Experiments 4.1 to 4.5, in Chapter 4, detail the screening experiment, the path of steepest ascent experiment, the central composite design experiment, the one-stage experiment, and the two-stage experiment, respectively.
[b] Bio-struvite production was not quantified.
[c] Phosphate supplemented to 40 mg P/L with potassium phosphate buffer at pH 7.8.
[d] Magnesium supplemented to a molar ratio of 1.1Mg:1PO_4.
[e] Produced bio-struvite was identified with environmental scanning electron microscope (ESEM) and electron dispersive X-ray spectrography (EDX).
[f] Bio-struvite and biomass were not separated.
[g] Bio-struvite quantified by phosphate measurement after the crystals were separated from the liquors using density separation with 2000 g/L sucrose solution and dissolution in 0.05M HCl.
[h] Productivity was calculated as the quantity of bio-struvite divided by the incubation time.
H.s., *Halobacterium salinarum; B.p.*, *Bacillus pumilus; B.a.*, *Brevibacterium antiquum;* n.d., not determined.

Although the phosphate content of the sludge dewatering liquors ranged from 1-50 mg PO_4-P/L, bio-struvite production was quantified when the phosphate content was 40-50 mg PO_4-P/L. There was no clear relationship between phosphate content and amount of bio-struvite produced. Nevertheless, the highest amount of bio-struvite produced was observed when the sludge dewatering liquors had the highest content of phosphate (50 mg PO_4-P/L). This coincided with the highest productivity observed for *B. antiquum* over the incubation period (57.3 mg/L.d) but likely this value was high, compared with the other cases in Table 8-1, because of the short period of incubation (3 days). The productivity after 3 days of incubation, in this experiment of chapter 5, can be compared with the productivity at 3 days for *B. antiquum* in Chapter 3 which was 96.8 mg/L.d (Figure 3-5a). The lowest amount of bio-struvite was obtained with 41 mg PO_4-/L and with magnesium (74 mg/L) making a molar ratio of 2.3 Mg:1 PO_4. The lowest production and lowest productivity occurred in the 2-stage experiment of Chapter 4. Likely, the low amounts of bio-struvite produced relate with a two-stage experimental set-up used where >90% of the volume of sludge dewatering liquors was replaced at the end of stage 1, the produced bio-struvite was normalized to the total volume of sludge dewatering liquors used (in both stages). The low amount of bio-struvite observed suggests the phosphorus uptake for bio-struvite formation only took place in one of the stages.

Magnesium relation with bio-struvite production was not fully assessed because all the experiments done throughout this work were completed with a molar excess of magnesium relative to phosphate. Hence, bio-struvite production under magnesium limiting conditions was not studied. Because phosphorus is considered a pollutant in WWTP, there is a need to capture as much phosphorus as possible into the bio-struvite, which requires at minimum an equimolar amount of magnesium. Magnesium is not typically considered a pollutant in WWTP so supplementing excess magnesium to promote the removal of more phosphorus from the wastewater is a trade-off frequently done in struvite recovery processes. Thus, all experiments done in this work were run

with excess of magnesium in order to obtain the maximum possible amounts of phosphorus in the form of bio-struvite.

Half the samples of sludge dewatering liquors used throughout this work had an original molar amount of magnesium below the molar amount of phosphorus. The experiments using these magnesium-limited samples were supplemented with magnesium and run under excess magnesium conditions at molar ratio of 1.1Mg:1PO_4 in the experiments of Chapters 3, and 4, and of 1.1Mg:1TP in the experiments of Chapter 5.

Comparing the amount of produced bio-struvite obtained in the experiments with magnesium-excess sludge dewatering liquors (which were not supplemented with magnesium) indicates that more magnesium did not lead to more bio-struvite: in experiment 4.4, 76-170 mg bio-struvite/L were produced at 1.5Mg:1PO_4, while in experiment 4.5, 31-76 mg bio-struvite/L were produced at 2.4Mg:1PO_4 (Table 8-1). The experiments with magnesium-limited sludge dewatering liquors, which were then supplemented to run the experiment, produced comparable amounts of bio-struvite as seen in experiment 5, with 172 mg/L produced at 1.1Mg:1TP and 1.45 Mg:PO_4 ratios (Table 8-1).

The pH, ammonium and COD varied between the samples to pH 7.7-8.1, ammonium of 825-941 mg N/L and 302-595 mg COD/L.

The most evident trend in Table 8-1 was the progressive decrease in the incubation times used. This spawned from Chapter 3 where it was observed that bio-struvite production occurred within the first 4 days of incubation. Results in Chapter 4 confirmed that *B. antiquum* was able to reach the stationary phase of growth within 3-5 days for most of the conditions tested. This allowed for a reduction of the incubation time used.

Results in Chapter 4 showed that sludge dewatering liquors had a limited amount of carbon source available, and this limitation was observed to impact the growth rates of *B. antiquum* (Tables 4-4, 4-5, and 4-6). However, the remaining experiments on chapter 4 demonstrate a complex relationship between bio-struvite formation and availability of carbon source. The production

of bio-struvite decreased progressively from 170 to 76 mg/L when supplementing the sludge dewatering liquors with growing amounts of acetate, from 562 to 2248 mg COD/L (Figure 4-2). This implied that carbon availability had a direct effect on bio-struvite production. Moreover, bio-struvite formation was the highest on the two-stage experiment (72 mg/L) when supplementing 281 mg COD/L of acetate in the Stage 2, independently of the availability of carbon source in the Stage 1. On the other side, when no carbon source was added to Stage 2, the amount of bio-struvite produced bio-struvite was higher in the tests that had more carbon source added in the first stage. This complex relationship between growth rate, bio-struvite production and availability of carbon source suggests that biomineralization takes place through a complex biologically controlled mechanism.

The characterisation of different types of sludge dewatering liquors in Chapter 5 showed high organic and condensed phosphorus contents. Up to 276.1, 357.1, and 732.5 mg P/L of organic and condensed phosphorus were found in liquors from digester sludges from each of the studied WWTP (Table 5-3). It should be noted however, that no dewatering additives were used in the preparation of the sludge liquors tested, and that might justify the high amounts of total phosphorus found. Nevertheless, these liquors had phosphate content below what is reported to be necessary for economically viable conventional chemical struvite precipitation processes. Additionally, the organic and condensed phosphorus would not be available for P-recovery in conventional chemical struvite precipitation. The high amounts of organic and condensed phosphorus found represent an impressive opportunity for P-recovery, even if *B. antiquum* only recovered 50% (Figure 5-3). The potential P-recovery with a bio-struvite process on these liquors was higher than 100 mg PO_4-P/L which has been reported to be necessary for the conventional chemical struvite precipitation be economically viable (Dockhorn, 2009).

8.5 Bacteria growth in mixed-conditions

Results in Chapter 4 showed that *B. antiquum* was able to grow at rate of 3.44 1/d in sludge dewatering liquors supplemented with acetate (562-1686 mg COD/L) under sterile conditions (Table 4-4). This rate was compared with growth rates published for known groups of microorganisms in wastewater treatment processes. The growth rate of *B. antiquum* fitted in the middle of the range of values published for heterotrophs in activated sludge treatment processes. These were also comparable with the fastest of the denitrifying microorganisms and were higher than the growth rates of nitrifiers, enhanced biological phosphorus removal microorganisms and one order of magnitude faster than Anammox bacteria (Figure 4-1). These results suggested that *B. antiquum* can compete with other microorganisms found in wastewater.

However, results in Chapter 6 show that the inoculation of *B. antiquum* into a sequencing batch reactor using sludge dewatering liquors using acetate (562 mg COD/L) and high NaCl (3%) as selective pressure, was not successful towards achieving the enrichment of *B. antiquum*. The inoculation of *B. antiquum* into a SBR under open mixed culture conditions did not result in the establishment of a stable bacterial community composed mostly of *B. antiquum* bacteria. *B. antiquum* was found with a relative abundance of 0.9-1.5% at the end of 6 cycles of operation.

Further analysis of the relative abundance of *B. antiquum* and the cell counts in the mixed culture (Figures 6-3 and 6-4) indicated that likely the settled fraction of *B. antiquum* cells fell within the range of 10-62% and the growth rate within the range of 0-1.4 1/d (Table 6-3).

Hence, it was recommended to take measures to increase the retention of *B. antiquum* cells within the SBR and to increase the selective pressure to limit the growth the competing indigenous microorganisms and favour *B. antiquum* growth.

B. antiquum has been described as psychrophile (cold loving) and halotolerant (salt tolerant) (Gavrish *et al.*, 2004) both these extreme conditions can be used

to impose a more aggressive selective pressure on the competing indigenous microorganisms.

Bacterial community profiling in Chapter 6, was done with barcoded pyrosequencing of fragments of the 16S rRNA gene to assess the relative abundance of the bacteria species identified. The results showed that bacteria of the genera *Brevibacterium* were not present in the sludge dewatering liquors indigenous population (<0.01% relative abundance, Figure 6-4). On another side, these results suggest that *Brevibacterium* species are not well adapted to sludge dewatering liquors and that the enrichment *B. antiquum* in might be challenging.

On a positive note, the technique based on amplification of the 16S rRNA gene could be used to show the enrichment of *Brevibacterium* species in sludge dewatering liquors. This is a significant achievement as many other techniques were investigated, without success, during the course of the PhD studies, including: selective plating in Petri dishes, replica plating techniques, modification of *B. antiquum* with P-green fluorescence gene and phospholipid-derived fatty acids (PLFA) analysis.

Further results from the bacterial community profiling of the sludge dewatering liquors, in Chapter 6, identified 6 species of *Bacillus* genera with an overall relative abundance of 0.01% (*B. cereus, B. badius*, *B. weihenstephanensis, Bacillus* sp. *86348*, *Bacillus* sp. *MO-05*, and *Bacillus* sp. *S3-R6TC-BA1*). Other *Bacillus* species have been identified in wastewater samples and then shown to promote mineral formation in synthetic media rich in rich in aminoacids, phosphate and calcium or magnesium (Rivadeneyra Torres *et al.*, 2013; Gonzalez-Martinez *et al.*, 2015). This suggests that *Bacillus pumilus* might be good candidate for enrichment in sludge dewatering liquors, in order to produce bio-struvite.

No bacteria species was identified in the *Halobacterium* genera. As a comparison, only four bacteria genera had a relative abundance above 5%: an uncultured bacteria of the order *Rhizobiales* (10.1%), a bacteria from the genera

Simplicispira (9.78%), a bacteria from the Genera *Proteiniclasticum* (5.79%), and an uncultured bacteria from the genera *Smithella* (5.67%).

8.6 Value of bio-struvite

Results in Chapter 7 showed that bio-struvite and chemically precipitated struvite (chemical struvite) differed in their content of phosphorus and magnesium (Table 7-2). Both types of struvite were obtained from the same sample of sludge dewatering liquors, collected from a full scale WWTP. The bio-struvite was estimated to have 91% (w/w) of struvite crystals, while the chemical struvite had only 50% (w/w). Nevertheless, there was no difference between chemical and bio-struvite in terms of their impact on mustard seedlings growth. The presence of heavy metals, organic non-polar and organic polar micropollutants was found to be similar in both types of struvite, with differences only in the arsenic and mercury content (Table 7-3). The content of micropollutants was below existing and proposed regulations (Tables 7-3, and 7-5). Hence, these results support that bio-struvite should reach, or surpass, the market value of struvite obtained from conventional chemical precipitation struvite processes.

Estimates for the selling price of struvite found in literature ranged from 188-763 EUR/ton (Dockhorn, 2009; Molinos-Senante *et al.*, 2011; Ishii and Boyer, 2015) with the median price of 440 EUR/ton. However, Geerts *et al.*, 2016, reported a struvite selling price of 50-100 EUR/ton, and Kleemann *et al.*, 2015, used an indicative price of 100 EUR/ton. Recently, the selling price for bulk pure struvite, on offer by Jiangxi Rutom (Jiangxi, China), was of 260-300 EUR/ton (Alibaba Group, 2017).

The economic potential of a bio-struvite process will thus be limited by the bio-struvite production costs for which work is still necessary to detail the full requirements.

8.7 Future work

This section summarises the suggestions of further work spanned from the general discussion above.

Extend the work on this project for the remaining selected bacteria, in particular for *B. pumilus* and *H. salinarum*.

Results in Chapter 3 showed that *B. pumilus* and *H. salinarum* were able to induce bio-struvite formation up to 198 mg/L within 1 day. These results hinted at a possible different mechanism of biomineralization, possibly a biologically induced mineralisation (BIM) mechanism. Although BIM is a mechanism where the bacteria have less control over the mineralisation, there is potential for use as a way of triggering struvite formation. Moreover, *Bacillus* species have been said to promote mineralisation of carbonate and phosphate minerals in presence of high concentration of mineral ions, in several studies, and a gene cluster for carbonate biomineralization in *B. subtilus* has been reported (Barabesi *et al.*, 2007; Marvasi *et al.*, 2010; Rivadeneyra Torres *et al.*, 2013; Perito *et al.*, 2014; Gonzalez-Martinez *et al.*, 2015).

Expand on the understanding of the mechanisms of phosphorus uptake and use by *B. antiquum*, and its relation with carbon source availability.

Results in Chapter 4 showed that carbon source availability can impact dramatically the growth rate of *B. antiquum*, and can affect the amount bio-struvite formed by *B. antiquum* (Figures 4-2, and 4-4). *B. antiquum* is an aerobic heterotrophic bacteria, thus uses carbon compounds as a source of carbon and of energy. Understanding the connection between energy and bio-struvite production might prove a biologically controlled mechanism of biomineralization, and explain why the bacteria produces bio-struvite.

Further the understanding of the mechanisms of struvite biomineralization.

The results obtained in Chapter 3 suggested that *B. antiquum* follows a biologically controlled mechanism (BCM) of biomineralization while *H. salinarum* and *B. pumilus* follow a biologically induced mechanism (BIM). BIM does not require a high level of control of the biomineralisation process by the bacteria, while BCM requires that the bacteria actively promote the biomineralization. Understanding if the bio-struvite forming process starts intra- or extra-cellularlly, would provide evidence for which type of mechanism the bacteria use. Following the evolution of the microenvironment pH, inside and outside the cells, in sludge dewatering liquors, can provide the evidence necessary to answer that question. Also, analysing the culture media for the evolution of the particle size distribution can also contribute to elucidate the bio-struvite formation mechanism.

Investigate the operational conditions of mixed culture systems

A first attempt to cultivate *B. antiquum* in a mixed culture system was explored in this work (Chapter 6) but failed to promote the enrichment of *B. antiquum* in sludge dewatering liquors. Investigating the operational conditions necessary to run a mixed culture system as the used SBRs should provide insight of the reaction time, the decant volume, the settling time, and the sludge wasting amount and frequency, in order to achieve the conditions better suited to the enrichment of the bio-struvite bacteria.

Explore the role of food-to-microorganism ratio (F/M) and solids retention time (SRT)

The F/M ratio and SRT are parameters widely used in biological wastewater treatment processes as a handle to gain control over the biological treatment processes. By varying the F/M ratio, and sludge wastage, the available substrate and the SRT can be managed to reach conditions best suited the bacteria. In order for the bio-struvite producing bacteria to outcompete the remaining microbial population whilst producing bio-struvite, the amount of food substrate available and the time the bacteria cells spend inside the reactor, measured by SRT, will have to be better understood.

Identify methods of retaining *B. antiquum* cells within mixed-culture bio-reactors while washing out the competing bacteria with the spent sludge dewatering liquors.

Chapter 6 has highlighted that the fraction of *B. antiquum* cells that remained in the settled volume and were used to the next SBR stage is small when compared with other cases of enrichment. Other studies attempting the enrichment of a bacteria in wastewater under open mixed-culture conditions, used methods with full biomass retention (>90%) (Third *et al.*, 2005), this means that there is a wide margin for improvement that can be studied for *B. antiquum*.

Evaluate the impact of increasing the selective pressure to favour *B. antiquum* growth while limiting the growth of the competing indigenous microorganisms.

Results in Chapter 6, suggested that *B. antiquum* growth rate in open mixed-culture conditions was below what had been observed before in the work detailed in Chapter 4. It was recommended to increase the selective pressure to favour the growth of *B. antiquum* and inhibit the competing microorganisms.

8.8 References

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Chapter 9 Conclusions

The conclusions detailed are presented in respect to the project key objectives.

Objective A: Understand the limitations of sludge dewatering liquors pH variability on the ability of selected bacteria to grow and to produce bio-struvite.

- Sludge dewatering liquors can be used to produce bio-struvite but pH variability can impact the growth of the selected bacteria.
- The selected bacteria were able to grow in sludge dewatering liquors at a range of pHs from 5.7 to 9.1.
- Bio-struvite production was observed at pHs between 7.3 to 7.8, with productions between 135-198 mg/L of bio-struvite, above non-inoculated controls.
- At pHs above 7.3-7.8, chemical precipitation became significant and was not possible to differentiate between chemical and biological struvite production.

Objective B: Elucidate how the sludge dewatering liquors composition influences the growth rate the selected bacteria, and production of bio-struvite.

- The growth rate (μ) of *B. antiquum* in sludge dewatering liquors ranged from 0.93 to 1.21 1/d but this could be increased by 3 fold to 3.44 1/d when supplementing the sludge dewatering liquors with acetate (1124 mg COD/L) and NaCl (3.0% w/v).
- Acetic acid, oleic acid, sodium chloride, and ammonium were shown to have a positive impact on the growth rate of *B. antiquum* with average growth rates of 1.92, 1.77, 1.57, 1.69 1/d, in the tests with high levels, respectively. Calcium was found to have a negative impact (0.95 1/d) on the growth rate of *B. antiquum*.
- However NaCl was found to hinder the mineralization of the bio-struvite at the concentration favourable for growth (3.0% w/v). This implied that

the sludge dewatering liquors can be dosed with acetate and NaCl to improve the growth rate, but the reactor must be operated without NaCl for a proportion of the cycle to ensure precipitation of the bio-struvite.

Objective C: Understand phosphorus availability for the selected bacteria to use and make bio-struvite in sludge dewatering liquors.

- *B. antiquum* was found to be able to use >50% of the organic and condensed phosphorus fractions, to make bio-struvite. This opened the opportunity to recover two phosphorus fractions that were not available for recovery before.
- Different types of sludge liquors were found to frequently have a high content of organic and condensed phosphorus. Up to 276.1, 357.1, and 732.5 mg P/L in liquors from digester sludges from each of the WWTP used.
- The modified acid-hydrolysis method was not suitable to obtain a reliable and separated quantification of the organic and condensed phosphorus, instead the difference between total phosphorus and phosphate (TP - PO_4-P) can be used to quantify the aggregate of organic and condensed phosphorus.
- However, none the liquors were found to have all the conditions suitable for bio-struvite formation. Combining primary sludge with digested sludge was suggested to mitigate carbon source limitation without increasing the calcium content of the combined liquors. Managing the pH of the liquors to suit *B. antiquum* growth remained the most challenging factor to control.
- Bio-struvite produced with *B. antiquum* enabled the recovery of phosphorus that before were not available for recovery. This widened the range of sludge dewatering liquors that can potentially be used for phosphorus recovery.

Objective D: Gain insight on how to establish a stable culture of selected bio-struvite producing bacteria under open mixed-culture conditions.

- A onetime inoculation of *B. antiquum* into a sequencing batch reactor cycling sludge dewatering liquors with acetate and NaCl, followed by fresh sludge dewatering liquors 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* bacteria.
- Bacterial community profiling showed that *Brevibacterium* species were not present in the sludge dewatering liquors indigenous microbial population (<0.01% of relative abundance). This confirmed that techniques based on amplification of the 16S rRNA gene can be used to show the enrichment of *Brevibacterium* species in sludge dewatering liquors.
- The growth rate of *B. antiquum* was estimated to be <1.4 1/d. Hence, the growth rate observed did not reach the growth rate observed previously in sterile conditions (3.4 1/d).
- The settling conditions tested were estimated to retain less than 62% of the *B. antiquum* cells in the sequencing batch reactors.

Objective E: Understand and quantify the fertilizer value of bio-struvite produced from sludge dewatering liquors.

- No difference was found between synthetic struvite, bio-struvite and chemical struvite in terms of their impact on mustard seedlings growth.
- The presence of heavy metals, and organic non-polar and polar micropollutants was found to be similar in both types of struvite produced from sludge dewatering liquors collected in a full scale WWTP.

- Polar organic micropollutants were not found in re-dissolved chemical or bio-struvite, and poly-aromatic hydrocarbons were present at $<3.7\times10^{1} \pm 1.2\times10_{1}$ µg/kg, which is below existing and proposed regulations.
- Bio-struvite was found to at least reach the same market value as conventional chemically precipitated struvite which highlighted the potential economical viability of a bio-struvite process, for the recovery of phosphorus from wastewater.

APPENDICES

Appendix A Measurement of dissolved CO_2 in the sludge dewatering liquors to assess pH variability

In order to assess if high dissolved CO_2 concentrations were responsible for pH variability, tests were completed to measure the dissolved CO_2 in the studied sludge dewatering liquors.

A.1 Materials and methods

A.1.1 Degassing of CO_2 from sludge dewatering liquors

The amount of CO_2 available in the sludge dewatering liquors to be released as a gas was estimated according to the methodology described by Hafner and Bisogni, 2007. Twenty millilitres of sludge dewatering liquors were placed in serum bottles and hydrochloric acid 2 M was added at 4 different levels: no acid, 500 µL, 700 µL, and 1000 µL. After acid addition the sludge dewatering liquors were autoclaved in closed and open serum bottles and these were compared with serum bottles kept static outside the autoclave. After 4 hours, the pH and the CO_2 fraction of the headspace of closed serum bottles were measured in a CSi 200 series gas chromatograph setup as described by McLeod, Jefferson and McAdam (2013). All tests were completed in triplicate.

A.1.2 Release of CO_2 during post anaerobic digestion treatment

To assess the impact of post anaerobic digestion treatment on the amount of CO_2 release from the digestate and corresponding changes in pH, two column tests were setup to simulate the conditions in aerated and non-aerated storage. Digestate was collected from three anaerobic digesters treating primary and BNR sludge and mixed in equal proportions. The digestate was placed in two open Perspex plastic cylindrical columns (0.96 m x 0.10 m). One of the columns was left standing for 22 days to simulate the post anaerobic digestion storage conditions of the full-scale WWTP sampled. In the second column the digestate

was aerated at 0.3 L/min compressed air for 20 days simulating aerated post anaerobic digestion storage conditions predominant in other WWTPs. Digestate samples were collected after 11, 20 and 22 days for the non-aerated column and after 2, 11 and 20 days for the aerated column. Twenty millilitres of sample were placed in serum bottles, the pH and the dissolved CO_2 were also measured.

Sterilization of the sludge dewatering liquors the pH increased from 7.8 to 9.8 (Table A-1). Filter sterilisation was also tested and although this method led to a more moderate pH change (from 7.8 to 8.5), after inoculation with the selected bacteria and incubation by agitation, the pH raised to 9.4 ± 0.1.

A.2 Results

Sterilization of the sludge dewatering liquors the pH increased from 7.8 to 9.8 (Table A-1). Filter sterilisation was also tested and although this method led to a more moderate pH change (from 7.8 to 8.5).

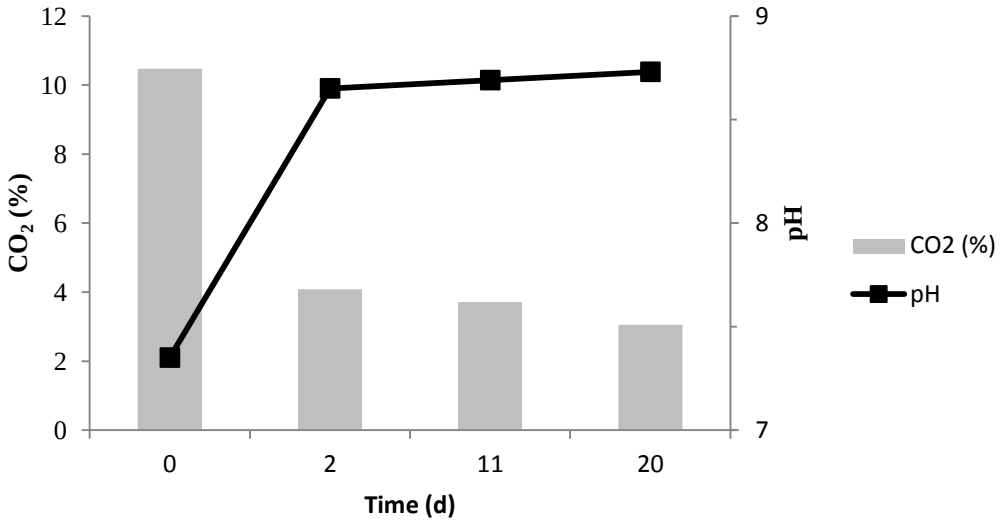
Table A-1 Characteristics of the sludge dewatering liquors collected from a full scale site before and after sterilization treatments.

	pH	**COD** (mg/L)	**NH_4** (mg N/L)	**PO_4** (mg P/L)	**Mg^{2+}** (mg/L)
Sludge dewatering liquors collected from full-scale site					
	7.8	455 ± 3	825 ± 66	44.5 ± 2	15.2
Sludge dewatering liquors after processing					
Centrifugation	8.3	327 ± 4	799 ± 14	27 ± 1	9.6
Sterilised by autoclave	9.7	449 ± 11	516 ± 6	58 ± 0	–
Sterilised by filtration	8.5	–	736 ± 11	23 ± 1	2.1

After 22 days the digestate kept in anaerobic conditions had a pH of 8.3 and released enough CO_2 to enrich the headspace to 4.7% CO_2, whilst the digestate from the aerated column released 4.1% CO_2 with a pH of 8.7 from day 2 onwards (Figure A-1). Results show that the potential for CO_2 release was higher in the un-aerated test (Figure A-1).

These results indicate that post anaerobic digestion treatment is a relevant factor for the implementation of a bio-struvite process and pH of the sludge dewatering liquors.

(a)



(b)

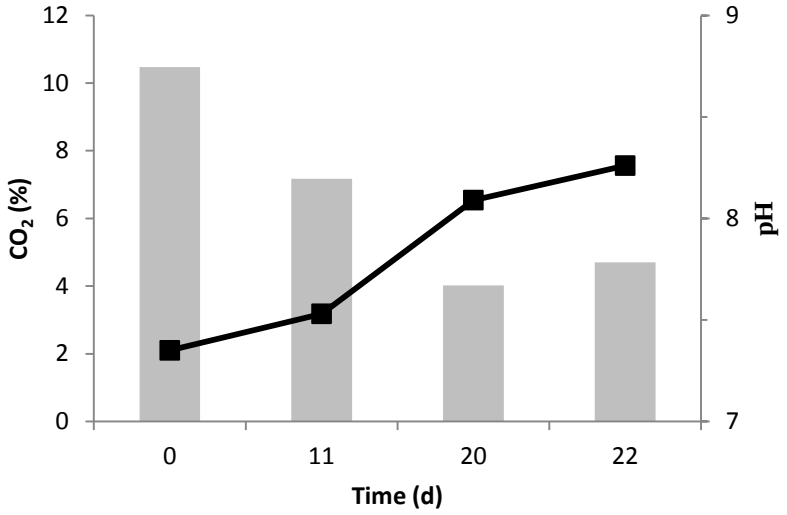


Figure A-1 Anaerobic digestate pH and released CO_2 fraction for two distinct post anaerobic-digestion treatments: a) aerated; b) non-aerated.

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Appendix B List of substances screened from samples of dissolved of bio-struvite, chemical struvite and synthetic struvite with a QTOF system

Table B-1 List of substances screened with a QTOF system and respective elemental formulas. Includes 196 individual substances and polyaspartic and polyacrylic acids polymeric families.

Name	Elemental formula
1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran	$C_{18}H_{26}O$
17α-estradiol	$C_{18}H_{24}O_2$
17α-ethinyl oestradiol	$C_{20}H_{24}O_2$
17α-trenbolone	$C_{18}H_{22}O_2$
2-ethyl-hexyl-4-trimethoxycinnamate	$C_{18}H_{26}O_3$
4-methyl-benzilidine-camphor	$C_{18}H_{22}O$
Acetaminophen	$C_8H_9NO_2$
Acetazolamide	$C_4H_6N_4O_3S_2$
Acetohexamide	$C_{15}H_{20}N_2O_4S$
Acetylsalicylic acid	$C_9H_8O_4$
Aminomethyl phosphonic acid	CH_6NO_3P
Amoxicillin	$C_{16}H_{19}N_3O_5S$
Ampicillin	$C_{16}H_{19}N_3O_4S$
Amprenavir	$C_{25}H_{35}N_3O_6S$
Androstenedione	$C_{19}H_{26}O_2$
Apricoxib	$C_{19}H_{20}N_2O_3S$
Atenolol	$C_{14}H_{22}N_2O_3$
Azamethiphos	$C_9H_{10}ClN_2O_5PS$

Name	Elemental formula
Azinphos-methyl	$C_{10}H_{12}PN_3S_2O_3$
Azithromycin	$C_{38}H_{72}N_2O_{12}$
Balofloxacin	$C_{20}H_{24}N_3O_4F$
BDE-100	$C_{12}H_5Br_5O$
BDE-153	$C_{12}H_4Br_6O$
BDE-28	$C_{12}H_7Br_3O$
BDE-47	$C_{12}H_6Br_4O$
Bentazone	$C_{10}H_{12}N_2O_3S$
Benzophenone	$C_{13}H_{10}O$
Benzophenone-3	$C_{14}H_{12}O_3$
Benzyl butyl phthalate	$C_{19}H_{20}O_4$
Bezafibrate	$C_{19}H_{20}ClNO_4$
Biochanin A	$C_{16}H_{12}O_5$
Bis(2-ethylhexyl) adipate	$C_{22}H_{42}O_4$
Bisphenol-A	$C_{15}H_{16}O_2$
Bosentan	$C_{27}H_{29}N_5O_6S$
Bumetanide	$C_{17}H_{20}N_2O_5S$
Caffeine	$C_8H_{10}N_4O_2$
Carbamazepine	$C_{15}H_{12}N_2O$
Carbutamide	$C_{11}H_{17}N_3O_3S$
Celecoxib	$C_{17}H_{14}N_3O_2SF_3$
Celestolide	$C_{17}H_{24}O$
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$
Chlorpropamide	$C_{10}H_{13}N_2O_3SCl$
Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$

Name	Elemental formula
Chlorthalidone	$C_{14}H_{11}N_2O_4SCl$
Cinoxacin	$C_{12}H_{10}N_2O_5$
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$
Clarithromycin	$C_{38}H_{69}NO_{13}$
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$
Clinafloxacin	$C_{17}H_{17}N_3O_3ClF$
Clindamycin	$C_{18}H_{33}ClN_2O_5S$
Clofibric acid	$C_{10}H_{11}ClO_3$
Clopamide	$C_{14}H_{20}N_3O_3SCl$
Coumestrol	$C_{15}H_8O_5$
Daidzein	$C_{15}H_{10}O_4$
Danofloxacin	$C_{19}H_{20}N_3O_3F$
Darunavir	$C_{27}H_{37}N_3O_7S$
Dasabuvir	$C_{26}H_{27}N_3O_5S$
DEET	$C_{12}H_{17}NO$
Delafloxacin	$C_{18}H_{12}N_4O_4ClF_3$
Delavirdine	$C_{22}H_{28}N_6O_3S$
Demeclocycline	$C_{21}H_{21}ClN_2O_8$
Di-2-ethylhexyl phthalate	$C_{24}H_{38}O_4$
Diazinon	$C_{12}H_{21}N_2O_3PS$
Dibutyl phthalate	$C_{16}H_{22}O_4$
Dichlorvos	$C_4H_7Cl_2O_4P$
Diclofenac	$C_{14}H_{11}Cl_2NO_2$
Diethyl phthalate	$C_{12}H_{14}O_4$
Difloxacin	$C_{21}H_{19}N_3O_3F_2$
Diisodecyl phthalate	$C_{28}H_{46}O_4$

Name	Elemental formula
Diisononyl phthalate	$C_{26}H_{42}O_4$
Diltiazem	$C_{22}H_{26}N_2O_4S$
Dimethyl phthalate	$C_{10}H_{10}O_4$
Dronedarone	$C_{31}H_{44}N_2O_5S$
EDTA	$C_{10}H_{16}N_2O_8$
Enoxacin	$C_{15}H_{17}N_4O_3F$
Enrofloxacin	$C_{19}H_{22}FN_3O_3$
Enrofloxacin	$C_{19}H_{22}N_3O_3F$
Equilin	$C_{18}H_{20}O_2$
Erythromycin	$C_{37}H_{67}NO_{13}$
Estriol	$C_{18}H_{24}O_3$
Ethoxzolamide	$C_9H_{10}N_2O_3S_2$
Fenitrothion	$C_9H_{12}NO_5PS$
Fenoprofen	$C_{15}H_{14}O_3$
Fleroxacin	$C_{17}H_{18}N_3O_3F_3$
Flucloxacillin	$C_{19}H_{17}ClFN_3O_5S$
Fluoxetine	$C_{17}H_{18}F_3NO$
Formononetin	$C_{16}H_{12}O_4$
Fosamprenavir	$C_{25}H_{36}N_3O_9PS$
Furosemide	$C_{12}H_{11}ClN_2O_5S$
Furosemide	$C_{12}H_{11}N_2O_5SCl$
Gatifloxacin	$C_{19}H_{22}N_3O_4F$
Gemifloxacin	$C_{18}H_{20}N_5O_4F$
Genistein	$C_{15}H_{10}O_5$
Glibenclamide	$C_{23}H_{28}N_3O_5SCl$
Glibornuride	$C_{18}H_{26}N_2O_4S$

Name	Elemental formula
Gliclazide	$C_{15}H_{21}N_3O_3S$
Glimepiride	$C_{24}H_{34}N_4O_5S$
Glipizide	$C_{21}H_{27}N_5O_4S$
Gliquidone	$C_{27}H_{33}N_3O_6S$
Glisoxepide	$C_{20}H_{27}N_5O_5S$
Glyclopyramide	$C_{11}H_{14}N_3O_3SCl$
Glyphosate	$C_3H_8NO_5P$
Grazoprevir	$C_{38}H_{50}N_6O_9S$
Hydrochlorothiazide	$C_7H_8N_3O_4S_2Cl$
Ibafloxacin	$C_{15}H_{14}NO_3F$
Ibuprofen	$C_{13}H_{18}O_2$
Ibutilide	$C_{20}H_{36}N_2O_3S$
Indapamide	$C_{16}H_{16}N_3O_3SCl$
Indometacin	$C_{19}H_{16}ClNO_4$
Ketoprofen	$C_{16}H_{14}O_3$
Levofloxacin	$C_{18}H_{20}N_3O_4F$
Lincomycin	$C_{18}H_{34}N_2O_6S$
Lomefloxacin	$C_{17}H_{19}N_3O_3F_2$
Mafenide	$C_7H_{10}N_2O_2S$
Malathion	$C_{10}H_{19}O_6PS_2$
Marbofloxacin	$C_{17}H_{19}N_4O_4F$
Mefruside	$C_{13}H_{19}N_2O_5S_2Cl$
Methyl parathion	$C_8H_{10}NO_5PS$
Methyltriclosan	$C_{13}H_9Cl_3O_2$
Moxifloxacin	$C_{21}H_{24}N_3O_4F$
Musk ketone	$C_{14}H_{18}N_2O_5$

Name	Elemental formula
Musk xylene	$C_{12}H_{15}N_3O_6$
Nadifloxacin	$C_{19}H_{21}N_2O_4F$
Naproxen	$C_{14}H_{14}O_3$
Nemonoxacin	$C_{20}H_{25}N_3O_4$
Nonylphenol	$C_{15}H_{24}O$
Nonylphenol diethoxylate	$C_{19}H_{32}O_3$
Nonylphenol monoethoxylate	$C_{17}H_{28}O_2$
Nonylphenol triethoxylate	$C_{21}H_{36}O_4$
Norethindrone	$C_{20}H_{26}O_2$
Norfloxacin	$C_{16}H_{18}FN_3O_3$
Octocrylene	$C_{24}H_{27}NO_2$
Octylphenol	$C_{14}H_{22}O$
Ofloxacin	$C_{18}H_{20}FN_3O_4$
Oleandomycin	$C_{35}H_{61}NO_{12}$
Orbifloxacin	$C_{19}H_{20}N_3O_3F_3$
Oxolinic	$C_{13}H_{11}NO_5$
Oxytetracycline	$C_{22}H_{24}N_2O_9$
Parathion	$C_{10}H_{14}NO_5PS$
Parecoxib	$C_{19}H_{18}N_2O_4S$
Paritaprevir	$C_{40}H_{43}N_7O_7S$
Pazufloxacin	$C_{16}H_{15}N_2O_4F$
Pefloxacin	$C_{17}H_{20}N_3O_3F$
Penicillin	$C_9H_{11}N_2O_4S$
Phenobarbital	$C_{12}H_{12}N_2O_3$
Phosmet	$C_{11}H_{12}NO_4PS_2$
Pipemidic	$C_{14}H_{17}N_5O_3$

Name	Elemental formula
Piperacillin	$C_{23}H_{27}N_5O_7S$
Piromidic	$C_{14}H_{16}N_4O_3$
Polyacrylic acid (n = 1 to100)	$(C_3H_4O_2)_n$
Polyaspartic acid (n = 1 to100)	$(C_4H_5NO_4)_nH_2$
Progesterone	$C_{21}H_{30}O_2$
Propranolol	$C_{16}H_{21}NO_2$
Propyphenazone	$C_{14}H_{18}N_2O$
Prulifloxacin	$C_{21}H_{20}N_3O_6SF$
Rosoxacin	$C_{17}H_{14}N_2O_3$
Roxithromycin	$C_{41}H_{76}N_2O_{15}$
Rufloxacin	$C_{17}H_{18}N_3O_3SF$
Salbutamol	$C_{13}H_{21}NO_3$
Sarafloxacin	$C_{20}H_{17}N_3O_3F_2$
Simeprevir	$C_{38}H_{47}N_5O_7S_2$
Sitafloxacin	$C_{19}H_{18}N_3O_3ClF_2$
Sparfloxacin	$C_{19}H_{22}N_4O_3F_2$
Spiramicin	$C_{43}H_{74}N_2O_{14}$
Sulfacetamide	$C_8H_{10}N_2O_3S$
Sulfadiazine	$C_{10}H_{10}N_4O_2S$
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$
Sulfadimidin	$C_{12}H_{14}N_4O_2S$
Sulfafurazole	$C_{11}H_{13}N_3O_3S$
Sulfamethizol	$C_9H_{10}N_4O_2S_2$
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$
Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$
Sultiame	$C_{10}H_{14}N_2O_4S_2$

Name	Elemental formula
Tamoxifen	$C_{26}H_{29}NO$
Temafloxacin	$C_{21}H_{18}N_3O_3F_3$
Testosterone	$C_{19}H_{28}O_2$
Tetrachlorvinphos	$C_{10}H_9Cl_4O_4P$
Tetracycline	$C_{22}H_{24}N_2O_8$
Tipranavir	$C_{31}H_{33}N_2O_5SF_3$
Tolazamide	$C_{14}H_{21}N_3O_3S$
Tolbutamide	$C_{12}H_{18}N_2O_3S$
Topiramate	$C_{12}H_{21}NO_8S$
Tosufloxacin	$C_{19}H_{15}N_4O_3F_3$
Tributyl tin oxide	$C_{24}H_{54}OSn_2$
Triclocarban	$C_{13}H_9Cl_3N_2O$
Triclosan	$C_{12}H_7Cl_3O_2$
Trimethoprim	$C_{14}H_{18}N_4O_3$
Tris-(2-chloroethyl)-phosphate	$C_6H_{12}Cl_3O_4P$
Tris-(chloropropyl)-phosphate	$C_9H_{18}Cl_3O_4P$
Tris-(dichloropropyl)-phosphate	$C_9H_{15}Cl_6O_4P$
Trovafloxacin	$C_{20}H_{15}N_4O_3F_3$
Tylosin	$C_{46}H_{77}NO_{17}$
Valproic acid	$C_8H_{16}O_2$
Vancomycin	$C_{66}H_{75}N_9O_{24}Cl_2$
Xipamide	$C_{15}H_{15}N_2O_4SCl$
Zabofloxacin	$C_{19}H_{20}N_5O_4F$
Zonisamide	$C_8H_8N_2O_3S$