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Microbial phosphorus removal and recovery by struvite biomineralisation in comparison to chemical struvite precipitation in municipal wastewater

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ABSTRACT

Microbial biomineralisation is attracting significant interest as an innovative process to recover nutrients from wastes. Nevertheless, little is understood about the requirements to form struvite using biological pathways in wastewater and how does this compare with conventional chemical processes. To address this gap, *Halobacterium salinarum*, *Bacillus pumilus*, *Brevibacterium antiquum*, *Myxococcus xanthus* and *Idiomarina loihiensis* were grown in wastewater to explore the relationships between cell growth, nutrients levels and properties of the recovered precipitates. The microorganisms were capable of removing ortho-phosphate (PO_4-P) from municipal wastewater at concentrations ranging from 5.4 to 62.4 mg PO_4-P/L. Visible crystals of biological struvite (bio-struvite) (identified by morphology XRD and elemental analysis), were observed at PO_4-P $\geq$ 19.7 mg/L, compared to chemical struvite precipitation at 62.4 mg/L PO_4-P (with pH adjustment). The initial nutrient concentrations presented a strong positive correlation with bio-struvite production yields and crystal size distribution. *B. antiquum* distinguished itself by relatively stable PO_4-P removal (68–97%) independent of the initial nutrient concentration, with effluents containing as low as 1 mg PO_4-P/L. The recovered bio-struvite presented high purity with low heavy metal contents, meeting regulations for inorganic fertiliser. The microbial processes for phosphorus (P) recovery as bio-struvite presented several key advantages: bio-struvite crystals were released to the wastewater and recoverable by filtration at PO4-P $\geq$ 19.7 mg/L, no need to adjust pH, bio-struvite crystals had purity equivalent to 11.8–12.3% P and low heavy metal content, which was similar or better than that of chemical struvite (12.6% P). This study validates bio-struvite's relevance for low nutrient concentrations.

1. Introduction

P is an essential element to living organisms, and fertilisers containing phosphorus (P) are widely used in agriculture industry. The price of commercial inorganic P fertiliser has kept on increasing due to the world-widely variability of phosphate rock reserves [1]. On the other hand, to meet more stringent legislation regarding P discharges (e.g. European Urban Wastewater Treatment Directive, Water Framework Directive), the improvement of wastewater treatment plants (WWTPs) to remove P is imperative in many countries [2]. Processes that enable struvite (magnesium ammonium phosphate hexahydrate-$MgNH_4PO_4.6H_2O$) production therefore have gained considerable attention from the water industry due to their capability to remove and recover P from wastewater streams [3]. Furthermore, owing to its high content of P and nitrogen (N), low degree of impurities and excellent slow-release property [4], struvite has been recognized as a good alternative to most P-rich fertilisers on market.

Chemical struvite is already a well-known way to recover P from wastewater as value-added product [3]. Supersaturation is the main driving force of struvite precipitation: ortho-phosphate (PO_4^{3-}) and ammonium (NH_4^+) integrated with Mg^{2+} within a proper pH range to form struvite when supersaturation condition in waste streams was achieved. A minimum of 100 mg PO_4-P/L was reported to be required for readily spontaneous precipitation of struvite [5], which was 10 times higher than typical values of raw municipal wastewater [6]. It has been proposed that P recovery via struvite is only economically viable at P concentrations above 50 mg/L, when promoted by pH adjustment and seeding for crystal nucleation [3,7]. Yet the potential P recovery from WWTPs via struvite was quantified at a maximum of 30%, in relation to the P available in raw wastewater [6]. The requirement of high P concentration limited chemical struvite application at WWTPs because only P-rich waste streams (e.g. supernatant from anaerobic digester, centrate from sludge dewatering centrifuge) were economically rewarding [8,9]. Meanwhile, chemical usage for pH adjustment may lead to

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environmental issue that minimised the potential benefits of struvite as fertilizer.

Biological struvite (i.e., bio-struvite) has been proposed as an alternative way to recover P from wastewater. A wide spectrum of microbial strains have been reported to alter solution chemistry (e.g. pH, NH_4^+) by metabolic activities, promoting supersaturation conditions to form struvite [10]. The crystallization process of bio-struvite was also linked with electrostatic interactions between cations (e.g. Mg^{2+}, NH_4^+) in solution and microbial cells/ extracellular substance secretions (e.g. extracellular polymeric substances) with anionic nature [11,12], and between molecular structure of the crystal surface frameworks and specific substances (e.g. polysaccharides) secreted by microorganisms [13]. Specific microorganisms (e.g. *Brevibacterium antiquum, Paramecium tetraurelia*) were reported to absorb nutrients into cells for nucleation of intracellular struvite that was then assembled within cells or secreted by cells, where specific regulations (e.g. controlled chemical composition) in isolated compartments (e.g. membrane-bound lipid vesicles) applied [14–16]. Compared with conventional chemical precipitation, struvite biomineralisation involved various roles of microorganisms to facilitate crystallization process and to exert control over biomineral product (e.g., morphology, nucleation site), whereby it has several advantages such as no pH-adjustment and recovering P at low concentrations (e.g. 7.5 mg PO_4-P/L) [17]. Bio-struvite production in wastewater were observed up to 21.5 mg P as bio-struvite/L in sludge dewatering liquor, by pure cultures of *B. antiquum* [18].

However, the benefits of bio-struvite application to WWTPs in comparison with standard chemical struvite precipitation, and the quality (e.g., purity, heavy metal contents) of bio-struvite recovered from wastewater are still poorly documented. This study aims to further investigate the importance of nutrient concentrations, including low PO_4-P, on microbial capability of P removal and recovery from wastewater, and on bio-struvite production and crystal size distribution, along with an assessment of purity and heavy metal content of the recovered bio-struvite in comparison with its chemical counterparts to reveal a potential application of struvite biomineralisation at WWTPs.

2. Material and methods

2.1. Materials

The five microbial strains investigated in this study were wild-type strains isolated from soil or sea water, and were purchased from commercial culture collections: *Halobacterium salinarum* (DSM 671, German Resource Centre for Biological Material, Germany), *Bacillus pumilus* (GB43, LGC Standards, Middlesex, UK), *Brevibacterium antiquum* (DSM 21545, German Resource Centre for Biological Material, Germany), *Myxococcus xanthus* (CECT 422, Spanish Type Culture Collection, University of Valencia, Paterna, Spain) and *Idiomarina loihiensis* (MAH1/ CECT 5996, Spanish Type Culture Collection, University of Valencia, Paterna, Spain). All the microorganisms selected in this study have been proven to form bio-struvite in streams (e.g. synthetic solution, seawater, wastewater) [17,19].

Municipal wastewater was collected from outlet of primary lamella clarifiers of a municipal WWTP with 2840 population equivalent (PE) (Cranfield, UK). The wastewater was filtered by a 10 μm nylon-mesh filter (Plastok, UK), followed by microfiber (equivalent to 0.7 μm), and finally filtered sterilised (0.22 μm PVDF membrane, EMD Millipore, UK). Filter sterilised (0.22 μm) concentrated solutions of magnesium sulphate heptahydrate ($MgSO_4$•7 H_2O), di-potassium hydrogen phosphate (K_2HPO_4), ammonium sulphate ($(NH_4)_2SO_4$) were supplemented together with bovine serum albumin (BSA) [20] as nutrient resources. Different concentrations of PO_4-P (5.4–62.4 mg/L, 4-levels) and NH_4-N (35–332 mg/L, 3-levels) were applied to the filter-sterilised wastewater (Table 1) to explore the significance of initial nutrient concentrations on the microbial capability for P removal and recovery. The concentrations of PO_4-P, NH_4-N, Mg^{2+}, K^+ and BSA were selected based on properties of wastewater reported suitable for bio-struvite formation and eligible wastewater sources at WWTPs [17,21].

2.2. Microbial cultivation

Starter cultures were grown in 250 mL E-flasks containing 100 mL yeast extract solution of 4 g/L (additional 1% w/v NaCl and 1 g/L Mg^{2+} to grow *I. loihiensis*), incubated on an orbital shaker (Stuart model SSL1, Fisher Scientific, UK) at 150 RPM at room temperature (21–24 °C) for 96 h. For inoculation in wastewater, the starter cultures were centrifuged (Sanyo MSE Falcon 6/300 centrifuge, 2400 RCF, 4 °C, 10 min) and washed with sterile 0.9% w/v NaCl solution. The pure microorganism pellets were re-suspended in sterile wastewater and inoculated in 40 mL wastewater in 100 mL glass bottles (Pyrex, Fisher Scientific, UK). Additional 0.8% w/v NaCl was added to *I. loihiensis* culture to ensure its growth [19]. The bottles were capped with foam stoppers and incubated on orbital shaker at 150 RPM at room temperature for 196 h. The samples were taken at the start and at the end of incubation time. All tests were completed in duplicate and controls were maintained under identical conditions but without inoculation.

Table 1
Characteristics of the raw wastewater collected from a municipal WWTP and the wastewater with different concentrations of NH_4-N and PO_4-P (average ± standard deviation of duplicates) used to investigated struvite precipitation.

		Molar ratio [PO_4-P]:[Mg]:[NH_4-N]	Saturation Index of struvite	PO_4-P (mg/L)	NH_4-N (mg/L)	pH	Mg^{2+} (mg/L)	Ca^{2+} (mg/L)	K^+ (mg/L)	SCOD (mg/L)
Raw wastewater		1:2:14	-1.66	5.4 ± 0.3	35.0 ± 0.6	7.8 ± 0.1	8.2 ± 0.5	39.0 ± 0.6	21.0 ± 0.2	150 ± 5
Wastewater with different molar ratio [PO_4-P]:[Mg]:[NH_4-N]	①	1:13:14	-1.04	5.4	35	7.8–8.0	56	39	93	750[a]
	②	1:4:4	-0.43	19.7						
	③	1:2:2	-0.17	33.9						
	④	1:1:1	0.12	62.4						
	⑤	1:13:74	-0.35	5.4	180	7.8–7.9				
	⑥	1:4:20	0.21	19.7						
	⑦	1:2:12	0.44	33.9						
	⑧	1:1:6	0.71	62.4						
	⑨	1:13:136	-0.2	5.4	332	7.8–7.9				
	⑩	1:4:37	0.35	19.7						
	⑪	1:2:22	0.61	33.9						
	⑫	1:1:12	0.84	62.4						

[a] containing 0.5 g/L BSA, equivalent to 600 mg/L SCOD

2.3. Biological and chemical struvite preparation, isolation, purification and identification

An optical microscope (Division of GT vision Ltd., UK) was used for crystal morphology in fresh culture at the end of 196 h incubation. To prepare enough bio-struvite for identification, additional 4 L sterile wastewater with initial PO_4-P of 33.9 mg/L and NH_4-N of 332 mg/L was applied for each microbial strain under the identical condition (the same as described in Section 2.2) for 196 h incubation. At the end of incubation period, precipitants were separated from the liquid by filtration (10 μm nylon-mesh filter, Plastok, UK) and washed with deionized (DI) water twice, passed for air-dry at 37 °C for 4 h, and finally weighed to determine the yield. Only the extracellular biological precipitants and chemical precipitants more than 10 μm were collected for determination and further analysis.

Chemical struvite was prepared in filter-sterilised wastewater the same source as for bio-struvite: 100 mL solution containing 0.2 M $NH_4H_2PO_4$ and 0.001 M K_2HPO_4 was added to 200 mL solution of 0.05 M $MgSO_4 7\ H_2O$; both solutions were pre-adjusted to pH 9 by 1 M sodium hydroxide (NaOH) and mixed in a glass bottle (1 L, Duran™, Fisher Scientific, UK) [20]. The mixture was agitated at 150 RPM at room temperature for 24 h for chemical struvite precipitation, followed by filtration and purification that was the same as for bio-struvite.

The solid phases of purified biological and chemical precipitants were characterised by scanning electron microscope equipped with energy dispersive X-ray spectroscopy (SEM-EDS, XL 30 SFEG, Phillips, The Netherlands) where samples were placed on carbon tabs and sputter coated with gold. The X-ray powder diffractometer (XRD, D5000, Siemens/Bruker, Germany) analysis of precipitants were performed in the range of 20–90° with a step size of 0.04° and a step time of 1 s. Diffraction data were analysed by DIFFRAC.SUITE™ EVA software (Bruker AXS GmbH, Germany) and database from the International Centre for Diffraction Data (ICDD®). The chemical characterisation of both biological and chemical precipitants, including PO_4-P, NH_4-N, Mg^{2+}, Ca^{2+}, K^+, Al^{3+} and heavy metals, was investigated by SEM-EDS or crystal dissolution of 1.25 g/L (prepared in extra pure water pre-adjusted to pH 2 by 1 M hydrogen chloride).

2.4. Analytical methods

The intact cell counts in microbial culture were estimated with a flow cytometer (BD Accuri C6, BD Biosciences, US) analyses using SYBR Green I (SG) - propodium iodide (PI) co-staining method [22]. The flow cytometer was also used to distinguish inorganic nanoparticles from cells by SG staining method. Assuming there was no free deoxyribonucleic or ribonucleic acid in microbial cultures, the inorganic nanoparticles counts were estimated by using total nanoparticle counts minus total cell counts. The concentrations of PO_4^{3-} and NH_4^+ were analysed using a Smartchem (Smartchem200, AMS/Alliance Instruments, France) according to the manufacturer's instructions (Labmedics, Abingdon, UK). Magnesium and calcium (Ca^{2+}) were analysed by atomic absorption spectroscopy (AAS, Analyst 800, PerkinElmer, UK) equipped with flaming and electrothermal spectrometer. Aluminium (Al), potassium (K) and heavy metals including chromium (Cr), iron (Fe), nickel (Ni), copper (Cu), arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) were analysed by inductively coupled plasma mass spectrometry system (ICP-MS, NexION 350, PerkinElmer, UK). The pH was determined by digital pH-meter (Jenway 3540, Bibby Scientific, UK). Soluble chemical oxygen demand (SCOD) was analysed by Spectroquant® cell test kit (Merck, VWR, UK). To avoid loss of microbial cells and fine particles (<10 μm) in filtration (for determination of crystal production yield), that may affect the subsequent analysis of particle size distribution, qualitative assessment (QA) of crystal production was applied for each test. It was completed by transferring the 40 mL wastewater and crystal precipitates to a 500 mL glass beaker, stirring the wastewater clockwise to allow crystals settled at the bottom centre of the beaker, and photographs were taken. This was followed by volume-based particle size distribution (D_v) analysis using a Mastersizer (Malvern 3000, Malvern Instruments Ltd, UK) with a Hydro EV dispersion unit (stirring speed of 1200 RPM). A computer application, Visual MINTEQ ver. 3.1 [23], was used to quantify the saturation index of struvite ($SI_{struvite}$) as an indicator of struvite recovery potential. This application is based on the thermodynamic equilibrium consists of Mg^{2+}, Ca^{2+}, PO_4^{3-}, NH_4^+, etc. with a solubility product constant (K_{sp}) of $10^{-13.26}$ [3]. The $SI_{struvite}$ values determined by the investigated initial nutrient levels in wastewater were presented in Table 1.

2.5. Statistical analysis

The statistically significant difference in terms of P removal efficiency, crystal production and crystal size (by Dv_{50} - the particle median diameter for a volume distribution) was investigated by two-tailed T-test (significance level $\alpha = 0.05$) in relation to the different microbial strains, concentrations of initial NH_4-N and PO_4-P. Statistical tests were considered significant at $p < 0.05$. The 95% confidence intervals (CIs) were also examined to estimate the range of mean values of P removal efficiency, crystal production and Dv_{50}. The statistical correlation was applied to distinguish the significant relationship between eight variables, including initial parameters (pH, NH_4-N and PO_4-P, molar ratio of initial [PO_4-P] to [NH_4-N], solution $SI_{struvite}$) and PO_4-P removal efficiency, crystal production and Dv_{50}, where the critical significance levels (R) was applied: $0.6 \leq R < 0.8$ was considered strong correlation, and $R \geq 0.8$ was considered very strong correlation. All the statistical analysis was performed using Microsoft Excel 2010 (Microsoft, Redmond, Washington, USA).

3. Results and discussion

3.1. Microbial growth and crystal identification

Microbial growth of all the five selected microorganisms was observed in each of the twelve tests (①–⑫, Table 1) with different concentrations of PO_4-P and NH_4-N (Fig. 1). The microbial intact cell count increased overall by 1–2-fold at the end of 196 h incubation period (Fig. 1). As the initial NH_4-N increased from 35 to 332 mg/L, the final intact cell count of *B. antiquum* and *B. pumilus* increased by 9% and 28%, respectively (Fig. 1). The positive effect of NH_4-N on *B. antiquum* growth was in agreement with previous observation that NH_4-N (510–839 mg NH_4-N/L) contributed to growth rate by 0.38 1/d in sludge dewatering liquors [18]. The microbial growth was correlated with SCOD reduction, which varied with the microbial species: 54–57% for *B. antiquum*, 46–50% for *B. pumilus*, 37–43% for *M. xanthus*, 27–31% for *H. salinarum* and 26–28% for *I. loihiensis*. Neither intact microbial cell nor SCOD removal was observed in the non-inoculated controls.

By the end of incubation period, the crystals recovered by each of the five tested microbial strains from municipal wastewater possessed dominantly the same elongated trapezoidal platy shape (Fig. 2a-e, Fig. SA) as the struvite previously described [24]. No struvite precipitate was observed in non-inoculated controls with constant pH level (7.8–8.0). The XRD results (Fig. SB) demonstrated that the crystals biologically recovered from wastewater (initial PO_4-P of 33.9 mg/L and NH_4-N of 332 mg/L) had similar peak profiles to the standard struvite (pattern COD 9007674), and thus they were of the same orthorhombic crystal structure as struvite. Furthermore, the SEM-EDX and crystal solution showed the stoichiometric ratio [Mg]:[P]:[N]:[O] of the crystals was 1:1:1:4, corresponding to the chemical formula of struvite in dehydrated phase ($MgNH_4PO_4$). The observed loss of oxygen (by molecular water) and N (by ammonia) within crystalline framework of both bio-struvite and chemical struvite in this study was due to the air-drying method which resulted in volatilization of ammonia and molecule water generally on crystal surface, as previously reported [25].

Both biological and chemical struvite crystals were observed to have

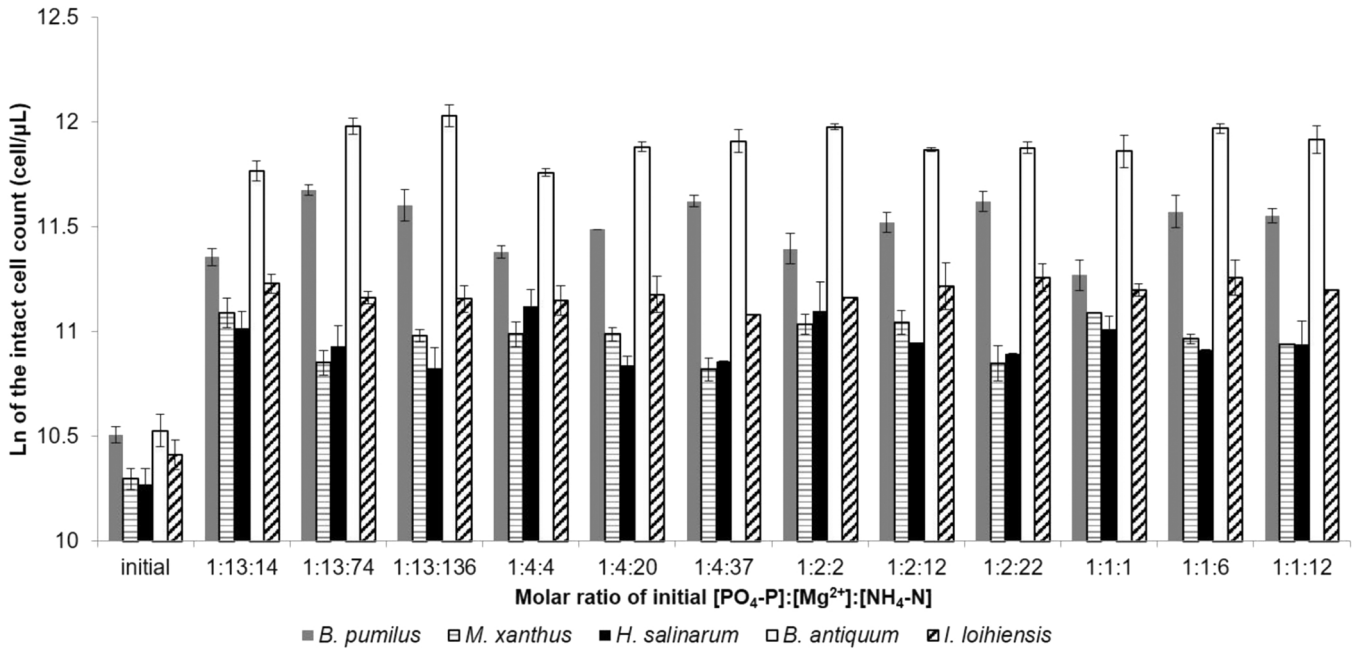

Fig. 1. Natural logarithm of intact cell counts of the *H. salinarum*, *B. antiquum*, *B. pumilus*, *M. xanthus* and *I. loihiensis* in municipal wastewater at initial and by the end of 196 h incubation at different molar ratio of initial nutrient concentrations within PO_4-P range of 5.4–62.4 mg/L in wastewater. Error bars represent standard deviation obtained from duplicates.

porous surface (Fig. 2g-i), which was similar as the previously reported surface structures of chemical struvite [26] and bio-struvite [13]. Porous surface originated from oriented aggregation and embedding of crystalline subunits into the crystalline framework. The comparison of the particle's matrix on the crystal surface in this study showed slight difference in terms of the porosity and building units: wide gap between subunits (high porosity) was observed on the bio-struvite crystal surface structure. And the particle size of the small building units for bio-struvite was relatively larger (1.5–2 μm x 1–1.5 μm) (Fig. 2g-h) than the chemical struvite's (0.8 μm x 0.4 μm) (Fig. 2i). Supersaturation affected crystal size and size distribution [27]. High supersaturation boosts nucleation rate, and thus produces large number of fine particles. In contrast, low supersaturation is typically associated with crystals of large size and lower content of fine particles [27]. The observation of relatively larger subunits of bio-struvite crystal surface indicated the crystallisation process occurred under lower supersaturation where more component ions were used for crystal growth other than nucleation. Furthermore, the high porosity of bio-struvite crystals increased sites where electrostatic interaction between crystal surface structure and charged particles (e.g., ions, cell out-layer, extracellular polymers) may occur, and therefore benefited crystal growth.

Low content of Ca^{2+} less than 0.5 wt percent (wt%) was detected by SEM-EDX on the bio-struvite struvite surface, whereas the Ca^{2+} on chemical struvite crystal surface was found 1.67–8.24 wt%. The Ca^{2+} was reported to affect the morphology and purity of chemical struvite by competitively combining with PO_4-P at pH $\geq$ 9 to aggregate on the struvite surface [28]. A similar structure as previously reported calcium phosphate (Ca-P) precipitation in struvite−water interfacial layer [29] was observed on the chemical struvite in this study (Fig. 2i). However, such Ca-P mineral layer was not observed on the bio-struvite surface in this study.

3.2. Phosphate removal, crystal production and size distribution

The capability of microorganisms to remove PO_4-P from wastewater varied with microbial strains. The maximum P removal at an initial PO_4-P concentration of 5.4 mg/L was 26% by *M. xanthus*, and all the way up to 97% by *B. antiquum* (Fig. 3a). The initial concentrations of PO_4-P and NH_4-N correlated with PO_4-P removal and removal efficiency (Fig. 3a). Within an initial PO_4-P range of 19.7–62.4 mg/L, the P removal efficiency was increased by 22% (*H. salinarum*), 23% (*B. antiquum*), 24% (*M. xanthus*), 28% (*B. pumilus*) and 66% (*I. loihiensis*) as NH_4-N concentration increased (Fig. 3a). The variation of P removal efficiency within range of initial PO_4-P between 5.4 and 62.4 mg/L were 29%, 38%, 65%, 71% and 77% for *B. antiquum, B. pumilus, I. loihiensis, M. xanthus* and *H. salinarum*, respectively (Fig. 3a). No PO_4-P removal was observed in non-inoculated control.

B. antiquum presented relatively constant P removal efficiency (68–97%) overall the investigated nutrients' concentrations when compared with the other four microbial strains (Fig. 3a), indicating that the bacterium's capability of P removal was less dependent on the initial PO_4-P level in solution. The highest PO_4-P removal efficiency by *B. antiquum* (87–97%) occurred at 5.4 mg PO_4-P/L, and achieved final $\leq$ 1 mg PO_4-P/L (Fig. 3a). The effluent met the EU standard for P discharge of 2 mg/L (WWTPs of 10,000–100,000 PE) or 1 mg/L (WWTPs >100,000 PE) [30]. Such performance was owing to a biological control mineralisation process where component ions (e.g., PO_4^{3-}, Mg^{2+}) were pumped into *B. antiquum* cell and accumulated in compartmentalized compartments such as vesicle-like structure, for integration with NH_4^+ to form bio-struvite under specialized regulations (e.g., controlled chemical composition) [31]. Such regulations ensure species-specific crystallo-chemical properties therefore the biomineral type and habit [32]. In contrast, in a biological induced mineralisation process (*B. pumilus, M. xanthus* and *H. salinarum*) where microorganisms exerted limited control on bio-struvite crystallisation, microbial growth and activity benefited the process mainly by heterogenous nucleation and metabolic activity (altering pH and NH_4-N) to achieve supersaturation condition [31]. The capability of P removal by microorganisms that were involved in bio-struvite producing via biological control mineralisation was theocratically independent on nutrient concentration, although the P removal efficiency by *I. loihiensis* varied from 16% to 82% overall the investigated PO_4-P and NH_4-N concentrations. It is noted that the intact cell counts of *I. loihiensis*, halophilic only doubled after 192 h incubation (within stationary phase of growth) (Fig. 1), and the utilisation of dissolved organic carbon was up to 210 mg/L (28%) SCOD removal. Whereas in a previous study where it was grown in

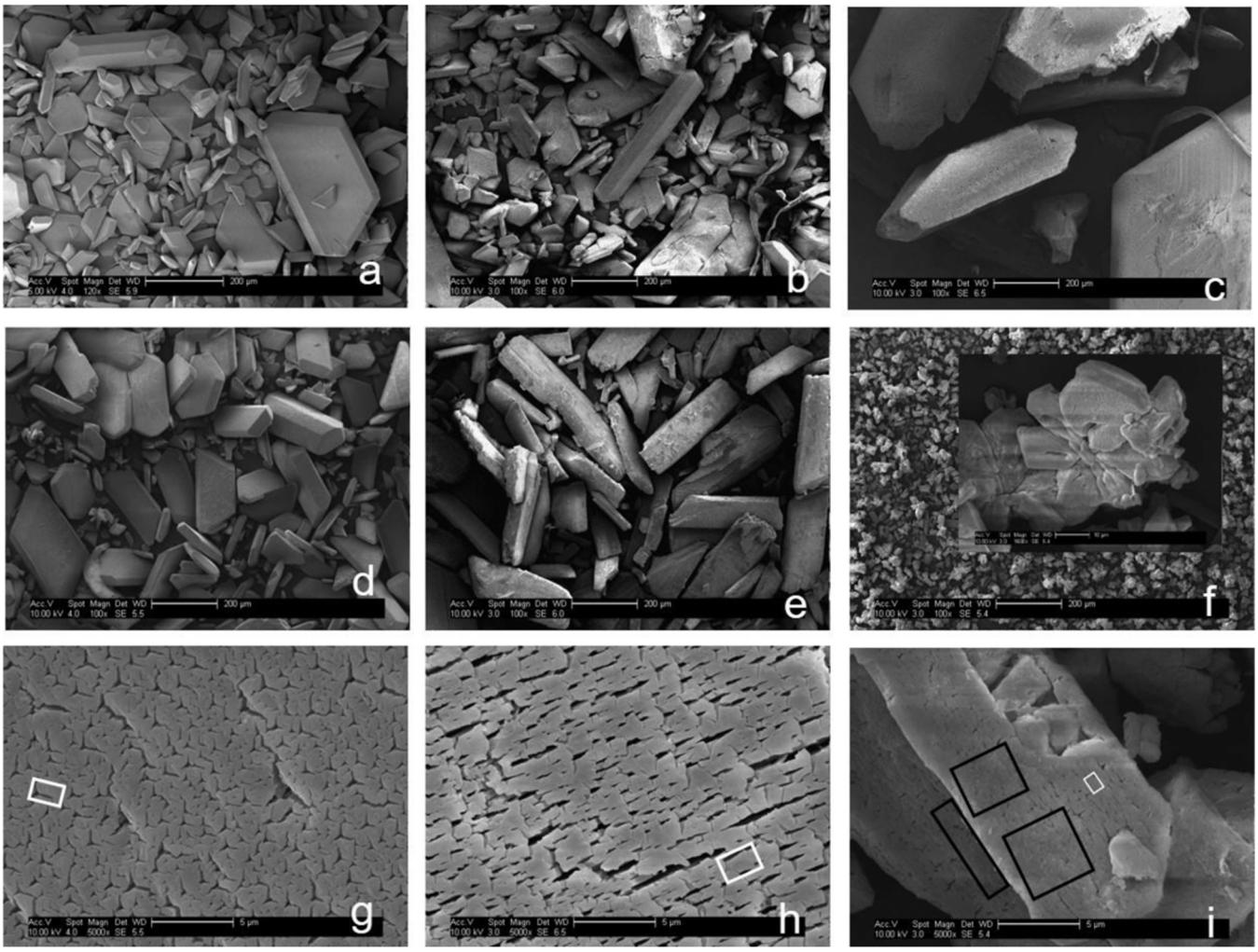

Fig. 2. SEM photo of the isolated bio-struvite crystals recovered from (a) *H. salinarums*, (b) *B. pumilus*, (c) *M. xanthus*, (d) *B. antiquum*, (e) *I. loihiensis* cultures in wastewater (196 h); (f) overview of chemical struvite (scale bar - 200 μm) and single crystal (white square, scale bar - 10 μm). Crystal surface of bio-struvite produced by *B. antiquum* (g), *M. xanthus* (h)) and abiotic struvite (i) (scale bar - 5 μm), white squares indicate the single building unit of struvite aggregated to the crystal surface and black square indicate the nano-calcium phosphate layer.

solution containing 1% NaCl and 4 g/L yeast extract, its intact cell counts 20-folded after 96 h by consuming 1680 mg/L (48%) SCOD [33]. Growth of *I. loihiensis* required NaCl of 0.7–20%., and with optimum NaCl between 2% and 6% [19]. It was suggested that the low NaCl level (0.8%) applied in this study slowed down its cell proliferation and usage of carbon source, thus limited this halophilic bacterium's capability of P removal.

The microbial strains were observed to produce bio-struvite crystal in wastewater containing initial PO_4-P of 19.7–62.4 mg/L (Fig. 3b). In particularly, only *B. antiquum* and *B. pumilus* produced bio-struvite at initial PO_4-P of 19.7 mg/L, NH_4-N of 35 mg/L and Mg^{2+} of 56 mg/L (corresponding to initial $SI_{struvite}$ of −0.43) (Fig. 3b). No precipitant was observed in non-inoculated controls. The presence of magnesium in solution is not only essential to microbial growth (especially *I. loihiensis*) but also significant for bio-struvite production due to the crystals' solubility equilibrium. For all inoculated wastewater in this study, the final concentrations of Mg^{2+} was observed between 15.0 and 54.7 mg/L, thus it did not limit bio-struvite production. A considerable amount of Ca^{2+} (19–21 mg/L) was removed from wastewater with initial molar ratio $[PO_4^{3-}]$:$[NH_4^+]$:$[Mg^{2+}]$:$[Ca^{2+}]$ of 2:2:2:1, and with final pH of 8.4–8.5. This was corresponding to an occurrence of an abundant of inorganic particles in microbial cultures (inorganic particles/total particles of 13–76%) and observation of amorphous nanoparticles. Calcium had potential to compete with struvite formation for PO_4^{3-}, typically producing amorphous calcium phosphates [3]. The presence of Ca^{2+} exerted negative impact on struvite purity within pH range of 7–10.5, where the struvite content decreased from around 70–30% as pH increased from 8.0 to 9.0 [34]. Calcium was also capable to interact with organic molecules for biological induced mineralisation where the increasing alkalinity, electron-interaction and heterogeneous surface might benefit formation of Ca-species [19].

Positive relationships between Dv_{50} and initial nutrient concentration were observed (Fig. 3c). Large bio-struvite crystal groups (Dv_{50} ≥100 μm) formed at initial NH_4-N of 180 mg/L and initial PO_4-P of 62.4 mg/L, and at initial NH_4-N of 332 mg/L and initial PO_4-P between 19.7 and 62.4 mg/L (Fig. 3c). At the end of 196 h incubation, neither PO_4-P removal nor crystal production was observed in non-inoculated controls. The pH of controls was then raised to around 8.5 by 1 M NaOH. After 72 h incubation, precipitates with orthorhombic crystal structure as struvite (Fig. 3b) were observed only in wastewater containing PO_4-P of 62.4 mg/L and NH_4-N of 332 mg/L, with PO_4-P removal of 38 mg/L (61%), chemical struvite production of 2.5 QA and Dv_{50} of 78 μm (Fig. 3a-c). The estimated initial $SI_{struvite}$ for chemical struvite precipitation in this study was 1.52, which was in the range of previously reported values from 1.5 to 10.6 for spontaneous precipitation [27], but higher than that for bio-struvite formation (−0.43 to 0.84)

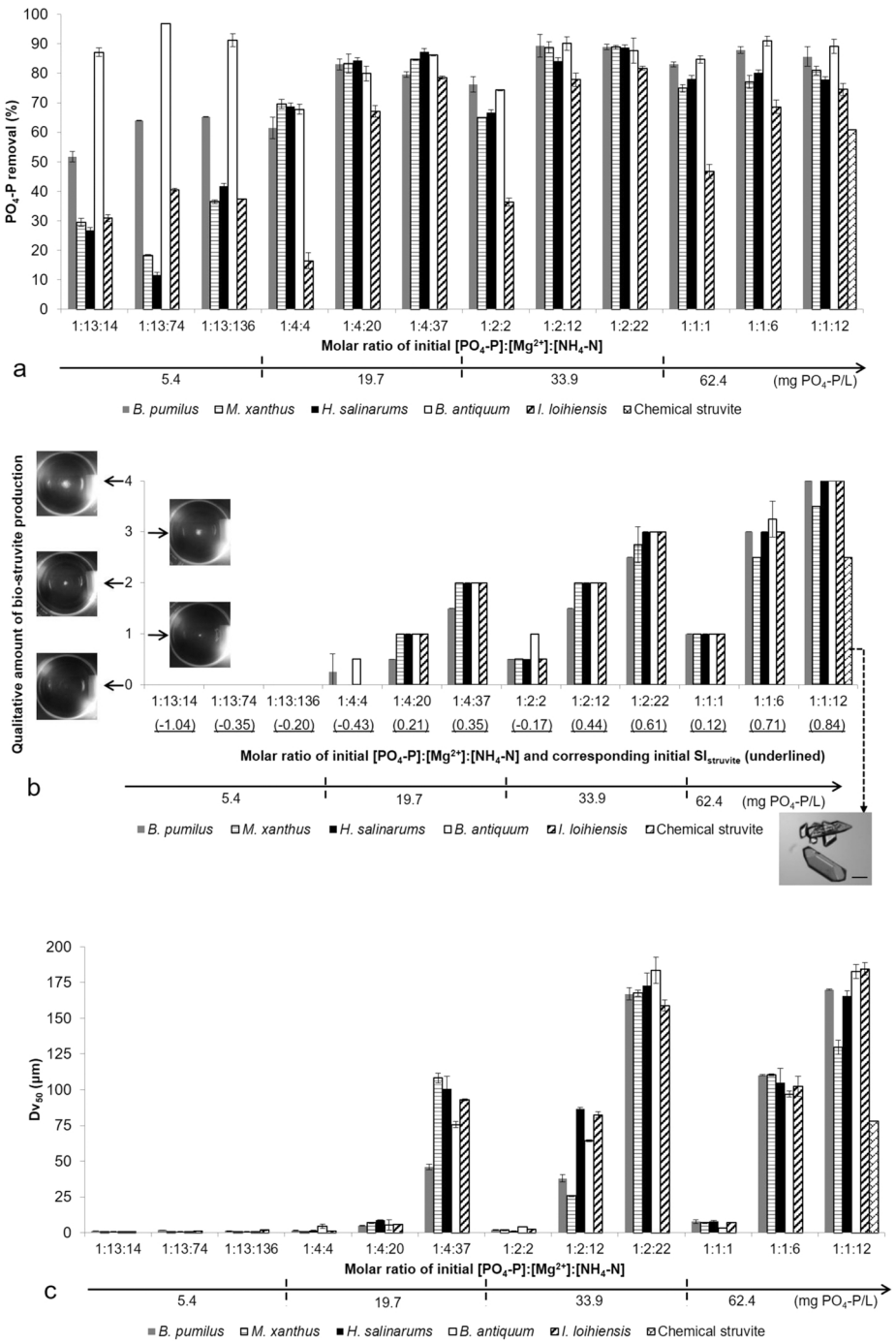

Fig. 3. The PO_4-P removal efficiency (a), struvite production (by qualitative assessment) (b) and Dv_{50} (c) at different molar ratio of initial nutrient concentrations and the corresponding $SI_{struvite}$ values (underlined) within PO_4-P range of 5.4–62.4 mg/L in wastewater. The five microorganisms shared same initial $SI_{struvite}$ values (underlined) for each molar ratio of initial nutrient concentrations. Error bars represented standard deviation obtained from duplicates. Dv_{50} represented the particle median diameter for a volume distribution. The PO_4-P removal and chemical struvite in non-inoculated control were only observed after raising pH to 8.5 by adding sodium hydroxide (photo with scale bar – 10.19 μm).

in this study (Fig. 3b), and the previously reported initial $SI_{struvite}$ of 0.6–0.8 in synthetic solution and wastewater [31,33].

When compared the struvite biomineralisation with chemical struvite precipitation in this study, the former recovered struvite by larger crystal groups, at lower initial PO_4-P and without chemical dose for pH adjustment. Although low level $SI_{struvite}$ slowed down the nucleation rate, specific metabolic pathways and organic cellular substance/structures involved in struvite biomineralisation reduced energy barrier for heterogeneous nucleation of bio-struvite and promoted its crystallization process [10]. The low $SI_{struvite}$ then benefited crystal growth (e.g., crystal size, morphology) by competitive for component ions to promote the crystals' settleability [27].

3.3. Importance of initial nutrient concentrations on P removal and bio-struvite product

B. antiquum was observed to distinguish itself from the other four microbial strains regarding P removal efficiency in wastewater (Table 2). There was no significant difference among the five tested microorganisms regarding crystal size (Dv_{50}) and production, but a significant difference between the inoculated and non-inoculated controls was observed (Table 2). With respect to different initial PO_4-P concentrations, there was significant difference in terms of P removal efficiency when initial PO_4-P increased from 5.4 and 19.7 mg/L, and in terms of Dv_{50} and crystal production when initial PO_4-P increased from 5.4 to 19.7 and to 33.9 mg/L (Table 2). Thus, for the investigated microorganisms, the significant initial PO_4-P concentration that impacted

Table 2
Statistical significance (two-tailed T-test) and 95% confidence intervals with respect to PO_4-P removal, Dv_{50} and crystal production at different concentrations of initial NH_4-N and PO_4-P in the five selected microbial cultures.

Two-tailed T-test				
Between groups		p values		
		PO_4-P removal efficiency	Dv_{50}	Crystal production
Microbial strain	*B. pumilus* and *M. xanthus*	0.233	0.980	0.937
	B. pumilus and *H. salinarum*	0.244	0.761	0.795
	B. pumilus and *B. antiquum*	0.048	0.834	0.681
	B. pumilus and *I. loihiensis*	0.010	0.754	0.970
	B. pumilus and control	0.000	0.033	0.008
	M. xanthus and *H. salinarum*	0.991	0.776	0.849
	M. xanthus and *B. antiquum*	0.024	0.850	0.729
	M. xanthus and *I. loihiensis*	0.234	0.768	0.969
	M. xanthus and control	0.000	0.026	0.005
	H. salinarum and *B. antiquum*	0.028	0.930	0.884
	H. salinarum and *I. loihiensis*	0.250	0.986	0.828
	H. salinarum and control	0.000	0.018	0.006
	B. antiquum and *I. loihiensis*	0.001	0.917	0.715
	B. antiquum and control	0.000	0.026	0.004
	I. loihiensis and control	0.000	0.022	0.009
Initial PO_4-P	5.4 mg/L and 19.7 mg/L	0.007	0.014	0.000
	19.7 mg/L and 33.9 mg/L	0.336	0.046	0.024
	33.9 mg/L and 62.4 mg/L	0.952	0.532	0.073
Initial NH_4-N	35 mg/L and 180 mg/L	0.068	0.001	0.068
	180 mg/L and 332 mg/L	0.606	0.003	0.085
95% confidence intervals (CI)				
		Range of mean values		
		PO_4-P removal efficiency (%)	Dv_{50} (μm)	Crystal production (QA)
Microbial strain	*B. pumilus*	68–84	4–88	0.4–2.1
	M. xanthus	51–82	7–87	0.5–2.1
	H. salinarum	50–83	11–98	0.5–2.3
	B. antiquum	80–91	7–96	0.6–2.3
	I. loihiensis	41–69	10–100	0.4–2.1
Initial PO_4-P	5.4 mg/L	34–63	1	0
	19.7 mg/L	63–83	8–54	0.6–1.4
	33.9 mg/L	71–87	36–118	1.2–2.3
	62.4 mg/L	73–85	55–133	1.8–3.3
Initial NH_4-N	35 mg/L	50–70	2–4	0.2–0.6
	180 mg/L	62–84	22–64	0.9–1.9
	332 mg/L	68–85	72–141	1.5–2.8

P removal efficiency and bio-struvite production and crystal size were 19.7 mg/L and 33.9 mg/L, respectively. Initial nutrient concentrations above the significant values did not significantly change the P removal efficiency, bio-struvite production and size. Moreover, Dv_{50} varied significantly as the initial NH_4-N increased from 35 to 180 and to 332 mg/L, but the changes of initial NH_4-N concentrations did not significantly correlate with the P removal efficiency and bio-struvite production (Table 2). Therefore, within the investigated range, the initial NH_4-N concentration presented a positive correlation with bio-struvite crystal size for all the tested microorganisms.

The 95% confidence intervals (CIs) of P removal efficiency (80–91%) by *B. antiquum* showed a relatively high PO_4-P removal within a narrow range, when compared with that of the other four microbial strains (68–84% of *B. pumilus,* 51–82% of *M. xanthus*, 50–83% of *H. salinarums* and 41–69% of *I. loihiensis*) (Table 2). This indicated a relative stable high P removal efficiency by *B. antiquum* within the investigated nutrient concentrations, which was in agree with the observation of P removal efficiency by *B. antiquum* in Section 3.2. The 95% CIs of Dv_{50} at initial 332 mg NH_4-N/L, 33.9 mg PO_4-P/L and 62.4 mg PO_4-P/L were of 72–141, 36–118 and 55–133 μm, respectively (Table 2). This indicated potential formation of large bio-struvite crystal groups (Dv_{50} >100 μm) at initial NH_4-N of 332 mg/L combined with initial PO_4-P ≥ 33.9 mg/L. The highest 95% CIs of crystal production occurred at initial 62.4 mg PO_4-P/L, followed by initial nutrient concentrations of 33.9 mg PO_4-P/L and then 332 mg NH_4-N/L (Table 2).

Initial PO_4-P, NH_4-N and $SI_{struvite}$ were identified to have significant positive correlations with P removal efficiency, bio-struvite production and Dv_{50} for the five tested microorganisms (Table 3). Compared with the initial PO_4-P and NH_4-N, the $SI_{struvite}$ was observed to have stronger correlations with Dv_{50} and bio-struvite production (Table 3). The P removal efficiency by *B. antiquum* was of less dependence on these three initial parameters than the other four microorganisms; whereas the P removal efficiency by *B. pumilus* highly depended on the initial $SI_{struvite}$ (Table 3).

The significant initial PO_4-P concentration of 19.7 mg/L for P removal and 33.9 mg/L for bio-struvite production and Dv_{50} suggested wider range of wastewater sources at municipal WWTPs for struvite biomineralisation than struvite chemical precipitation that frequently sourced from P-rich liquids with P above 50 mg/L [9]. A potential to produce large bio-struvite crystal groups with Dv_{50} more than 100 μm for readily settlement in wastewater at initial nutrient concentrations of high levels (NH_4-N = 332 mg/L, PO_4-P ≥ 33.9 mg/L) added benefits to struvite biomineralisation. The application of struvite biomineralisation at WWTPs was further discussed in Section 3.5.

Significant positive correlations were also observed among P removal efficiency, Dv_{50} and bio-struvite production, especially between Dv_{50} and bio-struvite production (Table 3). This indicated a potential of high P removal efficiency integrated with P recovery by bio-struvite of more production and larger size, as shown in a 3D scatter plot, where all the large 3D spheres (crystal production, QA≥3) were filled with colour representing high Dv50 (≥80 μm), and appeared with P removal efficiency more than 75% (Fig. SC).

3.4. Struvite purity and heavy metal content

No significant difference was observed regarding the purity between bio-struvite recovered from wastewater with PO_4-P of 33.9 mg/L and chemical struvite recovered from PO_4-P more than 2000 mg/L (Table 4).

Table 3
Significant correlations between the initial parameters and P removal, Dv_{50} and crystal production (R ≥0.6) in ● *B. pumilus*, ▲ *M. xanthus*, ◆ *H. salinarum*, ■ *B. antiquum*, ★ *I. loihiensis* cultures.

	P removal efficiency		Dv_{50} (μm)		Crystal production (QA)	
	R ≥ 0.8	0.6 ≤R < 0.8	R ≥ 0.8	0.6 ≤R < 0.8	R ≥ 0.8	0.6 ≤R < 0.8
Initial NH_4-N (mg/L)		▲◆★		●▲◆■★		◆
Initial PO_4-P (mg/L)		●		■		●▲■★
Initial $SI_{struvite}$	●	▲◆★	▲◆■★	●	●▲◆■★	
Dv_{50} (μm)		●★				

Table 4
Quantification of macronutrients and heavy/toxic metal in bio-struvite and chemical struvite (average ± standard deviation of triplicates).

		B. antiquum	*B. pumilus*	*H. salinarum*	*M. xanthus*	*I. loihiensis*	Chemical struvite	Theoretical struvite[a]	EU permissible levels
Macronutrient	PO_4-P (g/kg)	122.6 ± 3.3 (12.3% P)	121.9 ± 3.9 (12.2% P)	121.7 ± 1.9 (12.2% P)	118.1 ± 2.8 (11.9% P)	121.9 ± 3.3 (12.2% P)	127.2 ± 3.6 (12.7% P)	12.6% P	2% (total P_2O_5)[bd]
	NH_4-N (g/kg)	57.7 ± 0.2 (5.8% N)	56.2 ± 1.1 (5.6% N)	57.1 ± 0.4 (5.7% N)	53.94 ± 0.8 (5.4% N)	55.6 ± 0.3 (5.6% N)	52.42 ± 0.3 (5.2% N)	5.7% N	2.5% (total N)[bd]
	Mg (g/kg)	97.7 ± 0.9 (16.2% MgO)	96.2 ± 0.3 (16.0% MgO)	97.1 ± 0.7 (16.2% MgO)	93.1 ± 0.6 (15.5% MgO)	94.6 ± 0.6 (15.8% MgO)	96.0 ± 1.2 (16.0% MgO)	16.4% MgO	NYD
	K (g/kg)	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0	2% (water-soluble K_2O)[bd]
	Ca (g/kg)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.8 ± 0.0	0	NYD
Heavy/toxic metal	Al (mg/kg)	18.5 ± 0.7	12.0 ± 0.4	22.9 ± 0.8	24.8 ± 0.5	26.3 ± 0.3	31.2 ± 0.9	0	NYD
	Fe (mg/kg)	2.65 ± 0.23	2.19 ± 0.62	<LOD	3.25 ± 0.45	4.18 ± 0.07	8.69 ± 0.38	0	NYD
	Cr VI (mg/kg)	0.17 ± 0.02	0.23 ± 0.03	0.15 ± 0.01	0.26 ± 0.02	0.23 ± 0.01	0.38 ± 0.00	0	2 mg/kg[ce]
	Ni (mg/kg)	0.11 ± 0.00	0.08 ± 0.04	0.07 ± 0.01	0.11 ± 0.03	0.14 ± 0.01	1.19 ± 0.10	0	100 mg/kg[cd]
	Cu (mg/kg)	0.33 ± 0.19	0.53 ± 0.18	0.18 ± 0.02	1.57 ± 0.26	1.24 ± 0.03	0.42 ± 0.03	0	200 mg/kg[ce]
	As(mg/kg)	0.38 ± 0.01	0.30 ± 0.02	0.32 ± 0.01	0.27 ± 0.04	0.39 ± 0.04	0.07 ± 0.02	0	40 mg/kg[cd]
	Cd (mg/kg)	0.06 ± 0.05	0.66 ± 0.01	<LOD	0.04 ± 0.02	0.26 ± 0.02	0.00 ± 0.00	0	60 mg/kg (if P_2O_5 >5%)[cd]
	Pb (mg/kg)	0.18 ± 0.03	0.32 ± 0.04	0.11 ± 0.01	0.38 ± 0.16	0.36 ± 0.02	0.40 ± 0.02	0	120 mg/kg[cd]
	Hg (mg/kg)	0.03 ± 0.01	0.11 ± 0.05	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0	1 mg/kg[cd]

LOD - limit of detection; NYD – not yet defined
[a] - Theoretical struvite with chemical formula: $MgNH_4PO_4.6\ H_2O$; [b]- Minimum nutrient content in solid inorganic fertilisers; [c]- maximum heavy metal content in inorganic fertilisers; [d] - The European parliament and the council of the European union [36]; [e] - EU Fertiliser Regulation revision proposal 2014 [35]

Bio-struvite contained nutrients equivalent to 11.8–12.3% P, 5.4–5.8% N and 15.5–16.2% MgO, and the chemical struvite contained nutrients equivalent to 12.6% P, 5.7% N and 16.4% MgO (Table 4). The purity of both products was within the proposed acceptable purity range of struvite for fertiliser usage, with requirement of nutrients' content equivalent to 10.0–13.9% P, 4.6–6.3% N and 13.1–18.1% MgO [35]. Both P and N content of the bio-struvite met the EU minimum requirement as inorganic fertiliser, thus it has a potential use as P and N fertiliser alternative. Bio-struvite was also observed contain K (0.6–0.8 g/kg) and Ca (0.1–0.2 g/kg) that were recognized as essential macronutrients for organism growth (Table 4). Although the K content in bio-struvite was relatively higher than chemical struvite (0.2 g/kg), it was still far below than the EU regulated level of K in inorganic fertiliser [36].

Settled wastewater contained various heavy/toxic metals (Table SA), although the amount of heavy metal Cr, Ni, Cu, As, Cd, Pb and Hg in both bio-struvite and chemical struvite products (Table 4) met the EU maximum limitation [35,36]. Compared with the chemical struvite, the bio-struvite contained lower amounts of Ca, Al, Fe, Cr, Ni and Pb, but relatively higher amount of Cu, As, Cd and Hg (Table 4). In particular, bio-struvite produced by *M. xanthus* and *I. loihiensis* was observed contained higher Al (24.8–26.3 mg/kg), Fe (3.25–4.18 mg/kg), Cr (0.23–0.26 mg/kg), Ni (0.11–0.14 mg/kg) and Pb (0.36–0.38 mg/kg) than the other three microbial strains (Table 4). The highest Cd content (0.66 mg/kg) was found in the *B. pumilus* bio-struvite, compared with the other four bio-struvite (0–0.26 mg/kg) and the chemical struvite (<1 μg/kg) (Table 4).

The bio-struvite obtained in this study was found of similar purity as previously reported bio-struvite (11.9% P) which was recovered from sludge dewatering liquors, but lower of heavy metal contents, especially the Hg, As and Ni [21]. Furthermore, when comparing with the bio-struvite produced from sewage sludge ash (SSA), which was also proposed as a P-fertiliser alternative recovered from WWTPs, it was observed that the previously described SSA bio-struvite contained lower P content (9.4% P, 10.56% P) but higher toxic/heavy metal contents [37,38]. Thus, the bio-struvite recovered from primary settled municipal wastewater in this study is of better-quality regarding P and heavy metal content. The impurity of bio-struvite may due to the influence of the heavy metal-concentrated environment. Verma and Kulia, 2019 reported a great potential of EPS to bind heavy metal ions (e.g., Cd, Cu, Pd, Hg, Fe, Cr, Ni) by metabolic pathway. Such microbial biomineralisation may lead to precipitation of mineral containing inorganic pollutants under proper conditions [39]. Moreover, microorganism may possess resistance or tolerance to specific heavy metal (e.g. Cd) by accumulating and concentrating them within cells [40]. These microbial cells have potential to release the heavy metal to the mineral surface during struvite biomineralisation, via molecular interaction between cells and the crystal surface [10].

3.5. Implication for wastewater industry

Most of the previously reported struvite process were applied to waste streams rich of PO_4^{3-} and NH_4^+, such as side streams and liquors from anaerobic digestion [6,9,41]. Chrispim et al., 2019 suggested several preferred wastewater sources for struvite precipitation at WWTPs, mostly digester supernatant and sludge dewatering liquors, and highly dependent on the performance of sludge digestion to generate enough nutrient constituents. In this study, P removal and P recovery by biomineral forming microorganisms were observed at relatively low PO_4-P of 5.4 and 19.7 mg/L, respectively. Thus, compared with chemical struvite precipitation, struvite biomineralisation could be applied to wastewater streams with a much lower and wider range of PO_4-P concentrations. The eligible wastewater sources at WWTPs for bio-struvite formation could range from raw municipal wastewater (4–15 mg PO_4-P/L and NH_4-N > 35 mg/L) to settled wastewater (7.5 mg PO_4-P/L) and to digester supernatant (20–400 mg PO_4-P/L) [6,17]. However, aeration and nitrogenous organic compounds are also required to provide enough dissolved oxygen and carbon sources for microbial growth and specific metabolic pathways [20]. In particular, *B. antiquum* may have potential use to achieve high PO_4-P effluent quality due to the

observation of its capability in this study to reduce PO_4-P from 5.4 mg/L to less than 1 mg/L. The application of microbial strains for P removal and P recovery may vary with wastewater characteristics. In some WWTPs as the one described by Jaffer et al. (2002) for example, all the wastewater streams presented conditions suitable for microbial growth of the tested microbial strains [20], *B. antiquum* and *B. pumilus* can be applied for P removal from raw and settled wastewater (PO_4-P of 5.7–14.2 mg/L, NH_4-N of 16.1–23.9 mg/L, Mg^{2+} of 8–8.9 mg/L), and additional Mg^{2+} source might be required. In sludge dewatering liquors (PFT liquor, centrifuge liquor) containing 33.2–94.9 mg PO_4-P/L, 129–615 mg NH_4-N/L and 13–44 mg Mg^{2+}/L, all the five tested microorganisms can be applied for P recovery by bio-struvite. In particular, the centrifuge liquor was higher in pH, PO_4-P, NH_4-N and Mg^{2+}, and lower in Ca^{2+} [41], thus it is anticipated with better performance than PFT liquor regarding crystallization process and crystal purity.

In this study the concentrations of nutrients in the wastewater were manipulated to investigate the relationships between cell growth, wastewater composition in relation to nutrients, and the property of the precipitates recovered by filtration. Overall, bio-struvite production presented important advantages, in comparison with chemical struvite, and the process should be further developed for implementation at pilot and full scale where the organic matter and suspended solids in wastewater, in addition to its complicated ion components, exerted influence on the biomineralisation process. Further work will need to focus on the design of reactors/processes, operational conditions to ensure proliferation of the selected microorganisms, and even out-compete other microorganisms in mixed cultures, for eventual enhanced P recovery by bio-struvite from waste streams.

4. Conclusions

This study demonstrated the capability of selected microorganisms to produce visible bio-struvite crystals in wastewater containing PO_4-P $\geq$ 19.7 mg/L, much less than the PO_4-P (62.4 mg/L, with pH adjustment) for struvite chemical precipitation. *B. antiquum* distinguished itself from the other four microbial strains by relatively stable P removal efficiency (68–97%) independent on initial nutrient concentrations and $SI_{struvite}$, and reduced PO_4-P from 5.4 to less than 1 mg/L. Within the investigated range, a significant initial PO_4-P concentration for P removal efficiency was 19.7 mg/L, and was 33.9 mg/L for bio-struvite production and crystal size. Large bio-struvite crystal groups with Dv_{50} above 100 μm had most potential to occur at initial NH_4-N of 332 mg/L and PO_4-P above 33.9 mg/L. Compared with chemical struvite, bio-struvite recovered from wastewater with much lower PO_4-P was of higher surface porosity, and similar high purity and extremely low heavy metal content. It met the inorganic fertilisers regulation proposed by Fertilisers Working Group, which enable its potential use as a good alternative of P and N fertiliser. Biomineralisation benefited bio-struvite production by low initial PO_4-P requirement, large crystal size and no pH adjustment, whereby PO_4-P recovery through bio-struvite presents interesting benefits and opportunities to be applied to WWTPs.

CRediT authorship contribution statement

Yirong Leng: Conceptualization, Investigation, Writing – original draft. **Ana Soares:** Project administration, Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2022.109208.

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