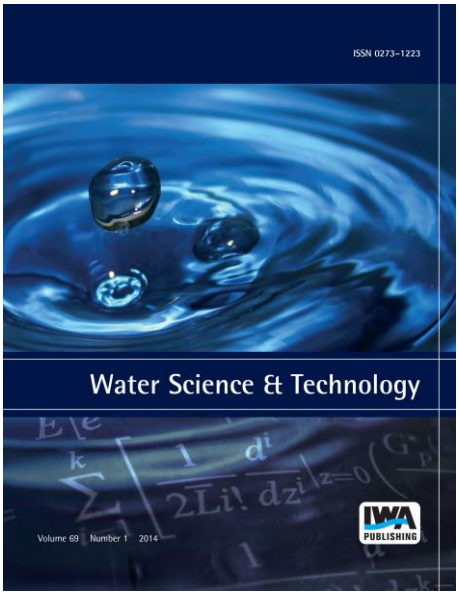

Ecology and performance of aerobic granular sludge treating high-saline municipal wastewater

Benjamin J. Thwaites, Ben van den Akker, Petra J. Reeve, Michael D. Short, Nirmala Dinesh, Juan Pablo Alvarez-Gaitan and Richard Stuetz

ABSTRACT

The successful development of aerobic granular sludge (AGS) for secondary wastewater treatment has been linked to a dedicated anaerobic feeding phase, which enables key microbes such as polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms to gain a competitive advantage over floc-forming organisms. The application of AGS to treat high-saline sewage and its subsequent impacts on microbial ecology, however, are less well understood. In this study, the impacts of high-saline sewage on AGS development, performance and ecology were investigated using molecular microbiology methods. Two feeding strategies were compared at pilot scale: a full (100%) anaerobic feed; and a partial (33%) anaerobic feed. The results were compared to a neighbouring full-scale conventional activated sludge (CAS) system (100% aerobic). We observed that AGS developed under decreased anaerobic contact showed a comparable formation, stability and nitrogen removal performance to the 100% anaerobically fed system. Analysis of the microbial ecology showed that the altered anaerobic contact had minimal effect on the abundances of the functional nitrifying and denitrifying bacteria and Archaea; however, there were notable ecological differences when comparing different sized granules. In contrast to previous work, a large enrichment in PAOs in AGS was not observed in high-saline wastewater, which coincided with poor observed phosphate removal performance. Instead, AGS exhibited a substantial enrichment in sulfide-oxidising bacteria, which was complemented by elemental analysis that identified the presence of elemental sulfur precipitation. The potential role for these organisms in AGS treating high-saline wastewater is discussed.

Key words | aerobic granular sludge, high-saline municipal wastewater treatment, microbial ecology, sulfide ecology

Benjamin J. Thwaites (corresponding author)
Michael D. Short
Juan Pablo Alvarez-Gaitan
Richard Stuetz
School of Civil and Environmental Engineering,
UNSW Australia,
Sydney, NSW 2052,
Australia
E-mail: *b.thwaites@student.unsw.edu.au*

Ben van den Akker
Petra J. Reeve
Nirmala Dinesh
South Australian Water Corporation,
Adelaide, South Australia 5000,
Australia

Ben van den Akker
Michael D. Short
School of Natural and Built Environments,
University of South Australia,
Mawson Lakes, South Australia 5095,
Australia

Ben van den Akker
Health and Environment Group, School of the Environment,
Flinders University,
Bedford Park, South Australia 5042,
Australia

Michael D. Short
Future Industries Institute,
University of South Australia,
Mawson Lakes, South Australia 5095,
Australia

INTRODUCTION

Aerobic granular sludge (AGS) has been shown to be a viable option for various municipal wastewater treatment applications, with recognised advantages such as increased hydraulic capacity and reduced physical footprint. AGS is commonly achieved using sequencing batch reactors (SBRs), with the technology utilising rapid-settling, dense microbial granules in place of floc-based conventional activated sludge (CAS). The conversion process has been shown to be highly dependent on the implementation of a dedicated anaerobic feed and short sludge settling times. The anaerobic feed selects for slow-growing microbes such as polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms, which have been shown to play a critical role in granule development. These microbes have been identified as being able to store the bioavailable organic carbon generated during the anaerobic feed (de Kreuk & van Loosdrecht 2004), with PAOs also playing a role in the removal of phosphate (PO_4) (Bassin *et al.* 2012). These groups of bacteria are predominantly found within the phylum *Proteobacteria* and class *Deltaproteobacteria* and *Gammaproteobacteria* (Diaz *et al.* 2003; Zhang *et al.* 2011).

Whilst AGS technology has been widely researched and applied in many locations worldwide, there is little known about the potential impacts of high-saline municipal

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wastewater on granule development and treatment performance. High-saline wastewater streams can result from groundwater infiltration into sewer networks (e.g. coastal locations) and often have high concentrations of sulfate and sulfide; this may lead to issues with granule formation and stability due to inhibition of extracellular polymeric substances (EPS) production, nitrite accumulation and PAO inhibition (Li *et al.* 2014; Welles *et al.* 2014). Previously, van den Akker *et al.* (2015) showed that development of AGS was possible in high-saline wastewater when using a dedicated anaerobic feed and long (≥2 hours) aerobic phase. Winkler *et al.* (2012b) showed that granules incubated for a short time in varied salinity concentrations (up to 40 g/L NaCl) had a reduced settling velocity that had the potential to cause biomass washout. However, once the salt concentration equalised there was no effect on settling velocity. This study was based on laboratory-grown granules under relatively short biomass salinity acclimation periods (up to 24 h). Further work on the impact of varied salinity concentrations on the granulation process was conducted by Li *et al.* (2017) who found that rapid granulation occurred when operating under the highest percentage (100%) of seawater. The ammonia removal efficiency was initially reduced; however it increased to 90% after 140 days of operation. This study also found that the presence of seawater severely reduced the maximum ammonium and nitrite oxidation rates.

To date, most of the published data on salinity impacts in AGS were largely derived from bench-scale reactors fed with synthetic wastewater and under periods of short-term exposure to high salinity conditions. Complementary field-based research at larger scale is, therefore, required to validate laboratory findings. Accordingly, this study investigated the impact of high-saline municipal wastewater on AGS formation, stability and microbial ecology at pilot scale. Secondly, the impacts of different feeding strategies were investigated to better understand the influence of anaerobic versus aerobic feed conditions. Characterisation of the AGS community was done based on whole-of-community 16S rRNA profiles and targeted analysis of functional genes specific for nitrifying and denitrifying microorganisms, with AGS microbial ecology compared to a neighbouring full-scale CAS SBR at functional and whole-of-community levels.

METHODOLOGY

The pilot-scale SBR was located at a large metropolitan wastewater treatment plant (WWTP) (Adelaide, South Australia), which receives high-saline (total dissolved solids of 6,000–7,000 mg/L) municipal wastewater, a result of high volumes of infiltration into the sewer network. The secondary activated sludge treatment at the full-scale WWTP consists of six SBRs with a design capacity of 32 ML/d. The pilot-scale reactor (63.9 L volume) was located within a weather-proof climate-controlled container and was controlled using programmable logic controllers allowing cycle times, volumetric exchange and air flow to mimic CAS maintenance or develop AGS (van den Akker *et al.* 2015). The pilot SBR was fed with screened (2 mm mesh) municipal wastewater (Table 1) sourced from the full-scale WWTP inlet and was inoculated with 3 g/L of flocculent biomass from the neighbouring full-scale CAS reactor.

The chemical oxygen demand (COD) loading rates and cycle times that were used in the operation of the AGS pilot and full-scale SBRs are given in Table 2. For the AGS trials, two feeding strategies were compared at pilot scale: a full 100% anaerobic feed (Strategy A) and a partial 33% anaerobic feed (Strategy B). Strategy A was assessed to investigate the impacts of AGS under high-saline conditions using operational parameters analogous to previous AGS studies (Morgenroth *et al.* 1997; Beun *et al.* 1999). Strategy B was assessed to understand the impacts of AGS operating under lower COD loads, which was comparable to the neighbouring full-scale CAS (100% aerobic feed) system (Strategy C). In light of the lower COD loads used in Strategy B, the impact of employing a reduced anaerobic feed duration was investigated as this further reduces the cycle time and makes AGS easier to retrofit within existing SBRs. The performance and stability of the AGS trials was monitored for 95 and 113 days.

Biomass and nutrient analysis

Nitrogen removal was examined throughout the trial periods by measuring COD, PO_4-P, NH_4^+-N, NO_2^--N and NO_3^--N in wastewater, mixed liquor and secondary effluent. Analysis was conducted using Hach colorimetric test kits 8000, 8048, 10031, 10019 and 10020. Suspended solids concentration and morphology were examined twice weekly, sludge settleability were determined using a 30-minute sludge volume index (SVI_{30}) (APHA 1998). Morphological changes were examined via light microscopy (Nikon (SMZ1000) and images were captured using Nikon Digital Sight (DS-U2, Japan) and NIS-Elements D 3.0 (Laboratory Imaging s.r.o.).

Table 1 | Median concentration of key parameters found in the high-saline municipal sewage ($n \geq 10 \pm 1$SD)

	Total COD (mg/L)	Ammonia (mg/L)	Total nitrogen (mg/L)	Sulfate (mg/L)	Conductivity (μS/cm)	Total dissolved solids (mg/L)
High-saline municipal wastewater	534.9 ± 64.7	35.1 ± 3.2	55.8 ± 7.7	668.6 ± 90.8	11393.5 ± 426.2	6535 ± 251.8

Table 2 | Operating parameters of the pilot AGS and full-scale CAS SBRs showing organic loading rates and cycle time phases

	COD loading ($kg/m^3 \cdot d$)	Anaerobic feed (minutes)	Aerobic feed (minutes)	Aeration (minutes)	Settling (minutes)	Decant (minutes)	Total cycle time (minutes)	Trial time (days)
Strategy A (100% anaerobic)	1.15	60	–	120	8	2	190	113
Strategy B (33% anaerobic)	0.76	20	40	80	15	10	165	95
Strategy C (100% aerobic)	0.80	–	54	108	54	54	270	∞

Microbial ecology analysis

Biomass samples were collected from each AGS feeding strategy (day 90). An additional sample was collected from the aerobically fed, full-scale CAS SBR. All biomass samples were stored at −80 °C prior to preparation for molecular analysis. A biomass sample from Strategy A was size-separated using increasing mesh sieves (300, 1,000, 1,400 μm) with the retained biomass washed off the mesh using sterile tap water. DNA was extracted using PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA.) with the biomass being washed in sterile phosphate buffer saline prior to following the manufacturer's extraction method. The extracted DNA was quantified using a NanoDrop 2000C spectrophotometer (Thermo Fisher, Delaware, USA). Analysis was conducted on the nitrogen removal functional gene groups (ammonia-oxidising archaea/bacteria (AOA/AOB), nitrite-oxidising bacteria (NOB) and denitrifying bacteria) using quantitative polymerase chain reaction (qPCR) targeting 16S rRNA/functional gene primer sets by Reeve *et al.* (2016). The reaction was carried out in duplicate using a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Each 25 μL reaction mixture contained 4 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 5 μM of oligonucleotide primers (Geneworks, Adelaide, Australia), 0.2 mM dNTPs (Promega, Madison, WI, USA), 1× GoTaq PCR buffer (Invitrogen), 1 U of GoTaq (Invitrogen) and 2 μM SYTO9 (Invitrogen). Thermal cycling conditions involved a primary denaturation at 95 °C for 6 min, followed by 55 cycles at 95 °C for 20 seconds, 52–66 °C for 30 seconds and 72 °C for 30 seconds.

High throughput sequencing was performed on the DNA extracted from the biomass samples collected on day 90. Genomic DNA was extracted using the same extraction method described above. DNA extracts were sent to the Australian Genomic Research Facility (Brisbane, Australia) where analysis was performed on an Illumina MiSeq sequencer using 16S rRNA gene specific primers targeting the region 341F to 806R.

SEM and EDS analysis

Biomass samples were collected and freeze dried for 72 hours. Samples were then coated with gold to a thickness of 20 nm. Scanning electron microscopy (SEM) was conducted using a Zeiss Merlin operated with a working distance of 6.0 mm, electron high tension of 10.00 kV. Samples were further analysed using an energy dispersive X-ray spectroscopy (EDS) detector mounted into the SEM chamber. This allowed for detection and identification of the elemental composition of user-specified field of the sample.

XFM analysis

Elemental X-ray fluorescence analysis was conducted to investigate granule structure, density and metals concentration using the X-ray fluorescence microscopy (XFM) beamline at the Australian Synchrotron (Paterson *et al.* 2011). Biomass samples from Strategy A were freeze dried and mounted on Kapton tape for analysis. Images were analysed as per the protocol of Donner *et al.* (2011) with co-localisation determined using tri-colour mapping.

RESULTS AND DISCUSSION

Start-up and performance

During start-up, there was a large increase in the mixed liquor biomass concentrations during the pilot trials with

the final steady-state concentration being 5–7 g/L (Supplementary Figure 1(a), available with the online version of this paper). The sludge settling performance achieved an SVI_5/SVI_{30} ratio of 1.1 in the full anaerobic feed within 37 days, and similarly the settling ratio of the partial anaerobic feed decreased to 1.2 within the initial 51 days of operation; this decreased ratio was consistent with previous findings by Liu *et al.* (2010). Analysis of the biomass morphology by light microscopy showed distinct changes in the biomass structure and development of clear granular formations. There was also an observable reduction in the filaments protruding on the surface of the granular structures which was consistent with previous findings by de Kreuk *et al.* (2005).

The ammonia removal performance of all mature feeding strategies ranged between 70 and 99.7% with total nitrogen removal typically >75% (Table 3). Analysis showed decreased PO_4 removal in the 33% anaerobic feed AGS (Strategy B), when compared to the 100% anaerobic contact operation (Strategy A), with median PO_4 removals of 9.2 and 20.7%, respectively. The PO_4 removal efficiency in the anaerobic contact investigations was greatly reduced when compared to the removal efficiency observed in the full-scale CAS operation and other AGS trials conducted in low-saline environments (Bassin *et al.* 2012). The nitrogen and PO_4 removal performance of AGS comparing both strategies A and B during start-up is provided in the Supplementary Figure 1(b) and 1(c) (available with the online version of this paper).

Microbial ecology

qPCR analyses compared key nitrifying and denitrifying microorganisms, with notable differences seen in the relative abundances of AOB, AOA and the NOB *Nitrobacter* sp. between the three feeding strategies (Figure 1(a)). The

Table 3 | Process performance summary comparing AGS (Strategy A and B) and full-scale SBR (strategy C), showing the range (min–max) of nutrient removal performance (%) and biomass characteristics

	PO_4 removal (%)	NH_4 removal (%)	Total nitrogen removal (%)	MLSS (g/L)	SVI_{30}	SVI_5/SVI_{30}
Strategy A (100% anaerobic)	5.4–49.7	77.8–99.7	16.0–97.5	1.7–8.8	37.5–238.2	1.1–2.1
Strategy B (33% anaerobic)	4.3–17.3	96.1–99.8	27.5–94.2	3.6–6.8	58.5–132.1	1.2–2.1
Strategy C (100% aerobic)	n.d.	70.8–99.6	75.7–92.9	2.7–3.9	216.0–360.0	n.d.

MLSS: mixed liquor suspended solids; n.d.: no data.

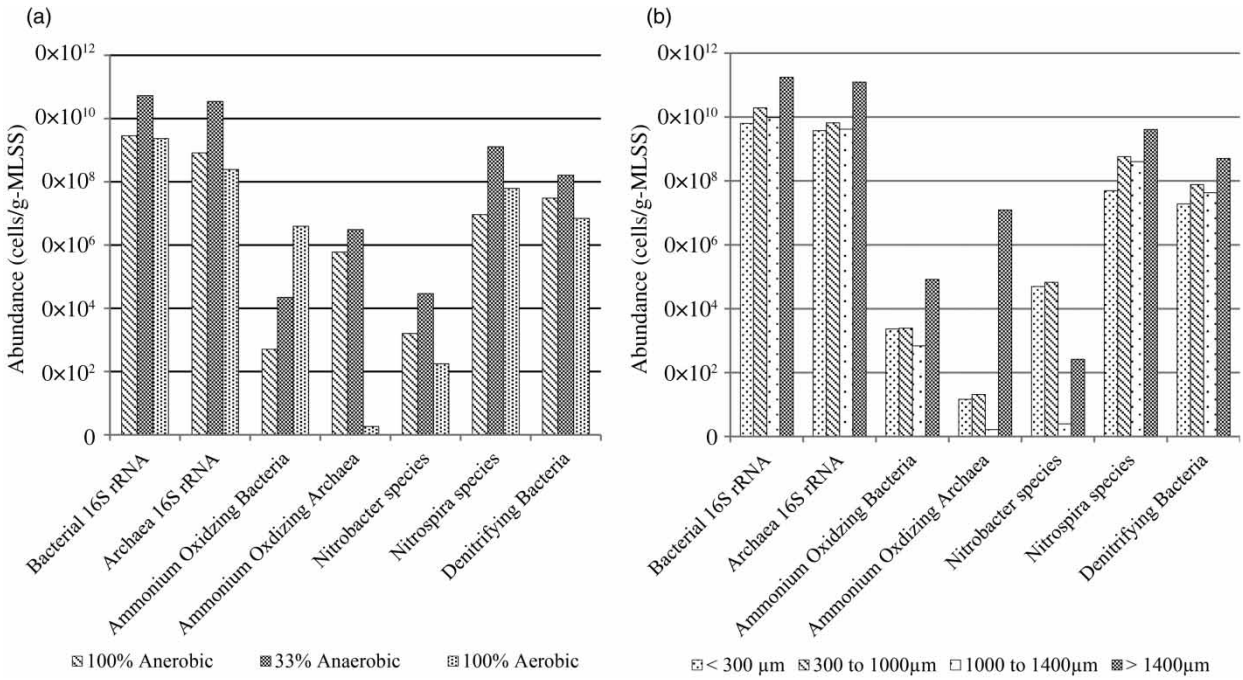

Figure 1 | Changes in the functional microbial ecology of biomass samples as determined by qPCR, comparing (a) Strategy A (100% anaerobic feed), Strategy B (33% anaerobic feed) and Strategy C (100% aerobic feed); and (b) the impact of AGS granule size (<300, 300–1,000, 1,000–1,400 and >1,400 µm) for Strategy B.

increased retention time (sludge age) of larger, denser granules can help explain the higher observed abundances of AOA within the AGS biomass relative to full-scale aerobic CAS operation, with the oxygen and nutrient gradient most likely contributing to niche-driven selective enrichment of slower-growing and less competitive archaeal ammonia-oxidisers over AOB (Short *et al.* 2013). Similarly, the development of the granular structures and increases in density and oxygen gradient may have driven the development of a nitrite-loop as seen by the increased abundance of *Nitrobacter* sp. (of 1–2 $\log_{10}$) and increased NOB/AOB ratio (by 20,000–50,000) within AGS. Similarly an increase in the proportion of NOB was also observed by Winkler *et al.* (2012a) which occurred when denitrifiers supplied NOB with nitrite through the reduction on nitrate, thereby forming a nitrite-loop. In this study, we did not observe an accumulation of nitrite or nitrate in the effluent or reduced total nitrogen removal performance. The size-separated biomass was also analysed using qPCR for the target functional genes (see Figure 1(b)). Notably, there was an increase in the abundance of AOA, *Nitrospirae* and denitrifying bacteria within larger granules (>1,400 μm), which was probably a result of the steeper oxygen gradient that exists across larger granules. Higher sludge age of the larger granules (>1,400; Figure 1(b)) may have further contributed to the increased abundance of AOA in this size fraction, given the slower growth rate of AOA relative to AOB (Pronk *et al.* 2015).

The increase in the abundance of *Nitrospirae* in the AGS samples seen within the qPCR was also confirmed through next generation sequencing (NGS), which showed an increase in abundance of phylum *Nitrospirae* organisms in samples taken from the two feeding strategies. Additionally, there was a clear increase in abundance of *Nitrospirae* in all granular morphologies when compared to the smaller floc-like biomass (data not shown). This increase in abundance has previously been linked to the development of the oxygen gradient whereby a study by Guimarães *et al.* (2017) showed the localisation of *Nitrospirae* towards the core as the granule increased in volume, which corresponded with an increase in their abundance.

Analysis of the high throughput sequencing data showed increases in phylum *Proteobacteria* from 55.1% in the 100% aerobic strategy C, to 66.8 and 72.5% in Strategy B (33% anaerobic) and Strategy A (100% Anaerobic) systems. This phylum contains class *Betaproteobacteria* and *Gammaproteobacteria* microbes, with these two classes including the PAO *Candidatus* phosphatis and glycogen-accumulating organisms, respectively (Lemaire *et al.* 2008). Relevantly, these microbes are known to be associated with AGS development and granular formation through the production of EPS (Figure 2). Further analysis of this class showed increased abundance of *Betaproteobacteria* in Strategy A (100% anaerobic), with the opposite in Strategy B (33% anaerobic). Within this class, NGS analysis showed the greatest enrichment of the PAO *Candidatus* Accumulibacter phosphatis occurred during Strategy A (1.3% total community abundance), compared with 0.13 and 0.17% for Strategy B (33% anaerobic feed) and strategy C (100% aerobic feed), respectively. This lower relative PAO abundance coincided with the reduced PO_4 removal performance observed within these systems. Furthermore, work by Wang *et al.* (2017) showed that the presence of increased abundances of *Proteobacteria* occurred within the high-saline granular sludge system while the abundance and metabolic activity of *Betaproteobacteria* decreased.

In comparison to the CAS flocs, the class *Gammaproteobacteria* had increased by 18.4 and 20.8% during Strategy A and B, respectively. This was largely attributed to a large enrichment in sulfide-oxidising bacteria (SOB) from the order *Chromatiales* and *Thiotrichaceae,* which collectively represented 23% of all operational taxonomic units (OTUs) compared to 6.6% within the CAS flocs. Size analysis of the separated granules found that the smallest granules had the highest representation of SOB (Figure 3(a)). It is possible that the uniquely high sulfate concentrations measured within the high-saline sewage (0.6–1.0 g/L), combined with the use of an anaerobic feed in the granular sludge pilot reactors, created conditions that favoured sulfate reduction, which provided a source of sulfide for the development of the SOB. Following this, investigation of the sulfate-reducing bacteria (SRB) population showed no large increase in abundance of SRB within the granular sludge biomass when compared to CAS (Figure 3(b)). SRB

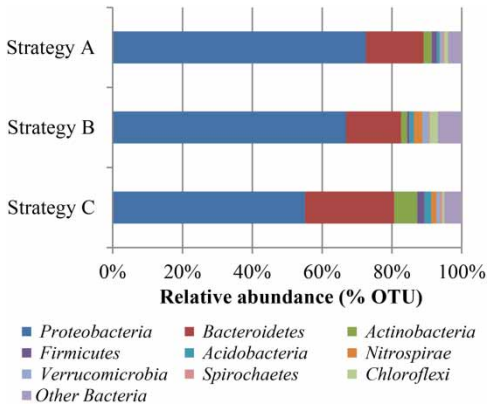

Figure 2 | Comparison of the relative abundances of microorganisms at the phylum level for each feeding strategy, which was determined using NGS of 16S rRNA.

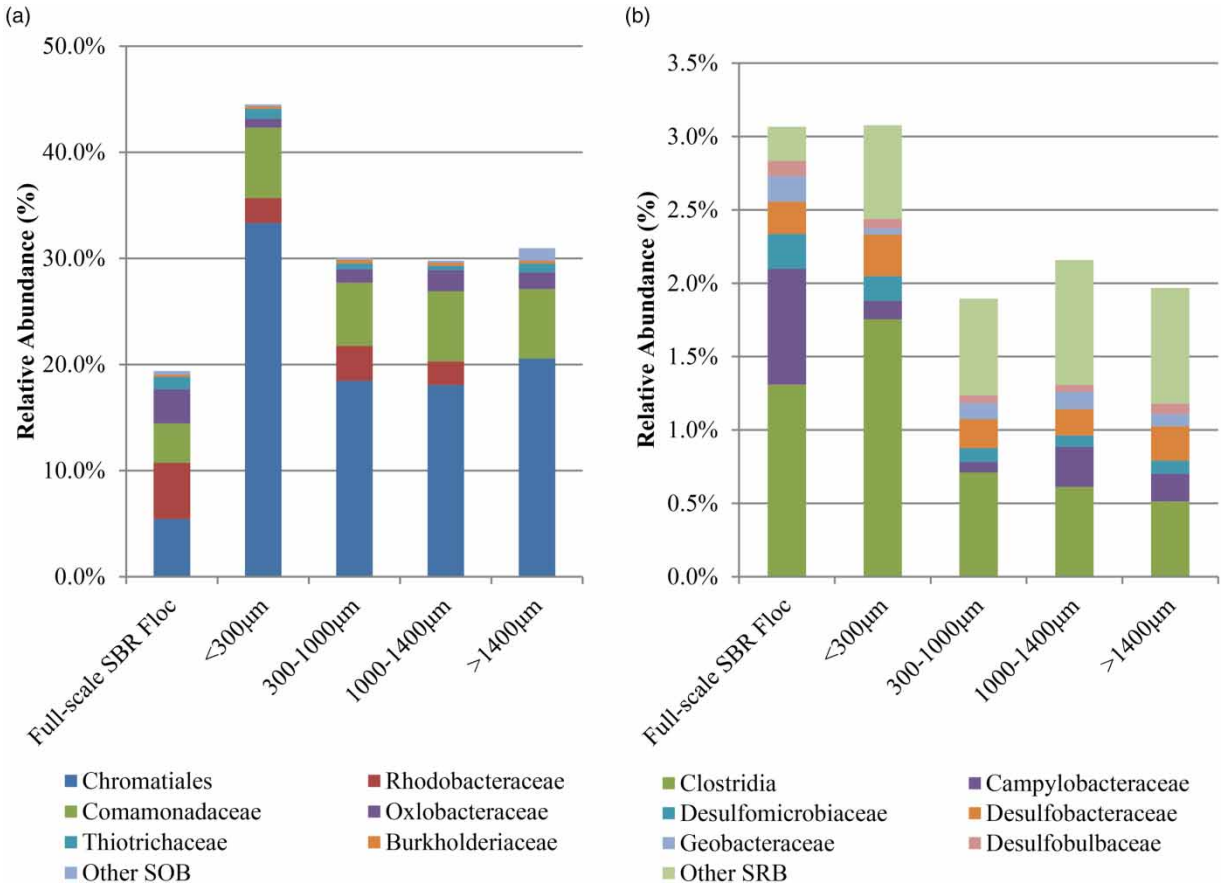

Figure 3 | Changes in abundance of sulfide-oxidising bacteria (a) and sulfate-reducing bacteria (b) of size-separated granules (<300, 300–1,000, 1,000–1,400 and >1,400 μm) that were sampled during Strategy B (33% anaerobic feed), with comparison to Strategy C (full-scale 100% aerobic feed).

were, however, well represented at >1.8% of OTUs within all floc and AGS samples. The increase in abundance of the SOB population in AGS may indicate that these organisms played important roles in the development and stability of AGS in high-saline and high-sulfide wastewaters. In this system sulfide may be oxidised by SOB under aerobic and/or anoxic conditions (i.e. autotrophic denitrification) due to the existence of an oxygen gradient within the AGS granules. Furthermore Rubio-Rincón *et al.* (2017), recently showed that the SOB *Thiothrix* (a genus in the order *Thiotrichaceae*) could also play an important role in biological phosphate removal under high-sulfide conditions. The role of SOB in AGS performance treating high-saline wastewater requires further investigation.

Scanning electron microscopy and X-ray florescence microscopy

SEM analysis comparing AGS and CAS samples identified very different external surface structures (Figure 4) and elemental composition. The AGS surface resembled microorganisms embedded in an exopolysaccharide-like crust. In contrast CAS was dominated by filamentous structures. EDS undertaken during SEM analysis indicated enrichment of sulfur on the surface of AGS, containing on average 2.9 At/wt% compared to 0.8 At/wt% detected on the CAS sample (Supplementary Figure 2, available with the online version of this paper).

Elemental analysis of AGS using XFM (Figure 5, full colour version at http://www.iwaponline.com/wst/toc.htm) complemented the EDS analysis (Supplementary Figure 2), which showed hot spots of elemental sulfur (red) precipitation as well as localisation of copper (blue) and zinc (green), which appeared to have strong affinity to co-localisation (cyan) with no apparent interaction between sulfur and both copper (purple) and zinc (yellow). Further analysis showed the sulfur concentration was 2.99 wt% with the zinc and copper forming 0.038 and 0.046 wt%, respectively. The evidence of elemental sulfur deposition within the granules can potentially be explained by the

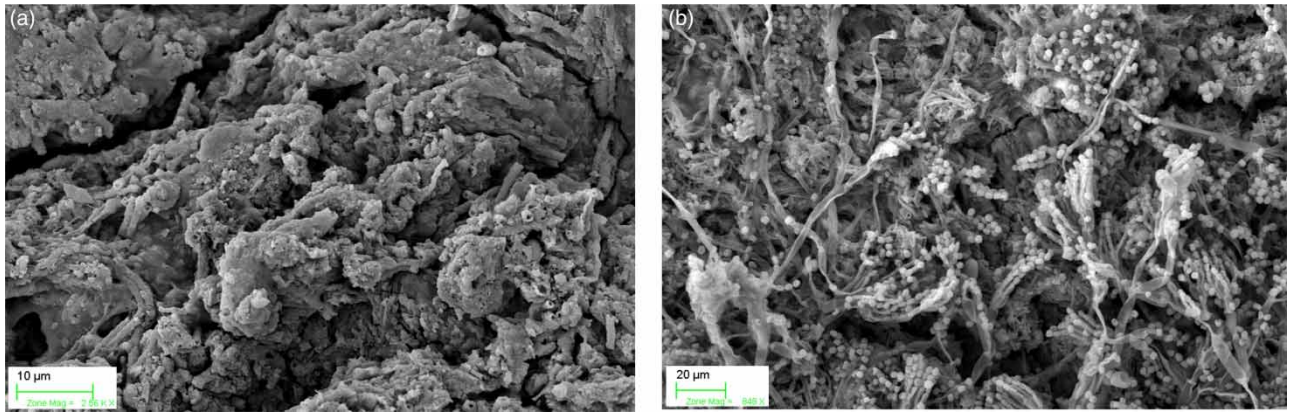

Figure 4 | Scanning electron micrographs comparing the surface structure of (a) AGS granules taken from Strategy B (33% anaerobic feed) and (b) biomass from strategy C (100% aerobic feed). Scale bar represents 10 µm and 20 µm respectively.

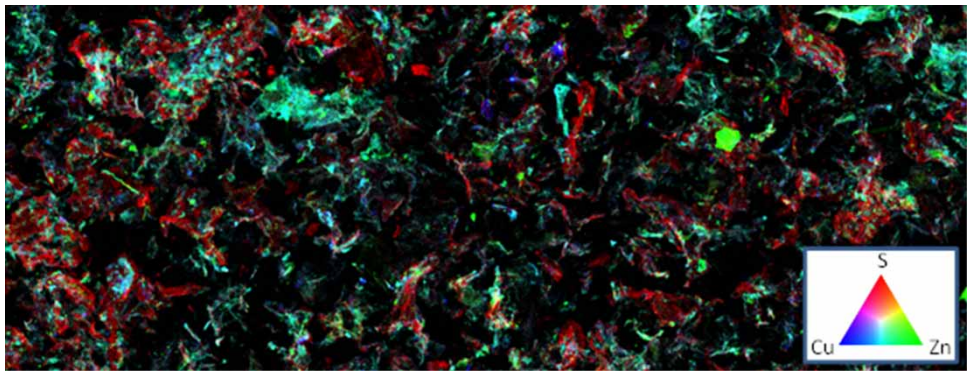

Figure 5 | Tricolor map from the X-ray florescence microscopy of AGS showing sulfur (red), zinc (green) and copper (blue). The full colour version of this figure is available online at http://www.iwaponline.com/wst/toc.htm.

increase in abundance of SOB within the AGS samples when compared to the CAS sample, given the oxidation of hydrogen sulfide by SOB can result in the production of elemental sulfur or sulfate. While only preliminary at this stage and lacking a CAS comparator, XFM results together with EDS and NGS observations suggest a potential role for SOB in AGS under high-saline (high-sulfate) wastewater applications, which warrants further investigation.

CONCLUSIONS

This pilot study showed that the formation and stability of AGS treating high-saline wastewater may not be as critically dependent on long anaerobic feeding conditions as previously published research suggests. The start-up time, stability and performance of AGS in the split anaerobic/aerobic feed (Strategy B) was comparable to that with a dedicated anaerobic feed (Strategy A). Analysis of the functional genes responsible for nitrification and denitrification showed changes in reactor functional microbial ecology between the two anaerobic feeding strategies, with no change in the nitrogen removal performance of the biomass. Reduced PO_4 removal performance was seen under partial anaerobic feed Strategy B relative to the 100% anaerobic feed Strategy A, with high throughput sequencing suggesting that this was probably the result of reduced PAO abundance. The increase in abundance of SOB in AGS indicates a potential role for these organisms in AGS development and stability, and warrants further investigation. This study has shown that AGS can be achieved and maintained with more challenging sewage characteristics such as those found in higher saline, high-sulfide municipal wastewater.

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