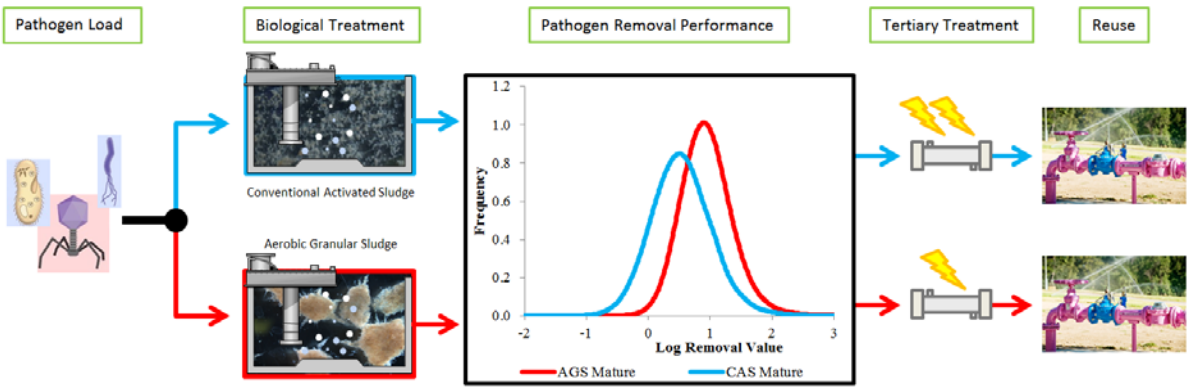
Comparing the performance of aerobic granular sludge versus conventional activated sludge for microbial log removal and effluent quality: implications for water reuse

Benjamin J. Thwaites[1], Michael D. Short[1,2], Richard M. Stuetz[1], Petra J. Reeve[3], Juan-Pablo Alvarez Gaitan[1], Nirmala Dinesh[3], Ben van den Akker[3,4,5]

1. UNSW Water Research Centre, School of Civil and Environmental Engineering, University of New South Wales, Sydney, NSW, 2052, Australia
2. Future Industries Institute, University of South Australia, Mawson Lakes, 5095, South Australia, Australia
3. South Australian Water Corporation, Adelaide, 5000 South Australia, Australia
4. Health and Environment Group, School of the Environment, Flinders University, Bedford Park, 5042 South Australia, Australia
5. School of Natural and Built Environments, University of South Australia, Mawson Lakes, 5095, South Australia, Australia

Graphical Abstract:



Abstract:

The application of aerobic granular sludge (AGS) technology has increased in popularity, largely due to the smaller physical footprint, enhanced biological nutrient removal performance and ability to perform with a more stable

operation when compared to conventional activated sludge (CAS) systems. To date, the ability of AGS to remove microbial pathogens such as; *Escherichia coli*, *Giardia*, and *Cryptosporidium* has not been reported. This study compared the log_{10} removal performance of commonly used pathogen surrogates (sulfite-reducing clostridia spores, f-RNA bacteriophage, *E. coli* and total coliforms) by AGS and CAS during the start-up phase, through to maturation. Results showed that AGS performed as well as CAS for the log_{10} removal performance of all microbial surrogates, except for spores which were removed more effectively by AGS due most likely to greater adherence of spores to the AGS biomass compared to CAS mixed liquor. Results suggest that AGS is capable of meeting or exceeding CAS-equivalent health-based targets for pathogen removal in the context of water recycling as well as not adversely affecting the secondary effluent water quality (suspended solids, turbidity and particle size) in terms of ultraviolet light transmissivity (254 nm). These findings confirmed for the first time that the adoption of AGS operation would not adversely impact downstream tertiary disinfection processes from altered water quality, nor would it require further pathogen treatment interventions in addition to what is already required for CAS systems.

Keywords: Aerobic granular sludge, biological nutrient removal, pathogenic indicator removal, wastewater recycling

1. Introduction

The use of aerobic granular sludge (AGS) in sequencing batch reactors (SBRs) has emerged as a viable alternative technology to traditional floc-based conventional activated sludge (CAS) for secondary level wastewater treatment. AGS conversion is achieved by modifying the operating parameters of SBRs to encourage CAS flocs to form dense, rapid-settling microbial granules. The conversion process has previously been shown to be heavily dependent on the application of an anaerobic feed combined with reductions in biomass settling time prior to decanting (Beun et al., 1999, de Kreuk, 2006, Pronk et al., 2015b). Together, these changes allow for the selection of slow-growing microorganisms (polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs)) and washout of slow-settling microbial flocs, respectively (Bassin et al., 2012, Liu and Liu, 2006). This increased settling velocity of granular sludge allows for reduced cycling times (i.e. increased hydraulic capacity) resulting in reduced wastewater treatment plant (WWTP) physical footprint (i.e. reduced infrastructure requirements) for new plants while increasing the hydraulic capacity of existing plants.

The role played by CAS processes for pathogen inactivation and removal from wastewater have been extensively studied (George et al., 2002, Koivunen et al., 2003, Lucena et al., 2004, Zhang and Farahbakhsh, 2007). These

studies have shown that pathogens are removed through two main methods: predation; and/or biomass adsorption. Predation can be highly dependent on biomass characteristics (Raboni et al., 2016), with bacterial removal previously shown to occur primarily by protozoan grazing during the aerobic phase (Curds, 1973). This is in contrast to virus removal, which occurs primarily as a consequence of biomass adsorption (Bales et al., 1993, Gray, 1990). Viruses adhere to the surface of the biomass and subsequently subjected to microbially-mediated inactivation, or physically removed via sedimentation (Arraj et al., 2005, Ng et al., 1993b, Ng et al., 1993a). Configuration of CAS operations can also influence virus removal, with Tanji et al. (2002) showing increased adsorption of phage (bacterial viruses) when the biomass was exposed to anaerobic–aerobic treatments.

While the benefits of adopting AGS have been extensively researched and documented over the last ten years, there is no current knowledge of how changes in the biomass structure (from CAS to AGS) affects pathogen removal performance. This has particular importance in the context of water recycling, where secondary treatment processes such as CAS also function as a critical treatment 'barriers' for pathogen removal, enabling water authorities and regulators to satisfy part of the health-based targets for water reuse (Wen et al., 2009, NRMMC et al., 2006) thus reducing the extent of additional tertiary treatment requirements. In Australia, for example, wastewater treatment processes—including CAS—are commonly assigned $\log_{10}$ removal values (LRVs) based on verified bacterial, protozoan and viral pathogen removal performance when configured as part of water recycling schemes under the national Guidelines for Water Recycling (NRMMC et al., 2006). These guidelines outline the required scheme-wide LRVs necessary for a given reuse scheme and recycled water end-use. Under these guidelines, elements with variable configuration and operation such as CAS systems require validation to demonstrate that the process can provide effective and reliable pathogen reductions. Validation is often based on characterising the LRV performance of reference pathogenic microorganisms, or their appropriate surrogates (e.g. spores, bacteriophage, faecal coliforms) and is typically performed under anticipated normal operating conditions. Validation studies have shown that pathogen removal during CAS treatment can typically achieve bacterial, protozoan and virus removals of 1.0–2.2, 0.7–2.5 and 1.9–2.5 $\log_{10}$ respectively (Wen et al, 2009).

For water recycling schemes in particular, it is important to understand the performance of AGS in regards to pathogen removal, as any loss in LRV capacity may require additional tertiary treatment interventions in order to meet stringent health-based targets for intended end use or discharge for environmental flows. Alternatively, there would be additional requirements placed on irrigators to implement different methods of irrigation to control dispersion. Additionally, there is no information currently available on the relative pathogen removal performance of AGS during start-up conditions, information that is important for WWTP operators to understand due to the significant changes that occur with regards to both biomass morphology and surface characteristics, and subsequent effluent water quality. Furthermore, the downstream impacts of AGS operation on effluent water quality also need to be considered, as any adverse change in the treated water quality parameters such as total suspended

solids (TSS), turbidity, colour, UV transmissivity (UVT) may impact on the performance and operational cost of UV and chlorine disinfection processes, which play an important role in delivering a robust multi-barrier approach to health risk management of recycled water. These impacts need to be investigated to ensure that any savings achieved by implementing AGS operation are not offset by the need for additional operational or capital expenditure due to loss in pathogen LRV performances or altered effluent water quality.

This study details the first investigation of pathogen LRV performance of AGS treatment (from start-up to mature operation), which was compared side-by-side with CAS operations at pilot scale. The variability in LRV performance was also characterised and linked to changes in effluent water quality, including UVT, SS and turbidity. LRV performance was based on the removal of four recognised pathogen surrogates in accordance with validation protocols of reuse schemes (Victorian Department of Health, 2013). This included sulfite-reducing clostridia (SRC) spores (conservative protozoan surrogate); f-RNA bacteriophage (human virus surrogate); *Escherichia coli (E. coli)*; and total coliforms (TC) (bacterial pathogen surrogates). These surrogates are widely used in validation exercises as they are typically more abundant in sewage than human pathogens and therefore allow high magnitude LRVs to be measured. The abundance and diversity of higher organisms in the two pilot reactors was also assessed, given the potential importance of predation on pathogen reduction in activated sludge systems.

2. Methodology

2.1 Pilot plant description

A pilot facility comprising two pilot reactors (Figure 1B) for CAS and AGS was established at a metropolitan WWTP in Adelaide, South Australia. The pilot plants used in this study have previously been described in detail by van den Akker et al. (2015). In short, pilot reactors had operating volumes of 61.5–63.5 litres and received untreated municipal sewage screened to ≈1 mm from the inlet of a neighbouring full-scale SBR plant. The typical feed sewage characteristics are shown in Table 1. SBR cycles of feed, aeration, settle and decant were controlled using a programmable logic controller. Both pilot plants were seeded with CAS harvested from a neighbouring full-scale SBR, to give an initial biomass concentration of 2 g/L when filled to operating volume.

Table 1: Typical influent sewage characteristics (in mg/L) of the high-saline municipal wastewater received at the full-scale SBR plant and pilot facility.

	TSS	NH_4^+-N	TN	PO_4^--P	COD
Mean ± 1 S.D.	150 ± 50	29.5 ± 2.8	30.6 ± 2.9	13.1 ± 1.71	414 ± 78.4
Range (min–max)	83–260	25.9–35.9	27.1–37.3	9.4–16.4	320–574

The CAS and AGS pilots operating conditions are given in Table 2. Briefly, the CAS pilot was operated with an aerobic feed and long settling time of 60 minutes to mimic the operation of the neighbouring full-scale SBR, whilst the AGS pilot was operated with an anaerobic feed and decreased settling time which had reached 12.5 minutes.

Table 2: Cycling conditions (in minutes) for both pilot plants.

	Anaerobic feed	Aerobic feed	Aeration	Settling	Decant	Total time
AGS Pilot	60	0	120	12.5–30	15–32.5	225
CAS Pilot	0	60	60	60	35	225

Loading and wastewater conditions for the CAS and AGS pilots are given in Table 3. The pilots were filled aerobically (CAS) or anaerobically (AGS) dependant on desired operating outcome with a volumetric exchange ratio of 25%. The dissolved oxygen (DO) concentration in the CAS and AGS pilots was maintained between 1.0–2.5 mg/L and 0.5–2.5 mg/L respectively for the duration of the trials (113 days). Settling time was reduced in the AGS pilot from 30 to 12.5 minutes over 74 days (immature phase days 0–73) with the pilot running 39 days (mature phase days 74–113) continuously at this settling time.

Table 3: Pilot plant performance data during the 113 day study (data in parentheses shows minimum and maximum performance range for each parameter)

	Dissolved oxygen (mg/L)	Organic load (COD kg/m^3/d)	Nitrogen load (TN kg/m^3/d)	MLSS (g/L)	Temperature (°C)
AGS Pilot	0.5–2.5	0.50	0.047 (0.042–0.057)	2.95 (1.77–4.67)	23.6 (18.8–27.2)
CAS Pilot	1.0–2.5	0.50	0.049 (0.043–0.059)	2.37 (1.66–2.75)	23.4 (15.2–25.9)

2.2 Monitoring and analysis

Wastewater treatment performance was monitored during the trial through the measurement of influent and effluent NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^--P and COD using commercial HACH® test kits (10031, 10019, 10020, 8048 and 8000 respectively). Total dissolved solids were analysed using HACH sensION 7 (USA). Mixed liquor was analysed for suspended solids (MLSS), effluent TSS and settleability using a 30-minute sludge volume index (SVI_{30}) following

standard methods (APHA, 1998). Changes in mixed liquor morphology were observed by light microscopy (Nikon, SMZ1000). Filamentous bacteria and higher organism identification and enumeration was conducted on CAS and mature AGS biomass samples (n = 3) at the Australian Water Quality Centre (Adelaide, Australia) according to the methods of Eikelboom (2000) and Jenkins (2003).

UVT analysis was conducted using a GENESYS 6™ UV-Vis spectrophotometer (Thermo Electron Corporation, Madison, Wisconsin, USA), with 5 mL samples placed into the quartz cuvette and absorbance analysed at 254 nm. Turbidity was measured using 2100N Turbidimeter (HACH). 10 mL samples were placed into the glass cuvette, inverted several times to mix, then analysed and reported as nephelometric turbidity units (NTU).

2.3 Sampling program

Grab samples of screened influent sewage, mixed liquor and treated secondary effluent were taken periodically during the trial phases. Influent sewage was sampled from an access point located immediately prior to the pilot reactors, mixed liquor samples were collected during aeration and effluent samples were collected from two locations: location A decant point (Figure 1, location A); and location B in to replicate a surface decant weir sample location used by full-scale SBRs (Figure 1, location B). Samples were collected between the hours of 0700 and 1100 and stored at <4°C prior to same-day microbial analyses.

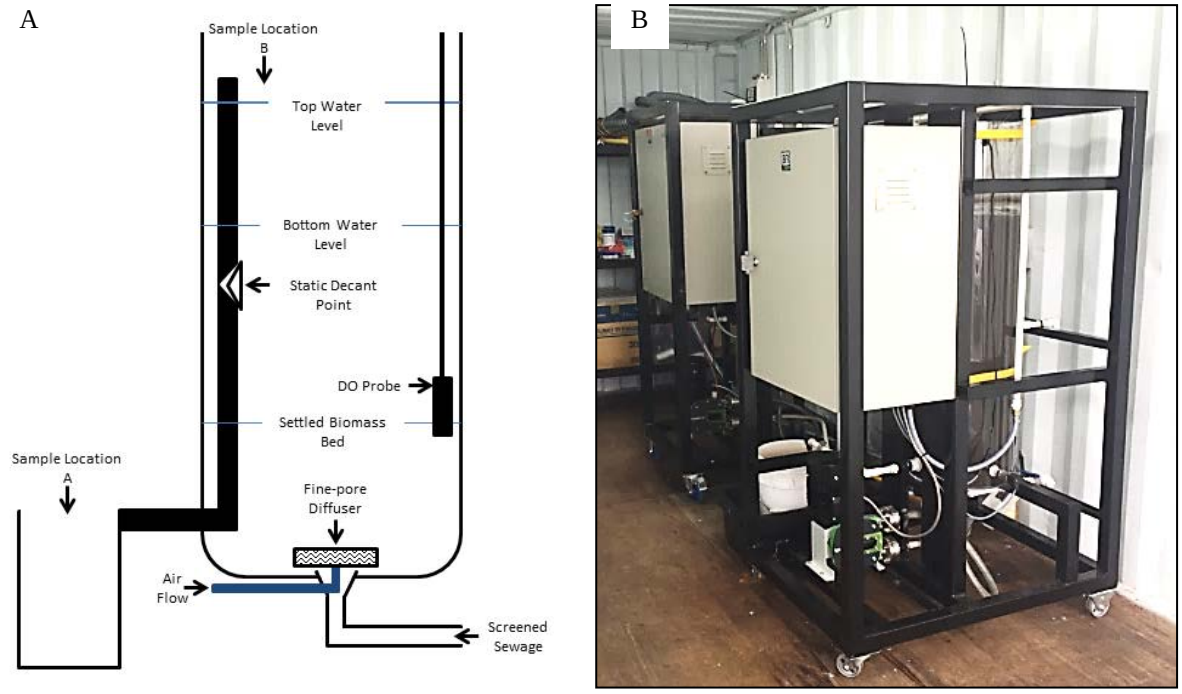


Figure 1: Schematic of the SBR pilot plants (A) operated for both AGS and CAS, showing the two effluent sampling locations (A and B) and (B) side-by-side pilots onsite

145

146 *2.4 Microbial indicator analyses*

147 Indicator bacteria (*E. coli* and TC) and the more conservative sulfite-reducing clostridia (SRC) spores were assayed
148 as outlined below after serial dilution of samples in sterilised UltraPure (Barnstead) water. For *E. coli* and TC, 100
149 mL of diluted sample was filtered through 0.45 µm gridded filter membranes (47 mm, Millipore, S-Pak), with the
150 membrane placed onto the surface of a dried plate of selective agar (Brilliance, Oxoid CM1046) and inverted and
151 incubated aerobically at 37°C for 24–36 h. *E. coli* was enumerated by counting blue colonies, while TC were
152 enumerated by counting both purple and blue colonies. *E. coli* colonies were confirmed by negative reactions to
153 Oxidase Test Strips (Oxoid, MB0266). SRC spore abundance was assessed on agar plates using Perfringens Agar
154 Base (Oxoid CM0587) plus Perfringens Selective Supplement (SR0088) for *Clostridium perfringens*. Samples were
155 filtered through 0.45 µm gridded filter membranes (47 mm, Milipore, S-Pak) and incubating inverted anaerobically
156 at 35 ± 1°C for 24–36 h. Prior to dilution a 50 mL aliquot of each sample was heated to 70°C for 20 minutes to
157 inactivate vegetative cells (Adcock and Saint, 2001). *E. coli,* TC and SRC abundance was expressed as colony-
158 forming units (CFU) per 100 mL.

159 f-RNA bacteriophage was quantified using the double agar layer technique as outlined in Noble et al. (2004). Briefly,
160 E. coli F-amp host (ATCC #700891) was cultured in TSB until growth phase was established (4-6 hours), this was
161 confirmed using absorbance spectrophotometry and pre-determined optimal range. 100 µL of *E. coli* culture was
162 added to molten tryptone soya agar (TSA) overlay containing 3mL of sample. Overlay samples were inverted to mix
163 and then spread evenly over dried TSA plates impregnated with 10 mg/L Ampicillin (Sigma-Aldrich). Agar overlays
164 were allowed to cool and set before being inverted and incubated aerobically for 24 h at 37 ± 1°C. Phage numbers
165 were determined by counting plaques (cleared zones) >2 mm and final abundance were expressed as plaque-
166 forming units (PFU) per 100 mL. All microbial indicator abundance data was $\log_{10}$ transformed for presentation and
167 further analysis.

168

169 *2.5 Filamentous and higher organism analysis*

170 Enumeration of filamentous bacteria was performed at the Australian Water Quality Centre (Adelaide, Australia)
171 using the in-house testing method TM-054, which was based on conventional microscopy of the morphological
172 features using the manuals by Eikelboom (2000) and Jenkins (2003). The relative abundance of 21 types were
173 assessed qualitatively using a scoring to characterise filament abundance ranging from 0 (none) to 6 (excessive).
174 Every sample was analysed by the same specialist to maintain consistency.

Higher organisms (namely protozoa and macroinvertebrates) were enumerated. Spirochaete and Zooglea sp. were analysed in parallel to filaments. Enumeration was based on conventional phase contrast microscopy of the morphological features using the methods described by Lindrea et al. (1999). The relative abundance of protozoan (free swimming ciliates, stalked ciliates, flagellates, suctoria, naked amoeba, testate amoeba and swimming amoeba), macroinvertebrates (rotifers and nematodes) and other organisms (spirochaetes and zooglea sp.) were scored semi-quantitatively ranging from absent (-); 1-2 observed (-/+); 5-10 observed (+); 10-100 observed (++); and >100 observed (+++).

2.6 Statistical analyses

Changes in nutrient removal performance were examined by nonparametric ANOVA (Brown-Forsyth ANOVA and Bartlett's t-test) analysis (immature AGS, Mature AGS and Mature CAS comparisons), unpaired t-tests (Mann-Whitney) were utilised to investigate the change in microbial indicator removals and effluent quality (suspended solids, UVT and Turbidity). Statistical significance was accepted at the $p<0.05$ level, with all analyses conducted using PRISM 6 (Version 6.07, GraphPad software, California, USA). Similarity of the filamentous bacteria and higher organism ecology in AGS and CAS were assessed using Bray-Curtis similarity analysis using Primer 6 (Primer-E, Pylmouth, UK) using average observed abundance values.

2.7 Probability density function (PDF) fitting

Microbial indicator data variability within the sampled influent, mixed liquor and decanted effluent was characterised by fitting exceedance probability plots. This form of analysis was utilised as it describes the data variability as used to inform quantitative microbial risk assessments. Indicator abundance was fitted with a normal probability distribution using @Risk software (Palisade Corporation, version 7.5) in order to perform a Monte Carlo simulation to determine the variation in indicator removal performance between immature AGS, mature AGS and the matured CAS pilots.

2.8 Biomass homogenisation

Mature AGS and CAS biomass samples were homogenised to detach microbial indicators in order to assess and compare the relative partitioning of microbial indicators between the liquid (wastewater) and solid (biomass) phases. Biomass samples (n = 4) were homogenised in the presence of a 1× Zwittergent 3-12 (Merc-Millipore) at 8000 rpm

(WiseTis Homogeniser, Thermoline Scientific) for a total of 4 minutes, with homogenisation being completed in one minute intervals (Caron et al., 2007). Indicators were measured before and after biomass homogenisation using methods outlined in Section 2.4 for un-homogenised samples.

2.9 Particle sizing

Particle size distribution (0.37–460.27 µm) was analysed using 100 mL CAS and AGS pilot reactor effluent samples collected from sampling location B (Figure 1) using a LISST-Portable instrument (Sequoia Scientific, USA). Full-scale secondary effluent was also collected and analysed to allow for direct comparison with the pilot scale data. Effluent samples were analysed in undiluted form, while non-homogenised and homogenised mixed liquor samples were diluted in sterile water to within acceptable analytical limits for the instrument. All samples were analysed in triplicate and the instrument chamber was rinsed with sterile ultrapure water (Barnstead) between samples. Particle size distribution was analysed using LISST Portable XR software, with final particle abundance given as number particles/L.

3. Results and Discussion

3.1 Pilot facility start-up and maturation

Figure 2 compares the SVI_{30} for both the AGS and CAS pilots over the study period (113 days). Analysis of the sludge settleability showed significant improvement in settling velocity for the AGS pilot over the initial 58 days of operation. During this time, AGS was achieved using the biomass washout technique whereby the settling time was reduced from 30 minutes down to 12.5 minutes. The formation of granules coincided with an increase in the biomass concentration (3.09 g/L to 4.67 g/L) with the AGS system and was considered to be mature from day 74 where it maintained an average SVI_{30} of 92.3.

In contrast to the AGS results, the SVI_{30} of the CAS system was consistently higher during the entire trial and was statistically similar to that of the full-scale SBR ($t_{(44)}$=0.291; p=0.134) with average SVI_{30} of 282.9 and 286.7 mL/g MLSS respectively. For comparison, Figure 2 also shows the full-scale SBR SVI_{30} over the same period, with results showing that both pilot and full-scale CAS plants were operating with similar sludge settling characteristics.

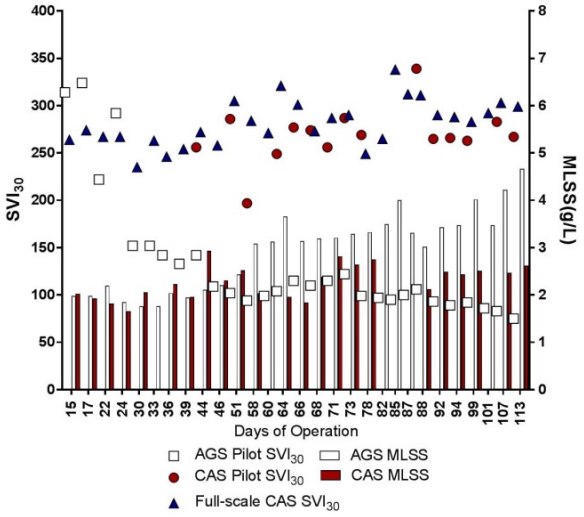


Figure 2: Biomass settleability (SVI_{30}; symbols) and mixed liquor suspended solids (MLSS; bars) for AGS, CAS and full-scale CAS SBR.

3.2 Nutrient removal performance and mixed liquor morphology

The nutrient removal performance of the pilots during mature (CAS) and immature (0–74 days) plus mature (75–113 days) (AGS) operation are summarised in Figure 3. For the AGS pilot, and despite distinct changes in biomass morphology from immature flocs to mature granules (Figure 4), nitrification performance was consistently high, as evidenced by the >95% ammonium removal performance throughout the trial. These NH_4^+-N removals are consistent with previously published studies (de Kreuk et al., 2005, Bassin et al., 2012) despite the change in morphology. Statistically, NH_4^+-N removal performance was similar between the AGS and CAS pilots, irrespective of operational maturity ($F_{(2,34)}$=1.03; p=0.368).

Total nitrogen removal efficiency, although statistically similar between AGS and CAS pilots ($t_{(35)}$=0.350; p=0.599), was quite variable and was likely attributed to the over-aeration in some cycles due to limitations with the pilot facility aeration control system, leading to impaired denitrification and oxidised nitrogen (NO_3^--N) accumulation similar to those observed in previous studies (Thwaites et al., 2017, van den Akker et al., 2015) (Supplementary Figure 1).

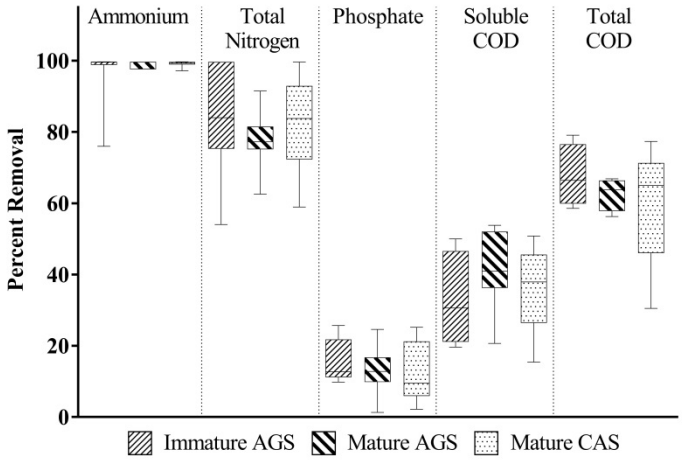


Figure 3: Treatment performance data (100–($C_{effluent}$/$C_{influent}$)×100) for immature (0–73 days) and mature (74–113 days) AGS and CAS pilots over the trial period (chart represented mean ± 1 S.D).

Soluble phosphate-phosphorus (PO_4^--P) removal was similar between both AGS and CAS pilots ($t_{(28)}$=0.880; p=0.386) regardless of biomass maturity. Net phosphate removals in the AGS reactor were markedly reduced when compared to the expected >90% removals seen in other studies (Bassin et al., 2012, de Kreuk et al., 2005, Pronk et al., 2014) and Winkler et al. (2011) who showed P-removal efficiency of 71 ± 5%. This may be indicative of the AGS being dominated by GAOs over PAOs (Winkler et al., 2011). Soluble COD removals were statistically similar between the AGS and CAS pilots ($t_{(24)}$=0.485; p=0.632) at around 35–40%, with the total COD removals also similar on average ($t_{(9)}$=0.676; p=0.52) at between 60–63%.

Morphological analysis of mixed liquor from pilot reactors via light microscopy revealed a distinct change in appearance with the conversion of CAS to AGS operation (Figure 4). Figure 4 shows the changes in mixed liquor morphology when examined using light microscopy with the distinct granular structure being observed by day 74 and maintained until the end of the trial. Figure 4A shows the mixed liquor that was used to seed the pilot operations, Figure 4B and C shows the biomass morphology at day 113.

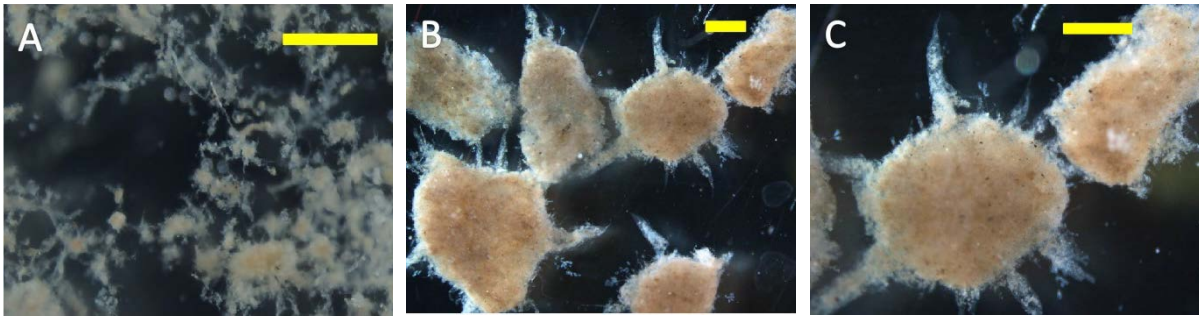


Figure 4: Mixed liquor suspended solids at day 0 (A) and at day 113 (B + C) from the AGS pilot. Scale bar represents 100µm

Morphological analysis of mixed liquor from pilot reactors via light microscopy revealed a distinct change in appearance with the conversion of CAS to AGS operation (Figure 4). Figure 4 shows the changes in mixed liquor morphology when examined using light microscopy with the distinct granular structure being observed by day 74 and maintained until the end of the trial. Figure 4A shows the mixed liquor that was used to seed the pilot operations, Figure 4B and C shows the biomass morphology at day 113.

3.3 Sampling location effects

It is important to note that both of the pilot reactors were built with a static decant outflow point that was situated above the settled sludge bed (Figure 1A). It was recognised that the effluent quality taken from this decant location may not be representative of treated secondary effluent that is collected from a surface decant weir which are common with most SBR installations. For example, there is potential for elevated suspended solids being discharged due to the close proximity of the decant point to the settled sludge bed, which can also result in increased abundances of viable pathogens and indicators. Parallel grab samples were therefore collected from the top of the reactors (Figure 1, Sample location B) to simulate off-take from a surface decant weir. Statistical analyses was then conducted comparing effluent concentrations of microbial indicators enumerated from the two decant locations and this showed that there were no significant differences between the two sample points for both CAS and AGS for all of the surveyed indicator organisms (Supplementary Table 1). Since both sample points produced statistically similar indicator organism abundances, data points from both sample locations were pooled for analysis and presentation hereafter.

3.4 Abundance of microbial surrogates

Abundances of microbial indicators were assessed within the influent sewage, biomass (MLSS) and treated secondary effluent of both CAS and AGS pilot plants using frequency distribution probability plots (Figure 5). Having data in this format provides an appreciation for inherent variability of treatment performance. Average concentrations of *E. coli*, TC and SRC spores in the influent sewage were 5.31×10^{7}, 5.15×10^{8} and 3.56×10^{5} CFU/100 mL respectively; while influent f-RNA phage concentration was on average 9.56×10^{4} PFU/100 mL (Figure 5). This analysis also showed that there was an average increase of approximately 1-log in abundance of SRC spores present in the AGS (1.38×10^{6}) and CAS (1.58×10^{6}) biomass samples when compared to the influent sewage. This accumulation was in contradiction to the other indicators analysed which showed approximately 1-log decrease in the biomass samples, indicating the potential for SRC spores to adsorb and accumulate within the

biomass. Analysis of the effluent samples taken showed concentrations of f-RNA concentrations were from the AGS pilot showed the average abundance of 2.72×10^2 (AGS) and 4.17×10^2 (CAS) PFU/100mL for f-RNA, 3.50×10^4 (AGS) and 7.54×10^4 (CAS) CFU/100 mL for SRC, 3.11×10^4 (AGS) and 8.43×10^4 (CAS) CFU/100mL for *E. coli,* 2.38×10^5 (AGS) and 6.63×10^5 (CAS) CFU/100 mL for TC

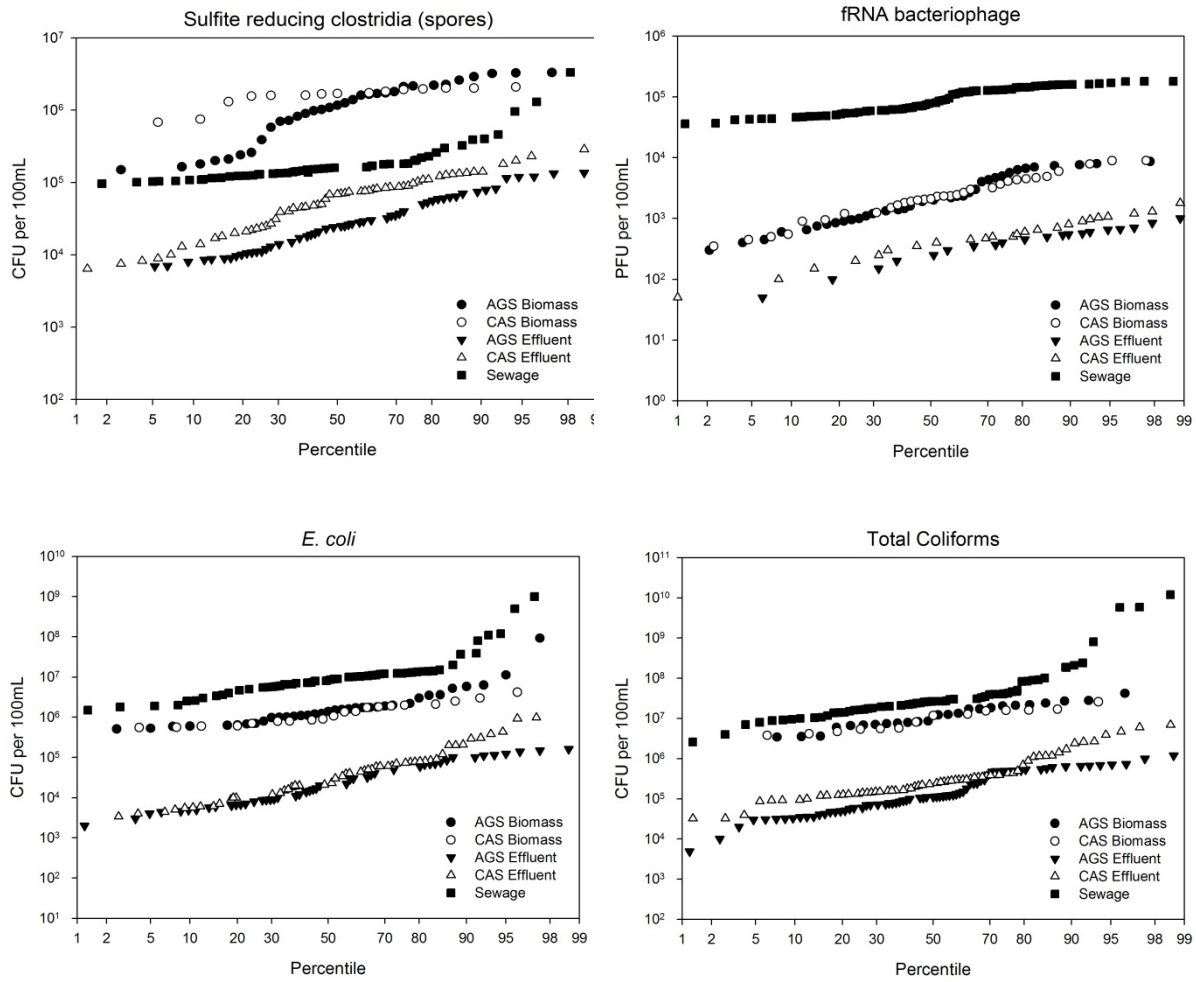


Figure 5: Frequency distribution plots showing the abundances of microbial surrogates within the sewage, mixed liquor and effluent of CAS and AGS pilot plants.

3.5 Log_{10} removal performance of microbial indicators

Log_{10} removal value (LRV) indicator performance PDF plots for both the CAS and AGS pilot (both immature and mature AGS) are presented in Figure 6. Monte Carlo simulation PDF fitting of all indicators enumerated from the raw sewage and decanted effluent was successfully achieved by applying a normal distribution. Median LRVs for *E.coli* and TC were only slightly higher for AGS (2.6- and 2.4-log_{10} respectively) when compared to CAS (2.4- and

2.0-log_{10} respectively). These values also compared well to the removal performance for CAS and secondary processes for f-RNA, *E. coli* and TC obtained from previously reported validation trials (Wen et al., 2009, NRMMC et al., 2006). Median LRV for f-RNA phage was the same for mature AGS and CAS at 2.4-log_{10} (Figure 6B). Interestingly, the median f-RNA phage LRV performance of the immature AGS (0–73 d operation) was 0.37 log_{10} greater than the mature AGS and CAS systems. To better understand the effects of AGS biomass morphology on pathogen removal, LRV performance was plotted over time from start-up to day 113 (Supplementary Figure S2). For SRC spores, LRV performance significantly improved around day 45–55 (Supplementary Figure 2B) which coincided with the time of granule maturation. An explanation for this improved LRV performance is unclear; however, it is possible that changes in biomass surface properties that occur during AGS start-up and maturation, such as increased EPS production (Liu et al., 2004, McSwain et al., 2005, Wingender et al., 1999), may have enhanced the potential for pathogen biomass attachment. For example, adsorption is considered a major removal mechanism of viruses in CAS systems (Wolfaardt et al., 1999), with previous work examining virus removal by CAS showing that virus interactions change with altered biomass morphology (Schijven et al., 2003).

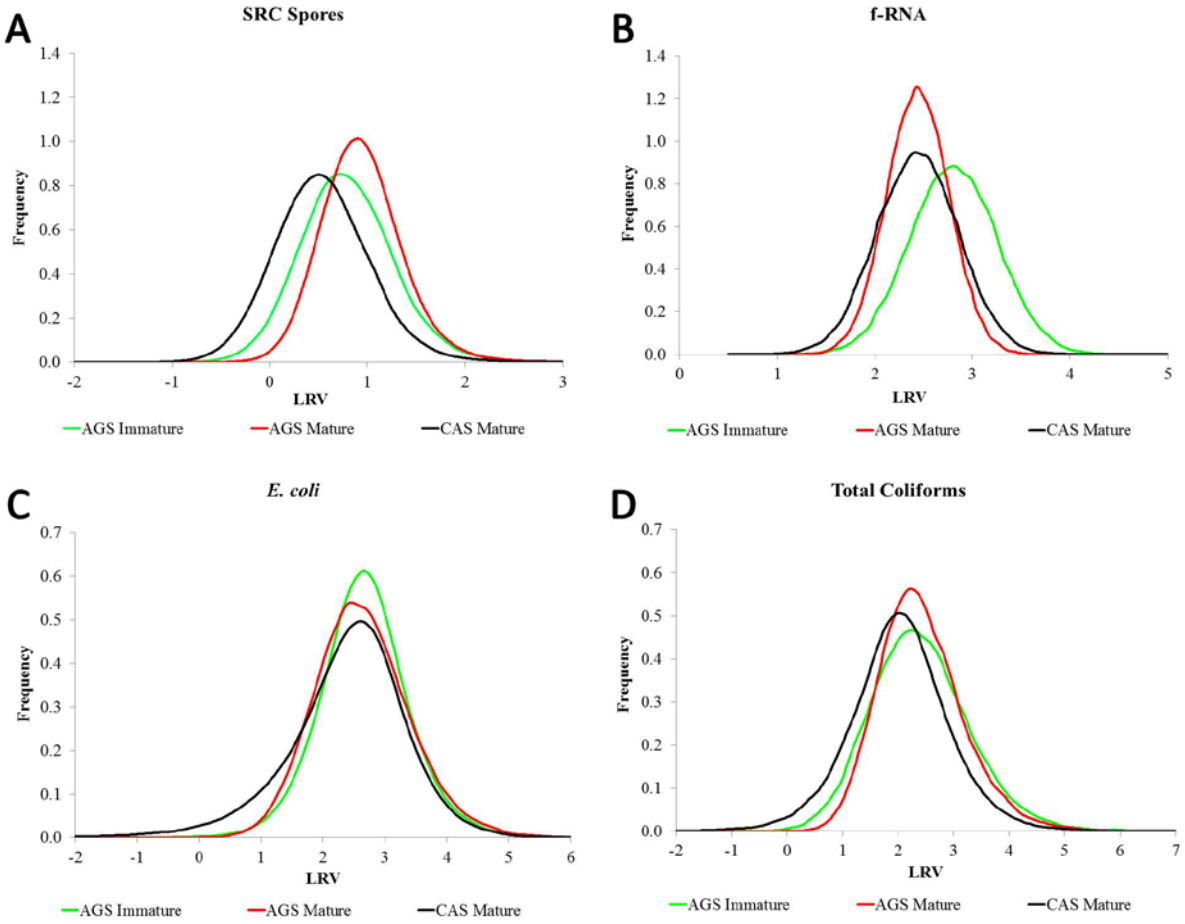


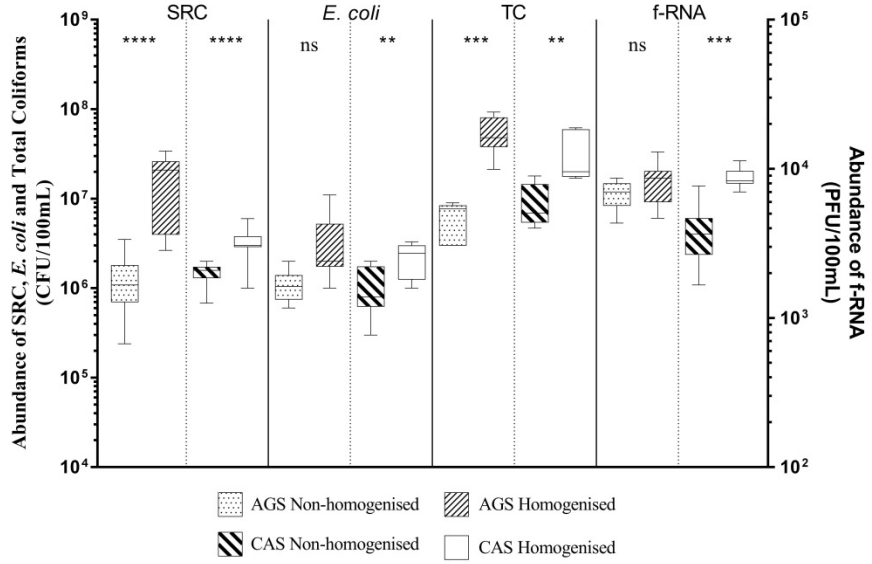
Figure 6: PDFs fitted using Monte Carlo simulations showing the log_{10} removal values (LRV) for immature AGS (green); mature AGS (red) and CAS (black) for all microbial surrogates.

The LRV of SRC spores (Figure 6A) was lower than that of the surveyed vegetative indicator (*E. coli* and TC) bacteria and phage in both CAS and AGS and therefore can be viewed as a conservative estimation for the worst-case removal performance benchmark for risk assessment purposes. The median LRV of SRC spores was however greater in the mature AGS (0.91-$\log_{10}$) when compared to CAS (0.46-$\log_{10}$) and immature AGS (0.78-$\log_{10}$) (Supplementary Table 2). Given anaerobic spores are known for their ability to strongly adhere to biomass and resist predation, the nature of this improved LRV performance of AGS over CAS was further investigated in Sections 3.6 and 3.7.

While mean LRVs for *E. coli* (2.64 and 2.38) and TC (2.42 and 2.02) were similar between CAS and AGS respectively, probability plots showed that LRV variability was moderately higher in the CAS system (Figure 6C and D). When comparing 5th percentile LRV, the mature AGS outperformed CAS with regards to *E. coli* (1.45 and 0.64*)*, TC (1.29 and 0.56) and SRC spores (0.31 and -0.27) by a LRV difference of 0.6–0.8-$\log_{10}$ (Supplementary Table 2).

3.6 Indicator organism adsorption potential of biomass

Initially, the biomass samples from CAS and AGS were homogenised in order to compare the role of adherence/adsorption of microbial indicators to biomass. Secondly, comparison of higher organism abundances in CAS and AGS was also done to see if the potential for predation differed between the two systems. Samples of AGS and CAS were homogenised to detach phage, spores and bacteria to investigate and compare the potential role of biomass adsorption and accumulation in the removal of indicator organisms. The abundances of indicator organisms were calculated and compared in both homogenised and non-homogenised samples (Figure 7). Homogenisation of biomass samples from both mature AGS and CAS showed increased abundances (release) of all microbial indicators following the homogenisation treatment (Figure 7). Following homogenisation of AGS mixed liquor, organism abundances increased by 1.05-$\log_{10}$ for SRC spores ($t_{(35)}$ = 5.17, p<0.0001), 0.92-log for TC ($t_{(16)}$ = 4.95, p<0.0001), 0.52-$\log_{10}$ for *E. coli* ($t_{(14)}$ = 1.84, p=0.09) and 0.08-$\log_{10}$ for f-RNA bacteriophage ($t_{(28)}$ = 1.81, p=0.08). Homogenisation of the CAS mixed liquor gave 0.35–0.36-$\log_{10}$ increases for SRC spores ($t_{(23)}$ = 4.79, p<0.0001), *E. coli* ($t_{(14)}$ = 3.17, p=0.007) and f-RNA phage ($t_{(28)}$ = 9.48, p<0.0001) and a 0.54-$\log_{10}$ increase for TC ($t_{(15)}$ = 3.03, p=0.008). Alternatively, the homogenisation process may not have been significantly aggressive enough in order to successfully detach the phage.



348

349 *Figure 7: Results from homogenisation of mixed liquor in the presence of a 1x Zwittergent showing significance*
350 **>0.05, **>0.01, ***>0.001 and ****>0.0001*

351

352 The higher release of SRC spores, *E. coli* and total coliforms measured following homogenisation of the AGS
353 biomass suggests that the AGS exhibited an increased potential of adherence of microbial surrogates when
354 compared to the CAS mixed liquor. This effect was greatest with SRC spores, with AGS displaying higher potential
355 for the entrapment of spores, which may explain the superior LRV performance by AGS (Supplementary Table 2).
356 These results indicate that higher partitioning of indicators into AGS biomass may occur, which may be a potential
357 advantage of AGS. While the homogenisation technique used here is an accepted method to dislodge
358 microorganisms bound to particulate material (Caron et al., 2007, Li et al., 2009), it is however unable to distinguish
359 between microbial indicator organisms that are attached to the surface of AGS versus those that might be entrapped
360 internally as the AGS develops. For the SRC spores, the possibility of internal entrapment is of interest and worthy
361 of further investigation, given the ability of spores to withstand long periods of dormancy. While increased
362 partitioning into the biomass is an advantage for secondary effluent disinfection, there is potential for the increased
363 load to be impacting downstream sludge treatments including thickening, digestion and biosolids
364 stabilisation/drying. Further work investigating the potential downstream impact of the increased pathogen removal
365 has on sludge treatment and the role EPS plays on the (ir)reversible adsorption of key pathogen groups is required
366 to better understand the public health performance of AGS systems.

367

368 *3.7 Higher Organisms*

As highlighted above, pathogen removal in activated sludge processes can be achieved through several pathways, including adsorption onto the biomass or predation by resident bacteriophages, protozoa or metazoa. The predatory role played by many of these organisms in attenuating activated sludge pathogens or their surrogates has been described by Curds and Fey (1969), Kim and Unno (1996), Ng et al. (1993b) and Seviour et al. (2010). Given the potentially important role of predation in pathogen inactivation and removal in activated sludge systems, it was important to measure and compare abundances of higher organisms in CAS and AGS, which could help explain any differences in indicator organism LRV performance. In particular, we sought to understand the abundance of those known to graze on activated sludge bacteria (i.e. amoeba, flagellates and ciliates).

Results showed that higher organism abundances remained stable throughout the course of the study, with no observable changes in the abundances of all amoeba, rotifers and Zooglea in samples from the pilot AGS and CAS and the full-scale CAS systems (Figure 8A). Dubber and Gray (2011) showed that higher organisms such as amoeba and ciliates abundances are not affected by periodic anoxic or anaerobic conditions, which is also supported by the findings of this study (Supplementary Figure 3) as such operational conditions which occur in both the AGS and CAS systems presented here and in other studies. Bray-Curtis analysis showed that there is no pronounced difference in higher organism diversity or abundance found within the AGS biomass when compared to both the CAS pilot and neighbouring full-scale SBR plant (Supplementary Figure 4). Given this similarity, the potential role for predation in AGS and CAS is similar and is therefore unlikely to explain differences seen in indicator organism LRV performances between the two systems.

In addition to the analysis of higher organisms, the presence and relative abundance of filamentous bacteria in the MLSS of AGS and CAS pilots and the full-scale SBR was investigated (Figure 8B). While filamentous bacteria may not play a direct role in the removal of microbial indicators via predation or adsorption, they do impact on biomass liquid separation performance and hence effluent water quality (Eikelboom and van Buijsen, 1993). For example, a healthy and balanced community of filamentous bacteria play an important role in floc formation and serve to catch and hold small particles during sludge settling, thereby yielding a lower turbidity effluent. Conversely, the proliferation of filaments will result in sludge bulking and elevated effluent solids. Given the relationship between effluent turbidity and effluent pathogen load, it was therefore necessary to consider differences in filamentous bacteria type and abundance between AGS and CAS. Figure 8b showed a reduction in filamentous bacteria Type 021N, Type 1851 and Beggiatoa abundance in AGS compared to CAS, however the diversity was similar. Data analysis using Bray-Curtis analysis found that the ecologies of filamentous bacteria in AGS and CAS samples were >70% similar (Supplementary Figure 4). The reduction in observable filamentous bacteria in the AGS biomass samples is consistent with previous research conducted by Tay et al. (2001) and van den Akker et al. (2015). This highlights the benefit of AGS limiting filamentous bacterial growth to facilitate superior sludge settling, however not to the extent that would be detrimental to the clarified effluent quality.

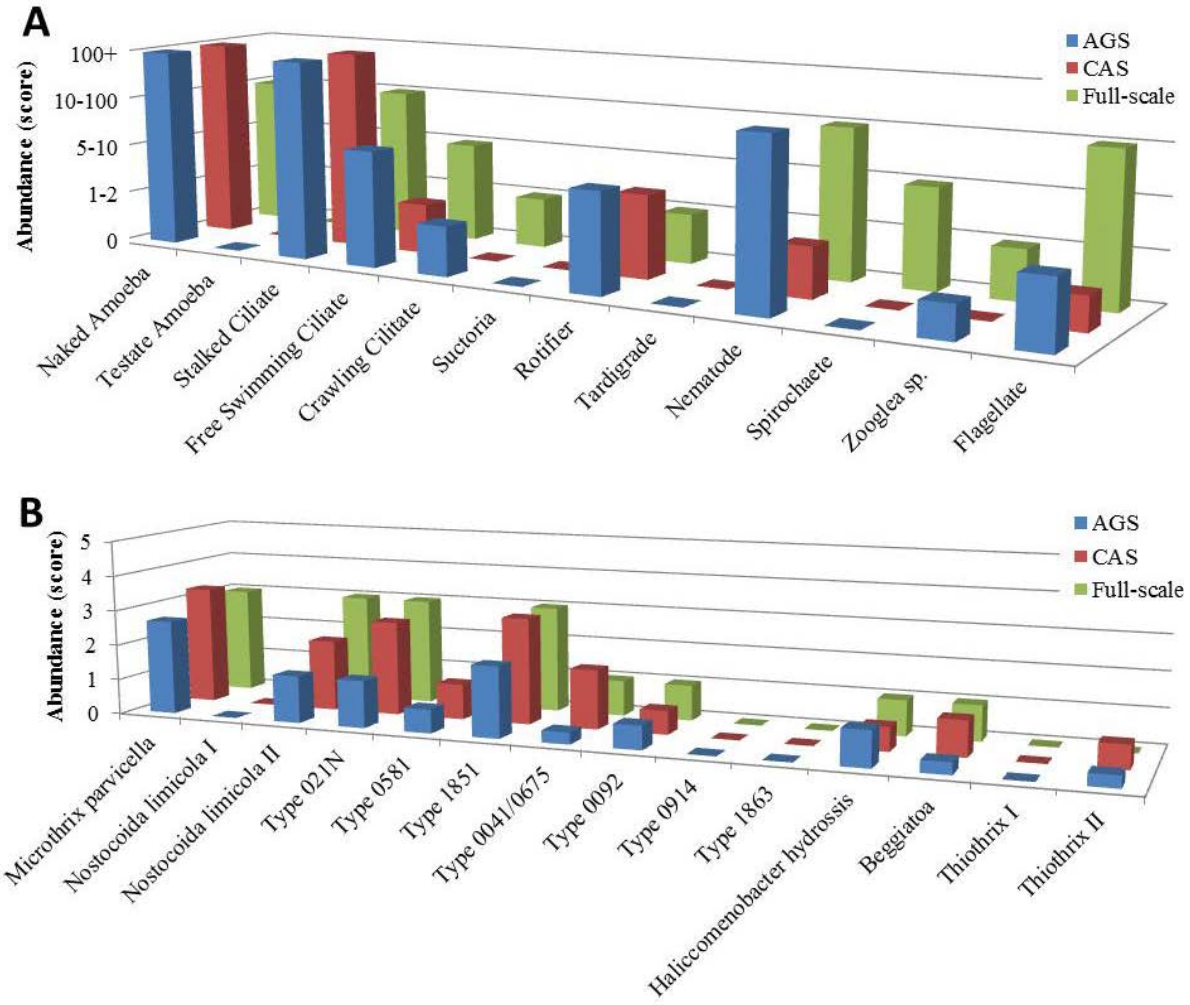


Figure 8: Comparative analysis for filamentous bacteria (A) higher organisms and (B) present in mixed liquor suspended solids collected from AGS and CAS pilot plants as well as full-scale CAS SBR. Mean abundances of organisms presented from samples (n = 3).

402

403 *3.8 Implications of AGS on secondary effluent quality and water reuse*

404 Secondary effluent samples were analysed for UVT (absorbance at 254 nm), turbidity and TSS (Table 4). TSS
405 analysis showed that the concentration was not significantly different ($t_{(39)}$=1.51; p=0.14) between the AGS and
406 CAS pilots. The turbidity results showed that there was a decreased turbidity of the effluent samples collected from
407 the AGS pilot ($t_{(14)}$=0.77; p=0.46) compared to the CAS pilot. Particle size profiling (Section 2.9) was completed on
408 secondary effluent samples from both pilot reactors as well as the full-scale SBR in order to better understand the
409 impacts of various un-retained biomass size fractions on the final effluent quality. Analysis was conducted on
410 samples drawn from sampling location A (Figure 1) to represent the worst case scenario, as this location collected
411 a greater fraction of washed out MLSS. Effluent particle size profiles (Figure 9) showed a greater similarity between
412 CAS operation (pilot and full-scale), with relatively more larger particles (≥100 µm) in the final effluent, while particles

washed out of the matured AGS pilot showed a decreased abundance of particles at larger sizes (≥100 µm) and a decreased total suspended solids concentration (Table 4). This indicates that while small supracolliodal (1–100 µm) particles are persistent in both biomass samples there was a divergence in profile around 30 µm with AGS having a reduction in the presence of larger material. In addition, there was a decreased abundance of the settleable material (>100 µm) and the large reduction in large (>150 µm) particles also suggests that the retained biomass was rapidly settling granular structures as the majority of washed out material is within the conventional activated sludge size spectrum. This shows that AGS operation is unlikely to negatively impact the downstream tertiary treatment processes such as chlorination and UV disinfection which are highly sensitive to increases in suspended solids and turbidity (Chahal et al., 2016). Additionally the secondary effluent quality would not be negatively impacted during AGS start-up and mature operation.

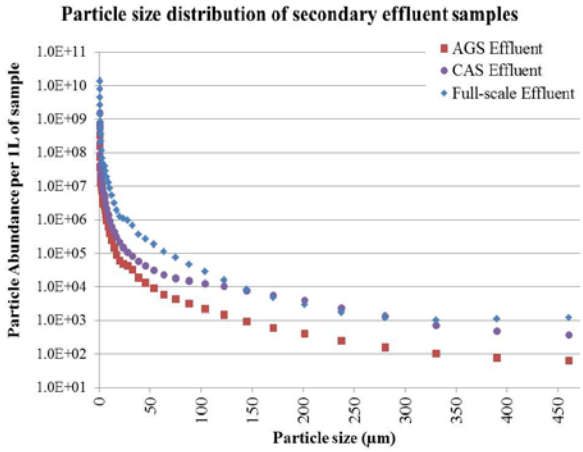


Figure 9: Analysis of secondary effluent particle size distribution (0.37–460.27 µm) from pilot AGS and CAS reactors alongside full-scale CAS effluent.

Table 4: Secondary effluent water quality data for AGS and CAS pilot reactors collected during the 113 days of operation (mean parameter data given ± 1 s.d.)

UVT (absorbance at 254 nm)		Turbidity (NTU)		TSS (mg/L)	
AGS	CAS	AGS	CAS	AGS	CAS
0.3 ± 0.02	0.3 ± 0.04	4.2 ± 1.6	5.2 ± 2.6	48.3 ± 18.3	58.4 ± 21.5

Results from microbial indicator monitoring have shown that AGS offers an increased capability to remove and/or retain SRC spores, *E. coli* and total coliforms. This increased removal performance has the potential to allow for a direct process saving through the reduction in the tertiary treatment requirements for water recycling applications. Nominally, as the largest increase in removal performance for AGS over CAS was identified in the SRC spores, the

greatest saving would therefore be in the reduction of disinfection. Additionally, since it was shown that the transition to AGS operation did not significantly impact secondary effluent water quality, it is unlikely to require any additional treatment in terms of filtration and disinfection power/reactors.

For wastewater recycling schemes, individual treatment barriers are typically conservatively accredited based on their validated 5th percentile LRV as a worst case performance indicator. This research has shown that for AGS operation, improvements in microbial LRV capacity are likely to translate into treatment operations and capital savings for downstream disinfection processes, while still meeting regulated recycled water quality guidelines (such as health-based targets). Increase in LRV by applying AGS may suggest that tertiary UV or chlorine doses could be reduced, or depending on the end-use, additional tertiary treatment or onsite exposure controls may not be required. Results also showed that immature AGS had no detrimental impact on LRV performance when compared to CAS performance, which provides confidence that there are no adverse impacts on exceeded health-based targets during start-up which involves active washout of poor settling biomass.

4. Conclusion

The application and performance of AGS has been widely investigated for many types of sewage and various performance characteristics; however, to date no reports of AGS pathogen removal performance exist. This study showed that microbial indicator LRV performance of AGS was equivalent to CAS for bacteria and viruses and significantly better for SRC spores (protozoan pathogen surrogate). Treated effluent water quality was also maintained during the pilot start-up phase for CAS to AGS conversion, suggesting that adverse impacts on downstream disinfection processes are unlikely to be experienced. This shows that AGS is capable of meeting CAS-equivalent health-based targets for pathogen removal.

During AGS start-up (0–74 days) there was more variability in LRV performance for all indicator organisms, owing to the washout of biomass and higher TSS in the effluent; nevertheless, LRV values were still higher than that recorded in the CAS systems which suggest that start-up of AGS is unlikely to pose an unacceptable level of risk of pathogen breakthrough and performance challenge to downstream disinfection and water recycling operations. Monte Carlo simulation also showed a lower variation in indicator removal performance for the mature AGS when compared to the CAS pilot.

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