Co-optimization of sponge-core bioreactors for removing total nitrogen and antibiotic resistance genes from domestic wastewater

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ABSTRACT

Inadequate sanitation can lead to the spread of infectious diseases and antimicrobial resistance (AMR) via contaminated water. Unfortunately, wastewater treatment is not universal in many developing and emerging countries, especially in rural and peri-urban locations that are remote from central sewers. As such, small-scale, more sustainable treatment options are needed, such as aerobic-Denitrifying Downflow Hanging Sponge (DDHS) bioreactors. In this study, DDHS reactors were assessed for such applications, and achieved over 79% and 84% removal of Chemical Oxygen Demand and Ammonium, respectively, and up to 71% removal of Total Nitrogen (TN) from domestic wastes. Elevated TN removals were achieved via bypassing a fraction of raw wastewater around the top layer of the DDHS system to promote denitrification. However, it was not known how this bypass impacts AMR gene (ARG) and mobile genetic element (MGE) levels in treated effluents. High-throughput qPCR was used to quantify ARG and MGE levels in DDHS bioreactors as a function of percent bypass (0, 10, 20 and 30% by volume). All systems obtained over 90% ARG reductions, although effluent ARG and TN levels differed among bypass regimes, with co-optimal reductions occurring at ~20% bypass. ARG removal paralleled bacterial removal rate, although effluent bacteria tended to have greater genetic plasticity based on higher apparent MGE levels per cell. Overall, TN removal increased and ARG removal decreased with increasing bypass, therefore co-optimization is needed in each DDHS application to achieve locally targeted TN and AMR effluent levels.

Keywords: Antibiotic resistance genes; sustainable wastewater treatment; wastewater bypass; denitrification; high-throughput qPCR

1. Introduction

Effective wastewater treatment and community sanitation are critical to global health and environmental protection. However, almost 2.5 billion people live without access to even basic sanitation (United Nations, 2015), which impacts infectious disease mortality and increases exposure to environmental antimicrobial resistance (AMR) via contaminated water (Hu et al., 2008; Manaia et al., 2016; Pruden et al., 2013; Quintela-Baluja et al., 2015; WHO, 2014; Zhang et al., 2009a). The impact of water- and waste-borne AMR releases is most profound in emerging and developing countries because waste management is not proceeding as rapidly as urbanisation, leading to declining environmental quality as development occurs. Accordingly, the United Nations has committed to reduce the lack of sanitation in half by 2030 (United Nations, 2016) and is espousing the One Health approach to combat AMR in the environment (Robinson et al., 2016; Singh, 2017). However, problems exist in expanding peri-urban environments because such locations often lack centralised sewage collection. As such, smaller, local-scale treatment options are needed to increase wastewater treatment coverage, although few reliable "small" technologies exist that reduce carbon (C) and total nitrogen (TN) levels as well as mitigate against waterborne pathogens and AMR releases.

Denitrifying Downflow Hanging Sponge (DDHS) reactors are a low cost and low maintenance wastewater treatment option that is suitable for smaller or decentralised applications (Bundy et al., 2017). DDHS systems can achieve high Chemical Oxygen Demand (COD), Ammonium-Nitrogen (NH_4-N) and TN removals by using bipartite aerobic-anoxic sponge layers and a raw wastewater bypass to supply extra carbon to lower submerged layers to promote denitrification (Isaacs & Henze, 1995; Schipper et al., 2010). The wastewater bypass is crucial to DDHS systems because, when carbon is

removed in top aerobic layers, lower layers become C-limited for denitrification, restricting conversion of nitrate to N_2, which is critical for application in places like China with tight TN discharge standards (Ministry of Environmental Protection (MEP), 2002). Further, DDHS systems use minimal energy because they employ passive aeration and also provide design flexibility in the sponge core (e.g. varying redox zones, reactor volumes and density ratios) that can be customised to local conditions. However, little is known about how DDHS reactors remove AMR genes (ARGs) and mobile genetic elements (MGEs) during treatment. There is reason to believe DDHS systems may be quite effective because sequenced redox conditions can enhance ARG removal (Christgen et al., 2015).

Here we used high-throughput qPCR (HTH-qPCR) to compare influent and effluent ARGs and MGEs in DDHS bioreactors as a function of wastewater bypass. Selected microbial culturing also was performed for Gram (-) Extended Spectrum Beta-Lactamase-producing bacteria (ESBL-producing) to compliment ARG and MGE data as well as TN and other treatment metrics. Such data is key for process optimisation, especially where TN and AMR reductions are both desired, such as places where improved decentralised treatment is urgently needed (e.g. China, India).

2. Material and Methods

2.1. DDHS reactor configurations

Four bench-scale DDHS bioreactors were set up as previously described (Bundy et al., 2017) and operated in parallel for 210 days. Each continuous-flow bioreactor was identical, made from PVC cylinders (0.5 m tall x 0.14 m internal diameter; working volumes = 3 L), and configured to include internal recirculation and a wastewater

bypass (also called “shunting”) to the submerged layer (Figure S1; see Supporting Information, SI). DDHS reactor cores consist of an upper hanging sponge layer exposed to air from above, below, and through side vents, which provide passive aeration for C-removal and nitrification; and a bottom anoxic sponge layer for denitrification, prospectively enhanced by wastewater shunting. Reactors were seeded with nitrifying return activated sludge (RAS) to encourage biofilm growth within the sponge matrix, and were operated in continuous-flow mode with an organic loading rate of 0.4 kg COD/m3-sponge/day (HRT = 0.6 days) and under room temperature environment (22-23 °C) (Bundy et al., 2017).

The reactors were designated R-S0, R-S10, R-S20 and R-S30, being defined by different bypass percentages; 0%, 10%, 20% and 30% (% of total wastewater by volume), respectively. R-S0 with no bypass was the control unit. Previous work showed TN removals were most efficient at bypass levels of 20 to 30% (Bundy et al., 2017). A 10% bypass was included to allow step-wise analysis from zero to 30%, to co-optimise the DDHS reactor for simultaneous TN and ARG removal.

2.2. Influent source, routine sample analysis and monitoring

Influent and effluent samples were collected and analysed to monitor treatment performance. Fresh settled wastewater (post primary settling; called ‘raw’ here) was collected weekly from a municipal wastewater treatment plant in northern England and stored at 4 °C prior to use as reactor influent. Raw wastewater was fed in parallel via influent pumps to all reactors from an 18-L carboy retained in a fridge located next to the reactors. Analyses on influent and effluents included Soluble COD (COD_s), Total COD (COD_t), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Total

Kjeldahl Nitrogen (TKN), Ammonium-Nitrogen (NH_4-N), Nitrite (NO_2-N) and Nitrate (NO_3-N), as previously described (Bundy et al., 2017). Mean wastewater and effluent characteristics are summarised in Table S1 (see SI).

2.3. Sample collection, DNA extraction and ARB enumeration

Sample collection for ARG, MGE, and antibiotic resistant bacteria (ARB; i.e., ESBL-producing isolates) quantification was conducted during quasi-steady-state conditions (based on C and TN removal data) during three biweekly sampling regimes. Altogether, 15 samples were collected for AMR-related analyses, consisting of five samples per sampling week: one influent from parallel feeding points and four DDHS final effluents from the respective final discharge points.

For ARG and MGE quantification, samples were collected and concentrated to obtain adequate biomass for DNA extraction. Effluent samples were collected, stored on ice (for 2 to 4 hours), and then filtered through 0.20 µm pore-sized Polyethersulfone filters (Pall Corporation, USA) to harvest the cells, whereas influent samples were collected and concentrated by centrifugation at 4000 x *g* for 10 minutes (Scientific laboratory, UK). Filtrates and centrates were discarded, respectively, and filter paper and pellets were stored at -20°C prior to subsequent DNA extraction, using the FastDNA SPIN Kit for Soil and a FastPrep-24 Homogeniser (MP Biomedicals, Santa Ana, CA, USA). Following extraction, DNA samples were checked for purity using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, UK) and DNA concentrations were quantified by using the Qubit 2.0 Fluorometer (Invitrogen, UK). DNA samples were stored at -80°C prior to downstream analysis.

In parallel, influent and effluent samples were screened for ESBL-producing *Enterobacteriaceae*, using ChromID ESBL selective chromogenic media (Biomerieux, UK). Raw wastewater samples were serially diluted in 1 x sterile phosphate buffer saline (PBS) solution and 100-uL aliquots were plated in triplicate per dilution per sample. Viable ESBL-producing *E. coli* and *KESC* isolates (i.e., *Klebsiella, Enterobacter, Serratia, Citrobacter*) were counted after 24 hours of incubation at 37°C and reported as CFUs/100 mL.

2.4. High-throughput quantitative PCR (HTH-qPCR)

Abundance and diversity of ARGs and MGEs were quantified by HTH-qPCR using the SmartChip Real-time PCR (Warfergen Inc. USA) (Su et al., 2015). A total of 296 primer sets (Table S2) were used to screen for ARGs and MGEs, including 293 validated primer sets targeting 284 ARGs, representing potential resistance to nine major classes of antibiotics. Eight transposase genes, two integron-associated genes (universal class I integron-integrase gene, *intI*; and the clinical class 1 integron-integrase gene, *cintI*); and one eubacterial 16S rRNA gene are also included. Target genes were originally identified with BLAST on the Antibiotic Resistance Genes Database (ARDB) or the National Center for Biotechnology Information (NCBI) database.

HTH-qPCR amplification was conducted as follows: 100-uL reaction containing (final concentration) 1 × LightCycler 480 SYBR® Green I Master Mix (Roche Inc., USA), nuclease-free PCR-grade water, 1 ng/μL BSA, 9 ng/μL DNA template, and 1 μM of each forward and reverse primer. The thermal cycle was as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and finally with a melting curve analysis auto-generated by the

programme. Corroborating 16S rRNA quantification targeting universal eubacteria for the same samples was performed using conventional qPCR. Standard curves and the same 16S rRNA primer sequences were used to quantify 16S gene copies for sample normalisation (Looft et al., 2012; Ouyang et al., 2015).

2.5. Genomic data screening and analysis

Raw HTH-qPCR data was cleaned using SmartChip qPCR Software (V 2.7.0.1), which removes data from wells with multiple melting peaks or inefficient amplification (i.e., outside 90% to 110%). Cleaned data from three independent samples (one per week per sampling location) were then screened according to their threshold cycle value (C_T). Samples with a C_T >31 were removed, which previous experience suggested are probable false positives (i.e., C_T = 31 was the detection limit).

Normalised gene copy numbers of ARGs and MGEs were calculated as described in previous studies (Chen et al., 2016; Ouyang et al., 2015). Bacterial cell numbers were estimated by dividing quantified 16S rRNA copy numbers by the average number of 16S rRNA per bacterium (estimated at 4.1 based on the Ribosomal RNA Operon Copy Number Database, rrnDB version 4.3.3) (Klappenbach et al., 2001).

One-way analysis of variance (ANOVA) tests were performed on the three biweekly ARG datasets and metadata, and statistical comparisons confirmed no significant variations existed among biweekly sampling events (i.e. $p > 0.05$). ARG and MGE levels from the three biweekly datasets were used for subsequent comparisons among influent and reactors effluents.

3. Results and discussion

3.1. Enhanced denitrification for decentralised wastewater treatment

Reactor performance data of the DDHS units is shown in Figure 1 and shows differences among bypass schemes. COD_s and COD_t removal efficiencies always were over 79% and 83%, respectively, and NH_4-N and solids (TSS and VSS; see Table S1) removals were consistently over 84% and 90%, respectively. Despite the addition of bypass wastewater in R-S10, R-S20 and R-S30, COD removal efficiencies did not significantly differ versus bypass levels ($p > 0.05$). However, TN removal rates improved dramatically with increasing bypass with significantly lower effluent NO_3-N levels in higher bypass units (see Table S1, paired t-test; $p < 0.001$). Gross TN% removals were 28.5%, 37.6%, 64.5% and 71.0% for R-S0, R-S10, R-S20 and R-S30, respectively, indicating wastewater bypass does enhance denitrification. Greater COD reductions in R-S20 and R-S30, and lower effluent NO_3-N levels (presumed converted to N_2) suggest increased denitrification is occurring as designed (Bundy et al., 2017).

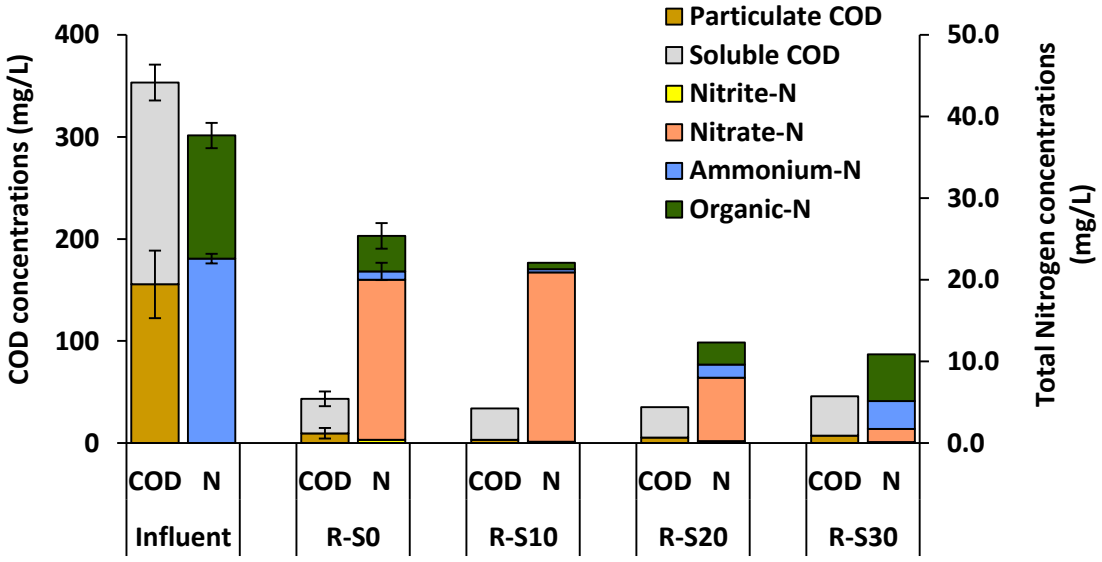


Figure 1. DDHS reactors mean performance as a function of wastewater bypass. Stacked bars present mean COD levels (particulate and soluble fractions) and nitrogen constituents (Ammonium; Nitrate; Nitrite; and Organic-N) in raw wastewater and the reactor effluents (n = 12 per reactor). Error bars show standard deviation around the mean; R-S10, R-S20 and R-S30 had minor standard errors.

3.2. Abundances and patterns of ARGs and MGEs

3.2.1. Total abundances

HTH-qPCR quantifies both ARGs and MGEs, including ARGs associated with nine different antibiotic classes, different resistance mechanisms (deactivation, protection, efflux pump, and unknown), and two MGE groups (transposases and integrons). A total of 59 unique ARGs ($2.2 \times 10^{10} \pm 3.7 \times 10^{9}$ copies/mL) and seven MGEs ($1.4 \times 10^{10} \pm 2.2 \times 10^{9}$ copies/mL) were detected in influent samples as shown in Figure 2, with "multidrug" ARGs being most abundant (MDR; 33.8%), followed by aminoglycoside (23.2%), tetracycline (19.6%), Macrolide-Lincosamide-Streptogramin B (MLSB; 12.9%) and β-lactam (9.5%). Detected influent MGEs were 58% and 42% for transposase and integrase genes, respectively. DNA was extracted from biomass concentrated from samples by filtering through 0.2um membrane filters, therefore ARG levels reported here are cell-associated. Extra-cellular ARGs were not included in this study.

Absolute ARG abundances significantly declined in all DDHS reactors (see Figure 2A), consistently achieving 1.0 to 2.0 log reductions (influent vs effluent paired test; $p < 0.05$). Effluent ARG levels ranged from 2.5×10^{7} to 4.5×10^{8} ARG copies/mL. Highest absolute ARG removals were seen in the reactors with 10 and 20% bypass as compared with no bypass (R-S0) and 30% bypass (R-S30). R-S30 had the highest effluent ARG levels, suggesting "excess" bypass negatively impacts ARG removal. MGE levels also

248 significantly declined in all reactors following similar patterns as for ARGs (Figure 2A).
249 Overall, the wastewater bypass improves TN removal and achieves efficient ARG
250 removal, which is co-optimized at ~20% bypass. Highest TN removals were seen at a
251 30% bypass, but Figure 2 shows ARG removal rates decline, presumably because more
252 raw wastewater bypasses the aerobic layer, suggesting the aerobic layer may be
253 particularly important to ARG removal as suggested previously by Christgen et al.
254 (2015).
255
256 Overall, Figure 2 shows DDHS reactors are "efficient" at reducing both ARG and MGE
257 levels. This is encouraging because DDHS systems use minimal energy compared to
258 other available options for ARG and MGE removal (Bundy et al., 2017). For example,
259 UV, advanced oxidation, and membrane bioreactor processes can effectively reduce
260 ARGs (Wen et al., 2018; Zhang et al., 2016), but they use copious energy and too
261 operationally complex for application where basic sanitation is lacking.

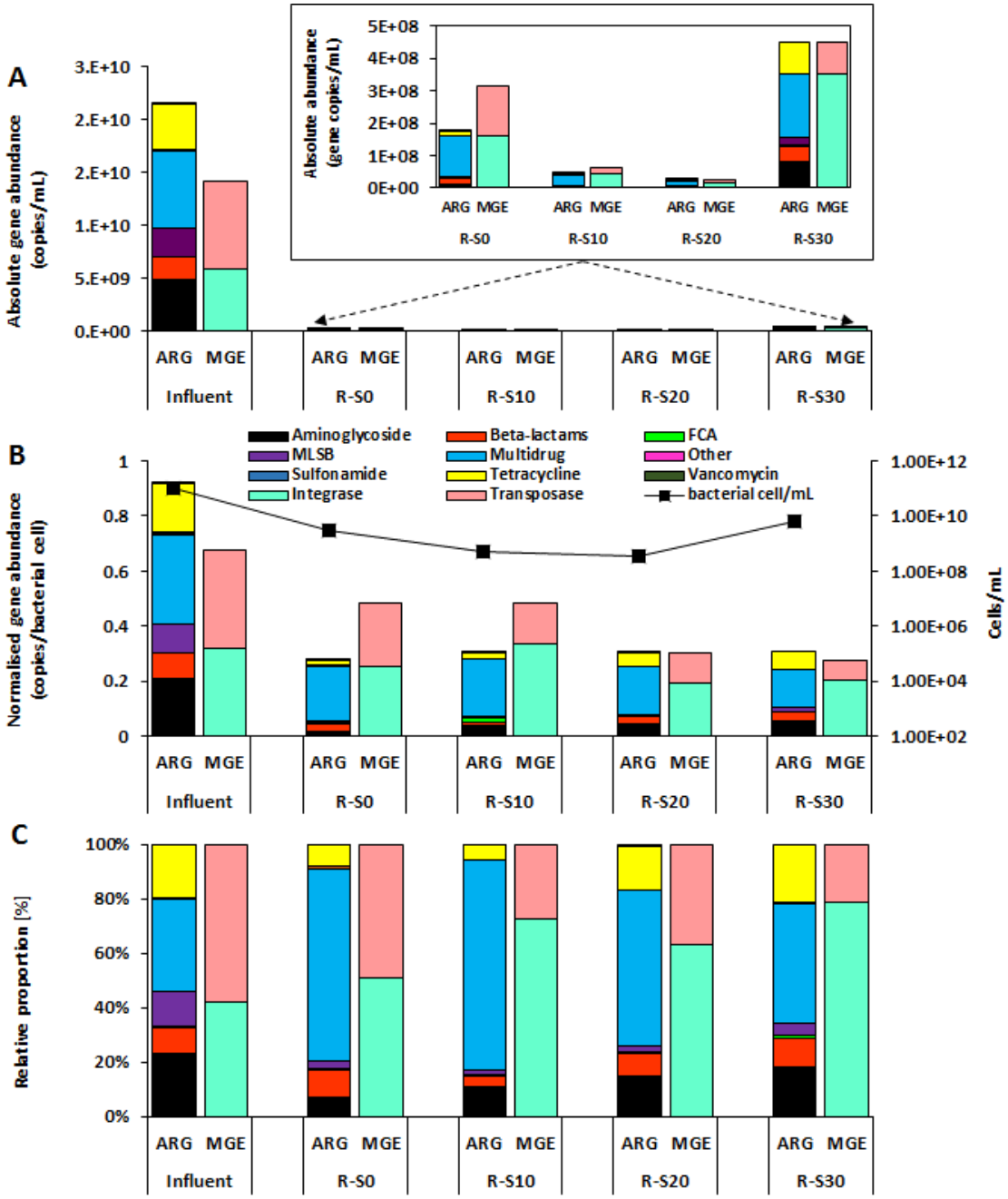


Figure 2. Total abundance of ARGs and MGEs detected in the raw wastewater and DDHS reactor effluent samples conferring resistance to specific class of antibiotics. (A) Absolute gene copy numbers per mL of wastewater; (B) Relative gene copy numbers normalised to bacterial cell numbers derived from ambient 16S-rRNA gene abundances; (C) Relative percentages of ARG abundances across samples. The line shows absolute bacterial cell levels in the influent and effluents, which reflects eubacterial abundances (error bars ~ small deviations concealed by marker). The blow-up insert shows subtle differences among ARGs and MGEs in different DDHS reactor effluents. FCA =

fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol ARGs; MLSB = Macrolide-Lincosamide-Streptogramin B ARGs.

3.2.2. Relative ARG and MGE abundances

Relative effluent ARG and MGE levels (normalised to bacterial cell abundances) display different removal patterns compared with absolute abundance data (Figure 2B). Relative ARG levels declined by ~70% in all four DDHS reactors, although dominant ARGs in effluents differed among bypass schemes. Specifically, relative effluent tetracycline and aminoglycoside ARG levels increased and MDR genes decreased with increased bypass, suggesting the aerobic top layer particularly enhances tetracycline and aminoglycoside ARG removal. In contrast, relative effluent MGE levels generally declined with increasing percent bypass, suggesting the anoxic layer may enhance MGE removal in DDHS systems.

DDHS reactors appear to be particularly effective at reducing medically important β-lactam and aminoglycoside ARGs. As examples, all DDHS configurations significantly removed ESBL- (e.g., $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{SFO}) and cephalosporin-resistsance (e.g., bla_{cepa} and bla_{AmpC}) ARGs, which are often associated with Gram (-) enteric bacteria (Alouache et al., 2014; Blaak et al., 2015; Willemsen et al., 2015). Further, 2.0 to 4.0 log reductions in culturable ESBL-producing *E.coli* and KESC (*Klebsiella, Enterobacter, Serratia and Citrobacter*) bacteria were observed in DDHS units (see Figure S2). Effluent ESBL-resistant isolate numbers increased with greater percent bypass and is consistent with ARG data.

DDHS reactors clearly reduce absolute ARG abundances from domestic wastewater. Estimated bacterial cell numbers in treated effluents showed 1.0 to 2.0 log reductions relative to influent levels (Figure 2B), with highest bacterial removals observed in R-S20. Further, bacterial removals parallel ARG removals, suggesting ARG reductions may be simply due to the removal of bacteria, which is greatest at intermediate bypass levels. This implies that ARG removal in DDHS systems may be primarily an ecological phenomenon, possibly including predation, which has been suggested previously for this type of reactor (Onodera et al., 2013). Conversely, TN removal increases with greater bypass, therefore an operational trade-off is needed to co-optimise TN and ARG removal for any application.

3.3. Broader observations on ARG removal in bioreactors from DDHS systems

Differences in ARG, MGE and bacterial removals across our DDHS systems permit some general observations about AMR removal in bioreactors. For example, data here suggest removal of common ARGs from wastewater is largely associated with removing bacteria, which in the case of DDHS systems, implies the top aerobic layer is particularly key to ARG removal. Previous work has shown aerobic processes may be better for ARG removal (Christgen et al., 2015), which data here suggest this may be due to greater bacteria removals. Specifically, as percent bypass is increased to a certain threshold (30% here), more influent bacteria (often anaerobes and facultative strains) "avoid" the aerobic treatment step, carrying and-or possibly exchanging ARGs in and through the lower anoxic layer. Therefore, although increasing percent bypass enhances denitrification, it allows bacteria to circumnavigate the aerobic layer. This is supported by the fact that relative ARG abundances are similar among effluents (Figure 2), suggesting absolute ARG in the effluents is mostly related to bacterial numbers.

In contrast, relative 'MDR' ARGs and also MGE abundances were lower in effluents when bypass is included (Figure 2B). The dominant ARG subclass in R-S0 effluent is MDR genes (~73%), whereas MDR only represents 44% of ARGs in R-S30 effluent (Figure 2C). Further, although absolute MGE levels increase with increasing bypass, relative MGE levels were highest in R-S0 and R-S10 with no or low bypass. This implies bacteria that survived both the aerobic and denitrifying layers tend to have greater genetic plasticity (i.e., higher MGEs per cell and potential for horizontal gene transfer, HGT), which may partially explain why such bacteria survive both redox environments.

An increase in MDR in aerobic processes has been seen previously (Czekalski et al., 2012; Pal et al., 2005; Yang et al., 2013), although a definitive explanation has not been provided. Higher MDR was previously explained by the presence of many micro-stressors in wastewater (e.g., metals, biocides etc.), which select for bacteria with multiple defence mechanisms (Christgen et al., 2015). However, our DDHS reactors had the same influent. Therefore, a better explanation is the change from an anoxic sewage environment to the aerobic treatment unit influences HGT, potentially selecting for MDR genotypes (Pal et al., 2005; Poole, 2012). This explanation is plausible because bacterial SOS stress responses cue HGT (Baharoglu et al., 2010) and a change in redox conditions would increase bacterial stress. However, a third explanation is that higher rates of HGT prevail under aerobic reactor conditions, possibly due to higher growth rates and greater bacterial densities. Suggesting aerobic units increase gross HGT is mildly controversial because others have found greater ARG HGT under anaerobic conditions (Rysz et al., 2013). However, data here imply the aerobic step in

DDHS systems is key to ARG removal, which is consistent with observations in other studies (Farkas et al., 2016; Leverstein-van Hall et al., 2003; Mokracka et al., 2012; Tennstedt et al., 2003; Zhang et al., 2009b).

3.4. Persistent and unique ARG and MGE subtypes, and practical implications

A Venn diagram of ARGs present in the influent and effluents is provided as Figure 3. It shows 10 "persistent" ARGs (i.e., not removed by any configuration) across all reactors and also unique ARGs among different effluents (see Table S4 for specific ARGs). Overall, effluent from R-S0 had the highest number of unique ARGs (10), whereas R-S30 effluents had lowest number of unique ARG numbers (2), although R-S30 also had the highest absolute bacterial and ARG abundances. ARGs in the central overlap were persistent in all effluents (see Table S3), including *tetQ*, *tetM*, *tetX*, *bl2d_oxa10*, and *qacEdelta1*; ARGs often associated with acquired resistance (van Hoek et al., 2011).

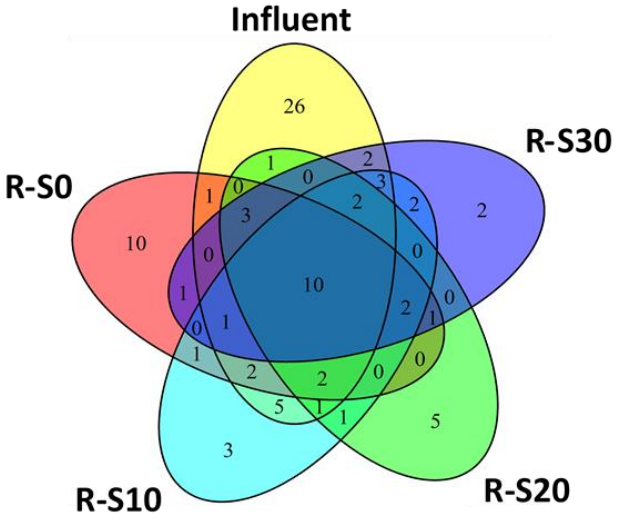
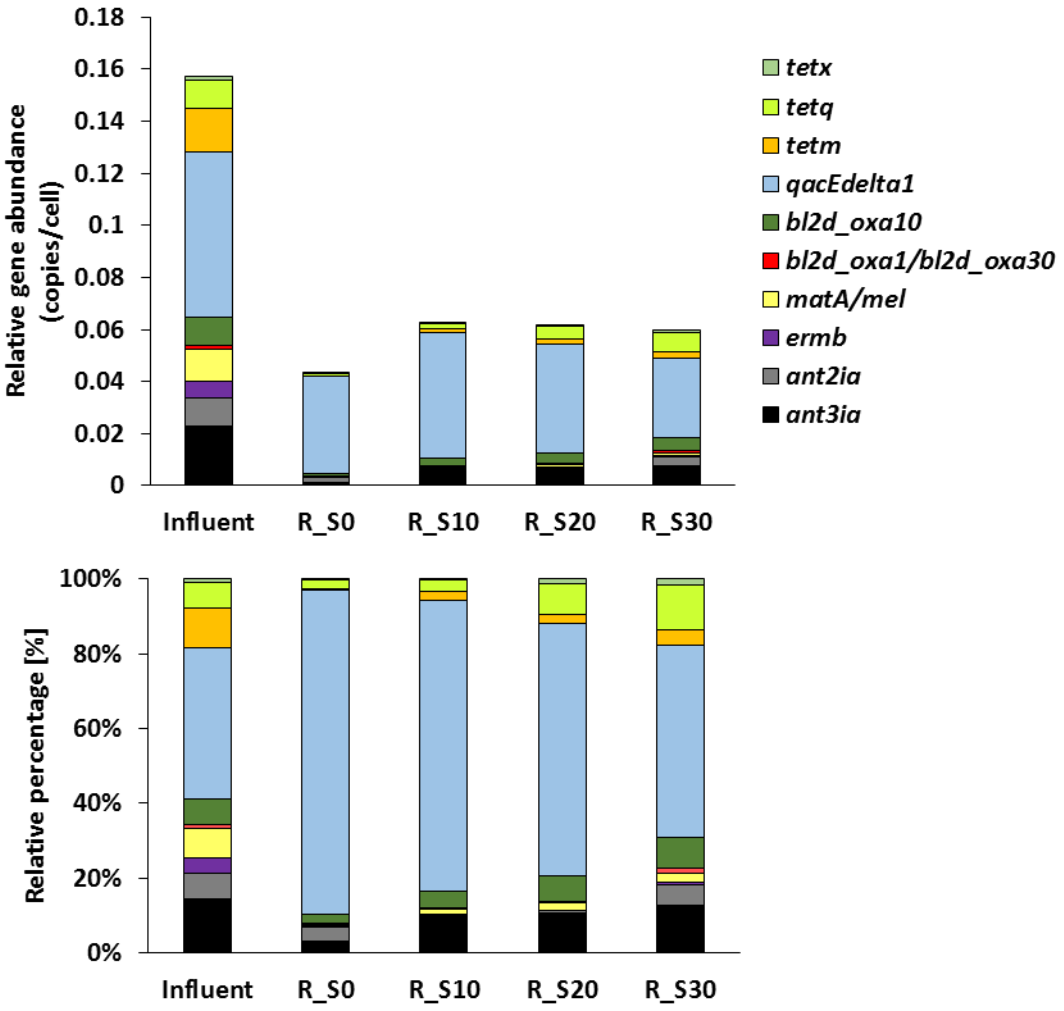


Figure 3. Venn diagram showing overlap of ARGs among influent and effluent samples from different DDHS configurations. Subsets represent number of genes detected in the wastewater influent (59 ARGs); R-S0 (35 ARGs); R-S10 (35 ARGs); R-S20 (28 ARGs) and R-S30 (30 ARGs). The central overlap represents the number of persistent ARGs.

All persistent ARGs are summarised in Figure 4 and statistical associations with persistent MGEs are provided in Table S5. First, persistence appears strongly associated with MDR genes, especially in no or low bypass reactors. However, if one looks at the implied MDR signal, only one ARG is apparent, *qacEdelta1*, which is closely associated with integron cassettes (Partridge et al., 2009) and only correlates with *int1* and *Cint1* (Table S5). In data here, more of the persistent ARGs statistically correlate with tp614 (especially tetracyclines and ESBL ARGs), which codes for a transposable element often linked to carbapenem resistance (Soki et al., 2006). This does not mean tp614 is carrying these ARGs, but implies integron genes are not directly associated with the most persistent ARGs in DDHS effluents.



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377 **Figure 4.** Persistent ARGs not removed in any DDHS reactor configuration. Relative
378 abundances of persistent ARGs in the influent and effluents of each reactor (top panel;
379 ARGs noted in the legend), and corresponding relative percentages of ARGs in reactor
380 influent and effluent based on proportion of total ARG copy numbers (bottom panel).

381

382 **4. Conclusions**

383 DDHS and other sponge reactors are an attractive option for small-scale wastewater
384 treatment. Kobayashi et al. reported sponge systems effectively remove pathogenic
385 viruses (1.5 to 3.7 log reduction for aichivirus, novovirus and enterovirus) (Kobayashi
386 et al., 2017), which complements results here on AMR removal. In particular, DDHS
387 systems can reduce both TN and AMR from domestic wastewater (contrary to other

sponge designs) and are suitable for small-scale applications due to low energy and maintenance needs.

Based on high ARG removal levels, the potential for TN removal, and low energy demands, DDHS systems show great promise at reducing environmental and health impacts of wastewater discharge on local scales. As such, they should be considered in locations where centralised treatment does not exist or would be costly, although co-optimization is needed to satisfy local priorities relative to ARG versus TN removal.

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