Short Communication

Accumulation of antibiotic-resistant genes in anaerobic biofilm reactor fed with household chemical products

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Abstract
This research aims to determine the presence of antibiotic-resistant genes (ARG) in anaerobic biofilm reactors (ABR) fed with household chemical products (HCP) such as laundry detergents and handwash without any influx of antibiotics. The ABR comprised a three-chamber design with bottom sludge, a middle chamber containing fluidized PVC spiral, and a top chamber with packed coir fiber as a biofilm support medium, respectively. Four different ABRs were simultaneously operated for a prolonged period (200 day) and subjected to variations in physicochemical conditions. The ABRs fed with HCP exhibited solitary accumulation of log (4.4–7.5) *ermC* gene copies/g VS whereas, ARG was undetectable in glucose fed ABRs indicating that HCP exhibited antimicrobial activities synonyms to Erythromycin. Accumulation of Erythromycin-C (*ermC*) was relatively higher on the biofilm inhabiting PVC support medium and further accentuated by effluent recycling to log 7.5 *ermC* gene copies at a ratio of *ermC*/16S gene copies of 0.65. Physico-chemical factors such as substrate composition, biofilm support medium, and effluent recycling simultaneously elevated the concentration of *ermC* genes. The results indicated that HCP augments the accumulation of ARG in the microbiome, subsequently, increasing the risk in ARG transmission from sewage treatment plants to the ecology and humans.

Keywords Antibiotic resistance genes · Anaerobic biofilm reactor · Erythromycin · Tetracycline · Household chemical products

1 Introduction

Synthetic chemical compounds are commonly used in daily household activities. They are termed as household chemical products (HCP), xenobiotics, personal care products and household consumables etc. The majority of HCP are discharged through greywater (GW) channels and further categorized based on their mode of actions such as; surfactants, mainly used for the removal of dirt; antimicrobials, provide non-specific disinfection against microbes; photoprotective agents protect against UV radiations; etc. [1]. Irrespective of their intended application, majority of HCP and its breakdown products accumulate in the environment mostly in water, sludge, and soil [2]. HCP inadvertently impede the microbial mode of decomposition, especially the anaerobes due to their recalcitrant and heterocyclic structures [3]. Long-term exposure of microbes to HCP is reported to imbibe resistances against the microbicidal activities [4]. Such mechanisms feature extensively in a pathogen's resistance against the antibiotics by either acquiring or evolving its genomic structure. These foreign genes imbibing resistances against antibiotics are called as antibiotic-resistant genes (ARG) [5]. The substantial and uncurtailed usage of antibiotics

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has led to wide-scale dispersal and accumulation in the environment [6]. The non-specific microbicidal activities of HCP are somewhat similar to antibiotics, hence, long-term exposure of nonpathogenic, ecologically innocuous microbes to HCP are perceived to develop widespread antimicrobial resistance by acquiring or evolving ARG [7]. For example, Triclosan, a microbicidal agent present in personal hygiene products inhibits microbes by blocking the enzyme enoyl-ACP reductase that is responsible for fatty acid biosynthesis [8]. Previous studies have shown that the dosages of Triclocarban in anaerobic digesters at concentrations of 30 mg/kg anaerobic sludge led to an increase in mexB gene (e.g. multidrug efflux pump), along with the ratio of tetracycline genes to 16S rRNA gene copies were higher by three orders of magnitude [9]. HCPs contain various forms of antimicrobial compounds, hence, their discharge through the sewerage channel exposes them to the surface waters, microbial consortia of sewage treatment plants, and agricultural lands via sludge disposal. The role of these antibacterials on increasing the antibiotic resistance of the environmentally important microbes needs further assessment. Moreover, the level of inhibition incurred on anaerobic biofilm digester needs to be understood as the biofilm structure is hypothesized to resist a relatively higher concentration of HCP compared to the sludge-based systems. This research aims to determine the occurrence and accumulation of antibiotic-resistant genes (ARG) in anaerobic biofilm reactors (ABR) fed with simulated greywater (GW) comprising HCP such as laundry detergents and handwash without any influx of antibiotics. Besides, explain the plausible scientific basis of ARG accumulation with notable reference to the role of physicochemical and biological factors such as substrate composition, effluent recycling, PVC biofilm support medium, and biofilm structure.

2 Materials and methods

2.1 Anaerobic biofilm reactor (ABR)

Four different anaerobic biofilm reactors (ABR1–ABR4, Fig. 1) with similar dimensions, biofilm support material, and a working volume of 10 L were fabricated, operated, and optimized as per earlier designs [10]. The top (TC) and middle (MC) chamber of ABR were filled with biofilm support medium namely coir fiber and PVC spiral and respectively. The bottom chamber (BC) was mainly composed of sludge. The ABRs (1–4) were commenced with identical anaerobic sludge collected from a large scale biowaste (i.e. kitchen waste, leaves, and grass) fed anaerobic digester. ABR1 and ABR3 were fed with synthetically prepared GW constituents simulating a typical household discharge. It consisted of food-grade starch (300 mg/L), KH_2PO_4, NH_2CONH_2, and two of the commonly used commercial HCP namely laundry detergents and hand wash gel doses at a concentration of 300 mg/L and 150 mg/L respectively. ABR2 and ABR4 were fed with standard substrate containing glucose (500 mg/L), micro, and macronutrients respectively. The detailed composition of synthetic GW and standard substrate have been described earlier [10]. The ABR1–ABR3 was operated at constant organic loading rates (OLR) of 550 mg/L·day, and hydraulic retention time (HRT) of 24 h whereas, ABR4 was subjected to a periodic increase in OLR up to 3936 mg/L·day at HRT down to 4 h. ABR3 and ABR4 were equipped with effluent recycling to enhance the biodegradation rates. The rates of COD removal (mg/L·day) in each ABR are as follows; ABR1: 160; ABR2: 214; ABR3: 627; ABR4: 3540 [10].

2.2 Molecular analysis

The microbial samples were collected from ABRs at the end of the reactor operation (i.e. 200 days). The microbial sludge samples were randomly grabbed from BCs whereas the biofilm samples were randomly scraped from the surface of PVC spiral and coir fiber with the help of a scalpel. The samples were stored at − 20 °C until analysis. The extraction and purification of DNA, phylogenetic, and bioinformatics analysis and qPCR analysis of 16S rRNA were conducted as per the methods described earlier [10]. Briefly, DNA extraction and purification were performed with MO-BIO PowerSoil® DNA isolation kit and OneStep™ PCR Inhibitor Removal Kit (Zymo Research) respectively. The V4 target region of the 16S rRNA gene was amplified with 515F and 806R primers, further sequenced on the Illumina MiSeq platform at ESPSF, Argonne National Laboratory, IL, USA. The default software package incorporated in QIIME was used for data analysis. The qPCR studies were conducted in a Mastercycler thermocycler (Eppendorf International, Hamburg, Germany). The DNA samples were screened for the qualitative and quantitative estimation of four different ARG gene copies namely Erythromycin-A (*ermA*), Erythromycin-C (*ermC*), Tetracycline-Q (*tetQ*), Tetracycline-X (*tetX*), and 16S rRNA sequences. The primers for the above-mentioned genes were procured from Invitrogen, USA, based on the sequences described in several research articles (Table 1). The experiments were conducted in a 96 well PCR plate, each well containing 20 µL qPCR mixture composed of KiCqStart SYBR Green qPCR ready mix (Sigma-Aldrich), primers, sample templates, and Sigma water. The previously prepared stock ARG gene copies were used as standards for the qPCR analysis [15]. The stock ARGs was diluted in the range of 10^0–10^8 copies/20 µL to generate the standard plot. The minimum and maximum detection limits of each gene were determined

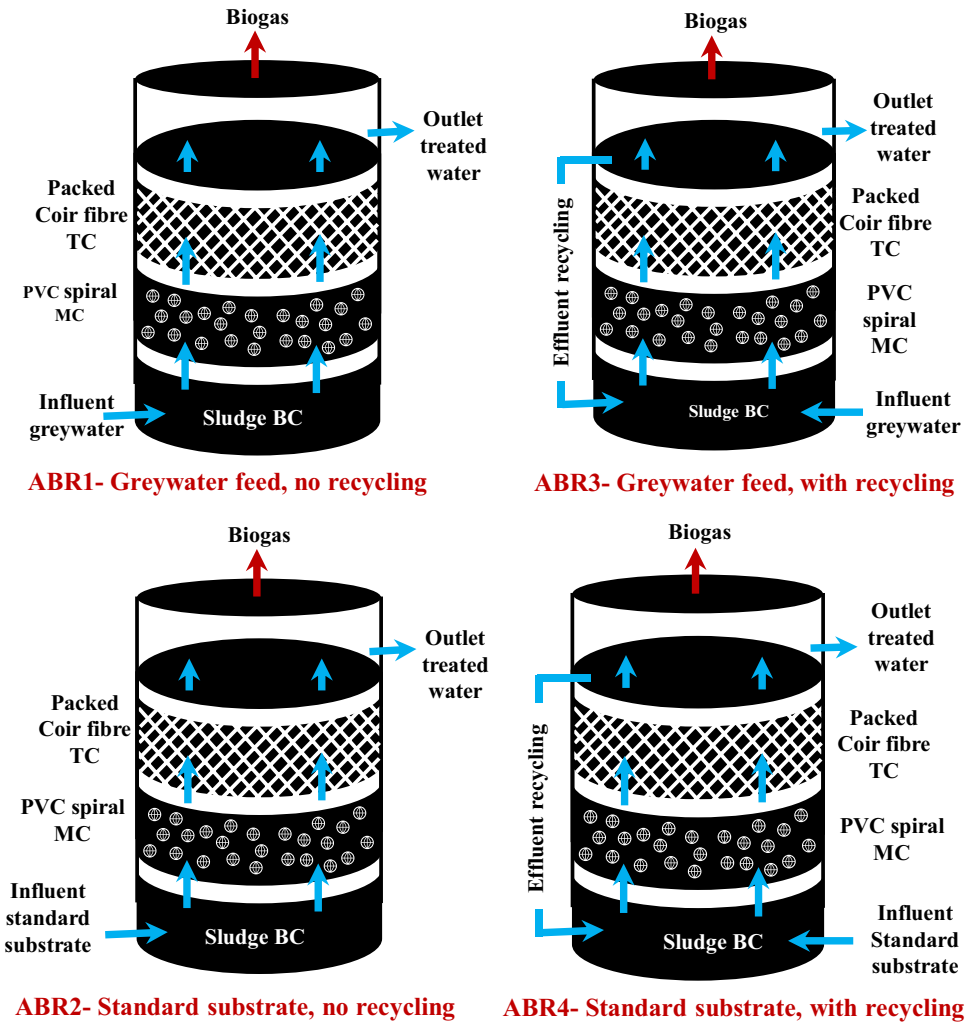

Fig. 1 Four different anaerobic biofilm reactors (ABR1-ABR4) for the treatment of GW and standard substrate. BC, MC and TC represents bottom chamber, middle chamber and top chamber respectively

Table 1 The sequence of qPCR primers, annealing temperatures, and detection range

Genes	Primer	Sequence (5′-3′)	Annealing Temp (°C)	Detection range (copies/20 µL)	R^2	Efficiency (%)	References
Erm A	Fwd	AGTCAGGCTAAATATAGCTATC	63	10^2–10^7	0.995–0.997	89–93	[11]
	Rev	CAAGAACAATCAATACAGAGTCTAC					
Erm C	Fwd	AATCGTGGAATACGGGTTTGC	63	10^2–10^7	0991–0.995	90–95	[11]
	Rev	CGTCAATTCCTGCATGTTTTAAGG					
Tet Q	Fwd	AGAATCTGCTGTTTGCCAGTG	63	10^1–10^7	0.990–0.997	101–108	[11]
	Rev	CGGAGTGTCAATGATATTGCA					
Tet X	Fwd	CAATAATTGGTGGTGGACCC	60	10^0–10^8	0.992–0.994	110–114	[12, 13]
	Rev	TTCTTACCTTGGACATCCCG					
16S	BACT1369F	CGGTGAATACGTTCYCGG	56	10^2–10^8	0.991–0.996	102–105	[14]
	PROK1492R	GGWTACCTTGTTACGACTT					

from the linear range of the standard plot. The qPCR operating conditions were similar for all the standard ARGs, sample genes, and no template control (distilled water, NTC) respectively, except for the annealing temperatures, that were specific to each ARGs (Supplementary data 1). A three-step template amplification procedure with subsequent melting curve analysis was conducted as per the methods outlined in the KiCqStart SYBR Green qPCR ready

mix. The qPCR amplicons of ARG standards and ABR samples were verified and compared a blank (distilled water) with the help of a 0.8% agarose gel electrophoresis containing agarose gel loading dye by Fisher BioReagents. Thereafter, DNA bands were stained with SYBR gold DMSO dye supplied by Fisher BioReagents followed by its visualization in a UV illuminator and image captured by a compact digital camera. The 16S rRNA gene copies of the ABRs have been described previously [10]. The total solids (TS) and volatile solids (VS) of the biofilm and sludge were estimated as per the standard methods [16].

3 Results and discussion

The HCP comprises a mixture of complex polyaromatic compounds that hinder microbial degradation, especially under anaerobic conditions. Hence, long-term exposure of microbes to these compounds has been reported to alter their genetic structure, which eventually leads to the development of resistance and modification of metabolic pathways to decompose those compounds [7]. The extracted DNA from microbial samples (biofilm and sludge) of ABRs were analyzed for the presence of four different ARG namely *ermA, ermC, tetQ, tetX*. The research specifically targeted the *tet* and *erm* classes of ARGs due to their ubiquitous presence in sludge, sediments, and all variants of wastewater treatment plants [17]. These classes of ARG's have also been reported to sustain and augment in the anaerobic digesters. Moreover, the majority of antimicrobial constituents in HCP possess a non-specific mode of action akin to erythromycin and tetracycline [18]. The qPCR results in Fig. 2, revealed the presence of only *ermC* gene copies, in GW fed reactors ABR1 and ABR3. On the contrary, ARG was undetected in glucose-fed reactors ABR2 and ABR4. The accumulated quantity of *ermC* gene copies in ABR1 and ABR 3 varied in the range of 7.5–4.4 log *ermC* gene copies/ gVS. Chronologically, the ermC gene copies was highest in ABR3-MC followed by ABR3-BC, ABR3-TC, ABR1-MC and ABR1-TC (Fig. 2). The results were also substantiated by analyzing the melting curve of the amplified genes with respect to the standards and confirmed by running the qPCR amplified genes in gel electrophoresis. The melting curves of the amplified *ermC* genes in ABR1 and ABR3 were identical to the standards within the range of 1 °C (Supplementary data 1) indicating an absence of experimental bias. Furthermore, parallel gel electrophoresis band patterns of the qPCR amplicons observed only in ABR1, ABR3, and *ermC* standards confirmed the presence of *ermC* genes in the samples ruling out any experimental anomalies (Fig. 3). Results indicated that the concentration of *ermC* gene copies was higher in ABR3 with respect to ABR1, although, both ABR1 and ABR3 were fed uniformly at equivalent OLR and HCP constituents (Fig. 2). These differences may be attributed to effluent recycling, as it is known to increase contact and simultaneously enhance the rates of substrate degradation and lead to selective accumulation of distinctive microbial species especially α-*Proteobacteria* sp., (Fig. 5) [10]. Moreover, the MC with PVC biofilm support contained a relatively higher quantity of *ermC* genes in both ABR1 (7.5 log gene copies/g VS) and ABR3 (4.8 log gene copies/g VS), hence, the synergistic role of PVC support media and biofilm structure in selective enrichment of the ARG accumulating microbe is plausible and cannot be ruled out. However, this research set up and methodology has inadequate scientific evidence to prove this hypothesis could not be taken up and will be addressed in future research. The ratio of *ermC*/16S gene copies varies in the range of 0.45–0.65 with the highest value determined in ABR3-MC (Fig. 4). The aforementioned ratio of 0.45–0.65, corresponds to group III and IV, ARG abundance levels in the environment reported in human feces, STP influents, feces, and wastewater from livestock farms respectively [6]. The higher ratio of *ermC*/16S gene copies as evidenced in this study suggests that a larger

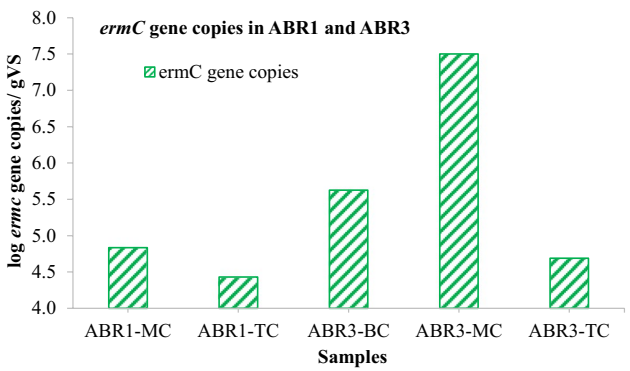

Fig. 2 The qPCR estimation of *ermC* gene copies in various chambers of greywater fed reactor ABR1 and ABR3

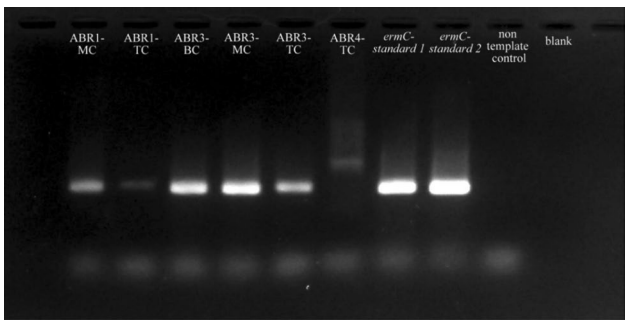

Fig. 3 The gel electrophoresis image of the qPCR amplified *ermC* genes in the greywater reactor samples (R1 and R3) with respect to the standards. The *ermC* genes amplicon size was 293 bp [11]

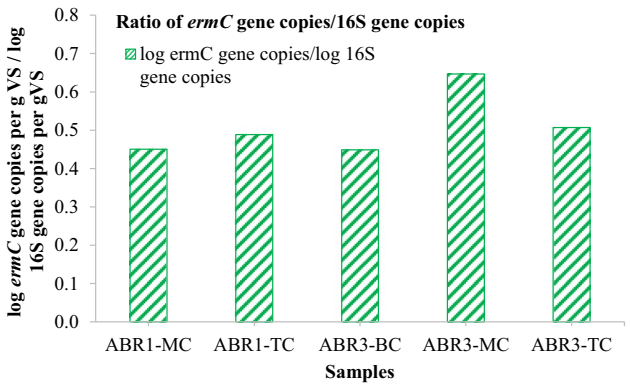

Fig. 4 The ARG abundance of *ermC* gene copies with reference to 16S gene copies per gVS in the various chambers of greywater fed reactor ABR1 and ABR3

fraction of the microbiome inhabiting ABR1 and ABR3 may be responsible for the magnification and accumulation of *ermC* [including the species belonging to the dominant classes, (Fig. 5)].

The presence of the *ermC* gene in GW fed ABRs depict the ability of microbial species to tolerate the antibiotic Erythromycin. Erythromycin inhibits protein synthesis in bacteria by binding to the 23 s subunit of the ribosome preventing the elongation of the amino acid chain [19]. The GW constituents, however, did not contain any known influx of antibiotics, hence, the accumulation of *ermC* can be solely attributed to the presence of HCP constituents in GW such as Triclosan, Triclorocarban, surfactants (LAS/SDBS). The result indicates that HCP constituents possess a specific mode of microcidal activity analogous to the antimicrobial activity of Erythromycin that would have led to the selective accumulation of *ermC* amongst several other ARGs. Heterogeneity in the microbiome of wastewater treating bioreactors/ digesters makes it extremely difficult to single out the species accumulating *ermC*. However, based on the results obtained from α-diversity of the microbiome (Khuntia et al. 2019) and available literature the following postulations are proposed, (i). HCP constituents in GW possess a bactericidal action similar to that of Erythromycin that may have compelled the bacteria to modify its genetic sequences to develop a resistance mechanism against Erythromycin, (ii). The results exhibited a significantly higher ratio of *ermC*/16S gene copies (i.e. 0.45–0.65) implying that the dominant microbial population in ABR1 and ABR3 must have been the carrier and proliferator of *ermC* (Fig. 5). A distinctive microbial population subsisted in ABR1 and ABR3 mostly dominated by the species belonging to the class of α-*Proteobacteria*, Γ-*Proteobacteria, Synergistia, Actinobacteria, Bacteroidia, Clostridia, Anaerolineae*, etc., whereas, significantly lower in ABR2 and ABR4 hypothesizing their collective role in the accumulation of *ermC* (Fig. 5). These results correspond to the reported study of the presence of ARG in α, β, and Γ classes of *Proteobacteria* [20]. In ABR3, the genus *Arthrobacter* was the most dominant, followed by genus *Kocuria* whereas in R1 the genus *Actinomyces* was followed by genus *Corynebacterium* respectively. It was also observed that these species were mainly concentrated in the MC (PVC substratum) of both ABR1 and ABR3. Several *Actinomycetes* sp., such as *Saccharopolyspora erythraea sp*., *Arthrobacter sp*., have been reported to inhibit both gram-positive and gram-negative bacteria by producing aminoglycoside-modifying enzymes (AME) [21]. AME also imbibes resistance to *Actinomycetes* sp., against a wide

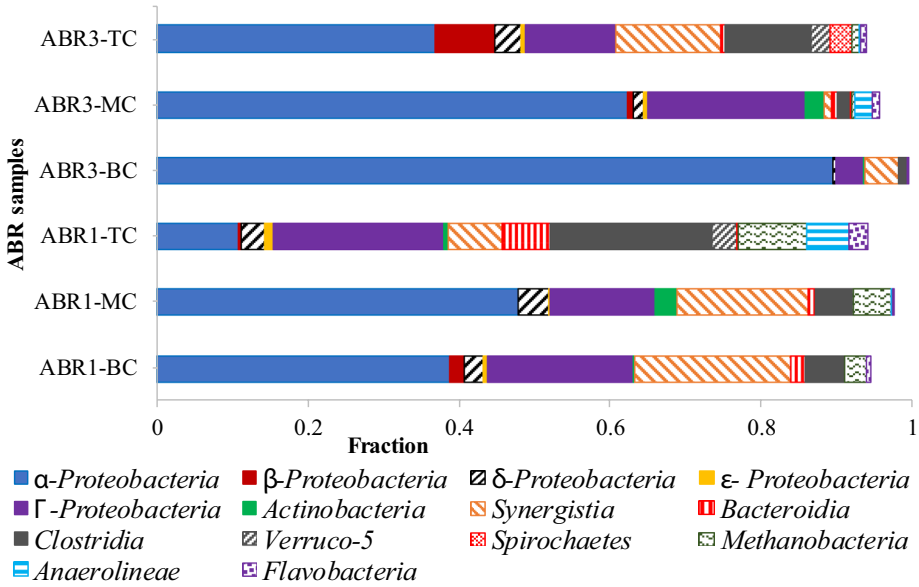

Fig. 5 The above figure shows only the most dominant classes of microbial species inhabiting ABR1 and ABR3. (Note: ABR2 and ABR4 possessed distinctive microbiome and absence of ARG hence, excluded from the above data. The phylum data in ABR-ABR4 has been previously reported [10])

range of antibiotics [21]. Moreover, Erythromycin and its derivatives are fermentation products of *Actinomycetes* sp., such as *Saccharopolyspora erythraea* sp., *Arthrobacter* sp.[22]. Woo et al. [23], hypothesized that the ARG of *Actinomycetes sp*., were responsible for being the origin of ARG in pathogenic bacteria. In contrast, the total population of class *Actinobacteria* in ABR1 and ABR3 does not exceed 2.5%, hence, the role of other classes of microbes in accumulating *ermC* cannot be ruled out. (iii) The accumulation of *ermC* was relatively more prominent in MC of ABR1 and ABR3 indicating the plausible role of biofilms and PVC substratum. The biofilm structure offers a unique advantage to the microbes to defend itself against the recalcitrant chemicals and antibiotics in the following ways, (a). The defiant physical matrix prevents the penetration of antimicrobial compounds, (b). Production of antibiotics and cell-wall modifying enzymes, (c). Release of extracellular DNA, (d). Transcriptional regulators, (e). Horizontal gene transfer, (f). Hypermutation, (g). Persister cells, etc. Conclusive evidence about the exact role of *ermC* genes in the biofilm and decomposition of HCP compounds in GW could not be drawn from this research. Moreover, the *Actinomycetes* sp., as a carrier of *ermC* genes is speculative, which would require further research to validate the hypothesis.

This study has revealed that long-term exposure of environmentally benign microbes to HCP, leads to the accumulation and proliferation of ARG, even in the absence of antibiotics. This phenomenon could be hypothesized as an alternative mechanism acquired by microbes to persist under a higher concentration of antimicrobial compounds such as HCP [24]. The situation can be alarming as the worldwide use of HCP especially for personal hygiene (e.g. handwash, surface sterilizers, disinfectants) has been increasing, furthermore, after the outbreak of SARS-CoV-2 pandemic, resulting in higher HCP concentrations in sewage. Hence, the microbial treatment of HCP ladened sewage would augment the ARG resistance. Additionally, the environmental discharge of treated sewage and ARG acquired sewage sludge could imbibe unacquainted resistance in benign microbes against antibiotics. Eventually, increasing the risk in the subsequent transmission of ARG from natural environments to humans and animals [6]. Effluent discharged from wastewater treatment plants in the river have been reported to simultaneously increase the ARG concentration and alter the bacterial communities [25].

4 Conclusion

The HCP constituents in GW feed led to selective accumulation of log 4.4–7.5 *ermC* gene copies/g VS in ABR1 and ABR3 respectively. On the other hand, ARG was undetectable in glucose fed reactors ABR2 and ABR4. The concentration of *ermC* gene copies was pronounced in ABR3 due to effluent recycling. A relatively higher concentration of *ermC* was present in the biofilm inhabiting PVC support medium in both ABR1 (log 4.4 gene copies/g VS) and ABR3 (log 7.5 gene copies/g VS). The ratio of *ermC*/16S gene copies was 0.45–0.65 that was typical of microbiome occupying human feces, STP influents, feces, and wastewater from livestock farm. A distinct microbial population and diversity in ABR1 and ABR3 especially belonging to the class of *α-Proteobacteria*, *Γ-Proteobacteria*, *Synergistia*, and its absence in ABR2 and ABR4, is speculated for the accumulation and proliferation of *ermC*. This research demonstrated that HCP augments the accumulation of ARG in the microbiome, subsequently, increasing the risk in ARG transmission from sewage treatment plants to the ecology and humans.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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