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INGENIERÍA AMBIENTAL

EVALUATION OF ANTIBIOTIC RESISTANCE IN MICROALGAE-BACTERIA SYSTEMS COUPLED TO ANAEROBIC DIGESTERS FOR WASTEWATER TREATMENT

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PRESENTA:

M. I. JULIÁN OSVALDO OVIS SÁNCHEZ

TUTOR PRINCIPAL:

DR. JULIÁN CARRILLO REYES, INSTITUTO DE INGENIERÍA

COMITÉ TUTORAL:

DR. GERMÁN BUITRÓN MENDEZ, INSTITUTO DE INGENIERÍA

DR. ENRIQUE ARTURO CANTORAL URIZA, FACULTAD DE CIENCIAS

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JURADO ASIGNADO:

Presidente:	Dr. Germán Buitrón Méndez
Secretario:	Dr. Daniel De Los Cobos Vasconcelos
1er vocal:	Dr. Juan Sebastián Arcila Henao
2do vocal:	Dr. Enrique Arturo Cantoral Uriza
3er vocal:	Dr. Julián Carrillo Reyes

Lugar donde se realizó la tesis: Laboratorio de Investigación en Procesos Avanzados de Tratamientos de Aguas (LIPATA). Instituto de Ingeniería, UNAM, campus Juriquilla, Querétaro.

DIRECTOR DE TESIS:

Dr. Julián Carrillo Reyes

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Lights will guide you home

Abstract

In this thesis, the fate of antibiotic-resistance genes was evaluated using microalgae-bacteria-based wastewater treatment systems, as well as the persistence of antibiotic-resistance genes and antibiotic-resistant bacteria during the anaerobic digestion of microalgae-bacteria aggregates. A series of experiments included high-rate algal ponds and flat-plate reactors. Section 2 demonstrates that high-rate algal ponds achieve significant removal rates of chemical oxygen demand and ammonia, as well as a significant removal of the resistome and antibiotic-resistance genes quantified by quantitative polymerase chain reaction. These results are consistent over time, highlighting the robustness and potential of high-rate algal ponds as viable technology for wastewater treatment in low to middle-income countries.

In Section 3, anaerobic digestion is considered as an alternative for the treatment of microalgae-bacteria aggregates generated during high-rate algal pond operation. This research focused on the effectiveness of this process for biogas production and the elimination of antibiotic-resistance genes and antibiotic-resistant bacteria. The results demonstrated that thermophilic conditions during anaerobic digestion resulted in a higher elimination of antibiotic-resistant bacteria and antibiotic-resistance genes than mesophilic conditions. This finding is significant as it underscores the potential of anaerobic digestion to improve treatment quality and manage the risks associated with antibiotic resistance. Additionally, the microbial community, mobilome, and functional genes significantly contributed to the resistome reduction in the microalgae-bacteria aggregates digestion process.

Section 4 focuses on the role of intracellular and extracellular fractions related to extracellular polymeric substances in the context of antibiotic-resistance genes. No antibiotic-resistance genes were detected in the final samples of free extracellular polymeric substances, and a significant decrease in the final samples of bound extracellular polymeric substances was observed. This decrease is consistent with a significant decrease in various mobile genetic element modules, including phages, indicating a decrease in horizontal gene transfer by transformation, implicating a decreased risk of antibiotic-resistance genes dissemination post-treatment. This section complements the results obtained in Section 2 and elucidates other mechanisms involved in the reduction of antibiotic-resistance genes in microalgae-bacteria systems.

This thesis contributes to wastewater treatment and the management of antibiotic resistance, and demonstrates the potential of microalgae-bacteria-based systems. This study proposes an integrated treatment approach that addresses the need for treatment and contributes to decreasing the spread of antibiotic resistance, a global public health problem.

Resumen

En esta tesis se evaluó el destino de los genes de resistencia a los antibióticos usando sistemas de tratamiento de aguas residuales basados en microalgas-bacterias, así como la persistencia de genes de resistencia a los antibióticos y bacterias resistentes a antibióticos durante la digestión anaerobia de los agregados microalga-bacteria. A través de una serie de experimentos que incluyen lagunas microalgales de alta tasa y reactores de placa plana. En la sección 2 se demuestra que las lagunas microalgales de alta tasa logran significativas tasas de remoción de la demanda química de oxígeno y amoníaco. Así como también la remoción significativa del resistoma y de genes de resistencia a los antibióticos cuantificados por reacción en cadena de la polimerasa en tiempo real. Estos resultados son consistentes a lo largo del tiempo, resaltando la robustez y potencial de las lagunas microalgales de alta tasa como una tecnología viable para el tratamiento de aguas residuales en países de ingresos bajos a medianos.

En la Sección 3, se considera la digestión anaerobia como alternativa para el tratamiento de los agregados microalga-bacteria generados durante la operación de las lagunas microalgales de alta tasa. La investigación se enfocó en la efectividad de este proceso para la producción de biogás y la eliminación de genes de resistencia a los antibióticos y bacterias resistentes a antibióticos. Los resultados demostraron que las condiciones termofílicas durante la digestión anaerobia resultaron en una mayor eliminación de bacterias resistentes a antibióticos y genes de resistencia a los antibióticos en comparación con condiciones mesofílicas. Este hallazgo es significativo ya que subraya el potencial de la digestión anaerobia en mejorar la calidad del tratamiento y la gestión de los riesgos asociados con la resistencia a los antibióticos. Además, la comunidad microbiana, el mobiloma y los genes funcionales contribuyeron de manera significativa a la reducción del resistoma en el proceso de digestión de los agregados microalga-bacteria.

La sección 4 se centra en el papel de las fracciones intracelulares y extracelulares relacionadas con sustancias poliméricas extracelulares en el contexto de los genes de resistencia a los antibióticos. No se detectaron genes de resistencia a los antibióticos en las muestras finales de sustancias poliméricas extracelulares libres y se observó una disminución significativa en las muestras finales de sustancias poliméricas extracelulares unidas. Esta disminución se alinea con una disminución significativa en varios módulos de elementos genéticos móviles, incluidos los fagos, lo que indica una disminución en la transferencia horizontal de genes por transformación, lo que implica una disminución del riesgo de diseminación de genes de resistencia a los antibióticos después del tratamiento. Esta sección complementa los resultados obtenidos en la Sección 2 y aclara otros

mecanismos involucrados en la reducción de genes de resistencia a los antibióticos en sistemas microalgas-bacterias.

Esta tesis contribuye al tratamiento de aguas residuales y al manejo de la resistencia a los antibióticos, evidenciando el potencial de los sistemas basados en microalga-bacteria. Este estudio propone un enfoque de tratamiento integral que atiende tanto la necesidad de tratamiento como contribuir en disminuir la propagación de la resistencia a los antibióticos, un problema de salud pública de alcance global.

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1. Introduction

Microalgae-bacteria systems as a potential solution for antibiotic resistance

1.1 Antibiotic Resistance in the Environment

Antibiotics are substances that inhibit or kill germs, particularly bacteria, by interfering with their metabolic processes (Vallero, 2016). They are widely used to treat human infections and also play a crucial role in agriculture, livestock, and aquaculture (Cabello, 2006; Economou and Gousia, 2015). Antibiotic contamination is rapidly increasing, primarily because of the intensive and widespread global use of antibiotics (Martínez, 2008). This has contributed to selective pressure, leading to the development of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in various receiving environments (Laxminarayan et al., 2013). Since the global commercialization of penicillin in 1941, antibiotic resistance has become a growing concern. One year later, a bacterium (*Staphylococcus aureus*) resistant to this antibiotic was reported, and in 1967, another bacterium resistant to penicillin (*Streptococcus pneumoniae*) was identified (CDC, 2019). Antibiotic resistance can be natural, either intrinsic (always expressed in the species) or induced (genes naturally occurring in bacteria but expressed at resistance levels after exposure to an antibiotic). For example, some bacteria prevent antibiotic access owing to the characteristics of their external walls. Acquired resistance involves the acquisition of genetic material-expressing mechanisms against antibiotics (ARGs). Additionally, these ARGs can be transferred to other microorganisms that have not been exposed to antibiotics (Reygaert, 2018). These mechanisms may change over time, leading to infections that are more resilient. Furthermore, ARB can transfer their resistance genes to other microorganisms not previously exposed to antibiotics (Figure 1.1) (CDC, 2020).

Antibiotics enter the environment through multiple pathways, and consequently, ARB and ARGs generally spread, as described in Figure 1.2. ARGs have been reported in wastewater, surface water, groundwater, and soil (Chen and Zhang, 2013; Lupo et al., 2012). This represents a One Health problem because human-animal-environment niches are associated with the interdependence of these pillars in the food chain and environment.

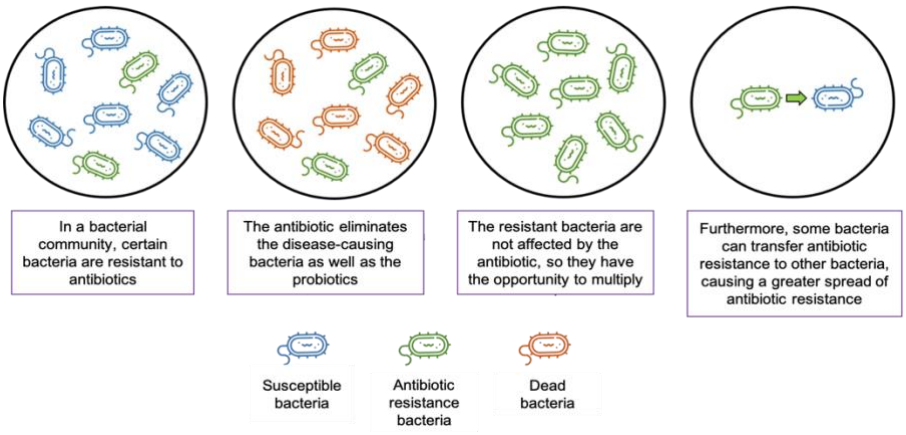

Figure 1.1 General mechanism of antibiotic resistance. Adapted from CDC (2020).

Overall, antibiotic resistance jeopardizes the ability to treat common infectious diseases; this has become a concerning cause of mortality, accounting for more than 700,000 annual deaths (WHO, 2018). Without coordinated proactive actions worldwide, it is estimated that by 2050, deaths due to antibiotic resistance could reach up to 10 million people, surpassing those caused by cancer or diabetes (O'Neill, 2016). The economic consequences of this problem are also of great concern, and a recent World Economic Forum report (2021) pointed out that disease causes worktime and productivity loss.

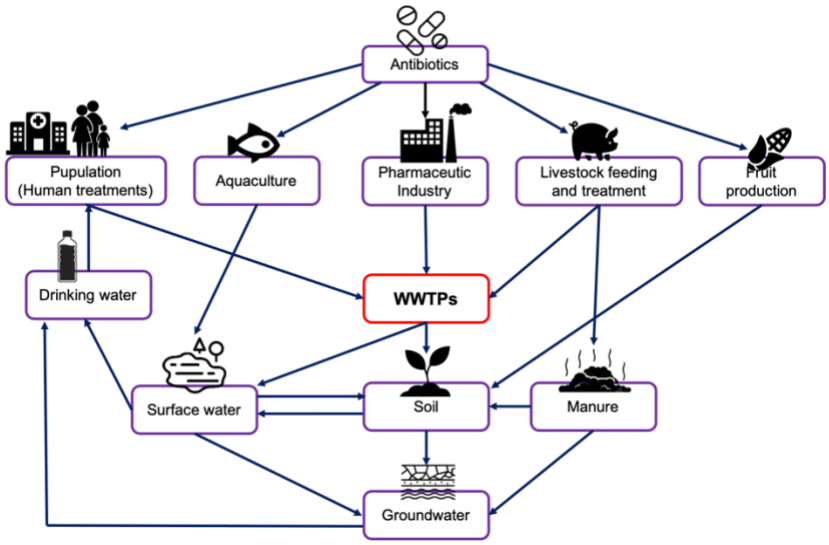

Figure 1.2 Transmission pathways for the dissemination of antibiotic-resistant bacteria and resistance genes in the environment. Adapted from a study by Pazda et al. (2019).

1.2 Horizontal gene transfer

The transfer of ARGs occurs in both gram-positive and gram-negative bacteria through two pathways: vertical gene transfer during reproduction and horizontal gene transfer when unrelated bacteria transfer genetic material (Durich, 2000). ARGs are primarily transferred through horizontal gene transfer (HGT), which refers to the non-sexual transmission of genetic material between unrelated genomes involving the transfer of genes across species boundaries. Most examples of HGT are well-documented in prokaryotes. In bacteria, three main mechanisms mediate horizontal gene transfer (Figure 1.3): transformation (uptake of free DNA), conjugation (plasmid-mediated transfer), and transduction (phage-mediated transfer) (Choudhuri, 2014).

During transformation, bacteria absorb DNA from the extracellular environment and incorporate it into their genomes. This process requires the bacteria to be naturally transformable or competent. Approximately 80 species of prokaryotes have been experimentally demonstrated to be naturally competent (Blokesch, 2016; Johnsborg et al., 2007). Natural transformation has been shown to results in the absorption of a wide range of DNA fragments, contributing to the development of antibiotic resistance in bacteria (Domingues et al., 2012).

Transduction is the transfer of chromosomal and extrachromosomal DNA between bacteria via bacteriophages (bacterial viruses). The main mechanisms of transduction are generalized and specialized. Together, these can mobilize any fragment of the bacterial genome. Generalized transduction occurs when bacteriophages in the lytic cycle incorporate sections of bacterial host DNA during capsid synthesis. In specialized transduction, the regions immediately flanking the integration site of a lysogenic phage are excised and packaged into the capsid (Chiang et al., 2019).

Conjugation, the most common HGT mechanism, involves transfer of genetic material via plasmids. Bacteria typically contain a circular chromosome, but some cells may harbor an additional circular DNA known as a plasmid along with transposons that can move between a chromosome and a plasmid or vice versa. Plasmids tend to copy and transfer to other cells. Conjugation begins when a pilus attaches its distal end to another bacterial or archaeal cell. The pilus then shortens, bringing the two cells into contact and forming a cytoplasmic bridge. The plasmid in the donor cell replicates, allowing a new copy of the plasmid to be transferred across the narrow cytoplasmic bridge to the recipient cell. After this transfer is complete, both plasmid strands build the missing half to form complete bicatenary plasmids in both cells.

Finally, the two cells disconnect and move in separate ways (Zeigler, 2014). This mechanism has been demonstrated to transfer ARGs among different bacterial genera (Devanga Ragupathi et al., 2019).

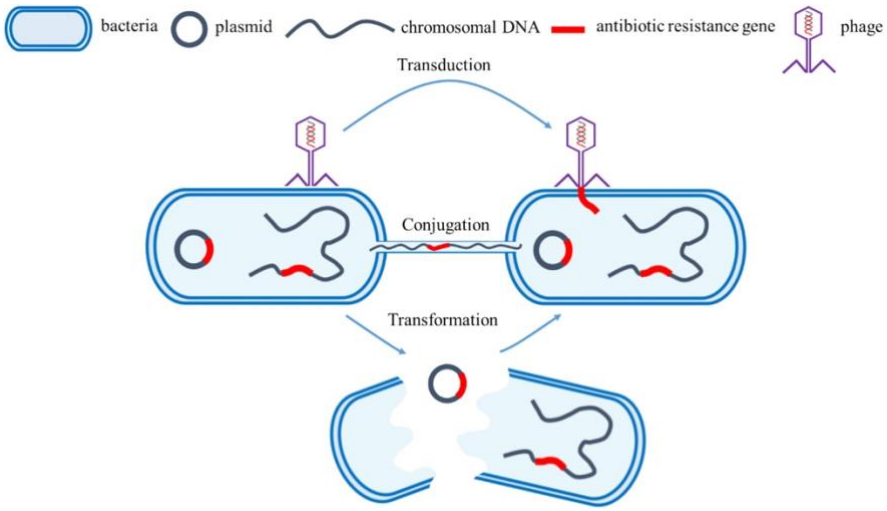

Figure 1.3 The three mechanisms of horizontal gene transfer: transduction, conjugation and transformation. Taken from Nguyen et al. (2021).

1.3 Mexican scenario of antibiotic resistance

Regarding antibiotic policies and regulations, in 2010, the Federal Commission for the Protection against Health Risks controlled the sale of antibiotics, strictly stipulating that they can only be sold with a medical prescription. However, prior to 2010, antibiotic sales occurred without a prescription despite being regulated by Article 226 of the General Health Law. Additionally, since 2016, the WHO has made efforts to report antibiotic consumption globally. However, Mexico's data are absent in the 2016-2018 report, possibly because the country has data but does not comply with the WHO's methodology.

To address issues related to antibiotic use and in alignment with international commitments, the National Strategy of Action against Antimicrobial Resistance was published in the Official Gazette of the Federation (DOF) on June 5, 2018. This strategy is mandatory for all institutions within the National Health System, outlining objectives and actions to control, reduce, or eliminate the risks associated with antimicrobial resistance while enhancing knowledge and scientific evidence on the issue (DOF, 2018).

The consumption and resistance to antibiotics have been extensively examined in the clinical context (Amábile-Cuevas, 2021; Camorlinga-Ponce et al., 2021; Miranda-Novales et al., 2020; Rosado-Rosado et al., 2021). For instance, Rosado-Rosado et al. (2021) investigated antibiotic

resistance in microorganisms isolated from clinical samples collected during patient hospitalizations (2015-2018) at a pediatric hospital in Mexico City. They noted an increase in *S. aureus* resistance to oxacillin from 0% in 2016 to 58% in 2018. Camorlinga-Ponce et al. (2021) studied antibiotic susceptibility in *H. pylori* strains isolated over 20 years (1997 to 2017) from Mexican mestizos and indigenous populations, and found an increase in resistance to clarithromycin (1.6-32.2%) and levofloxacin (9.2-58.1%) in indigenous populations of mestizo communities, which exhibited significantly lower antibiotic resistance in *H. pylori* strains, likely due to adherence to cultural practices, including traditional medicine, limiting antibiotic consumption.

There are limited reports on the presence of antibiotics, ARB, and ARGs in Mexican wastewater and surface waters. For instance, Rivera-Jaimes et al. (2018) identified pharmaceutical compounds in an urban wastewater treatment plant in Cuernavaca, detecting sulfamethoxazole and trimethoprim with influent concentrations of 1143 and 145 ng L^{-1}, respectively, and effluent concentrations of 730 and 143 ng L^{-1}, indicating limited removal in the treatment process. Regarding BRA in surface waters, Mondragón et al. (2011) isolated *Enterococcus faecalis* from the Mololoa River (Nayarit, Mexico), which showed high resistance patterns (100% for ciprofloxacin, 84.2% for kanamycin, 15.7% for vancomycin, 13.2% for gentamicin, and 7.8% for ampicillin), making clinical treatment challenging in cases of transmission to humans. Recently, a study was conducted on ARGs and *intI1* in four municipal WWTPs in Mexico City. In most cases, a significant decrease in the concentrations of these genes in raw wastewater has been observed during biological treatments, although not completely (Cuetero-Martínez et al., 2023). It is crucial to gain further insights into the fate of antibiotics, ARB, and ARGs in different environments to enhance our understanding of antibiotic resistance and promote effective solutions for controlling the dissemination of antibiotic resistance.

1.4 Antibiotic resistance in wastewater treatment plants

The persistence, fate, and removal of antibiotics, ARB, and ARGs in WWPTs have been extensively reviewed for different treatment systems such as conventional activated sludge (CAS), membrane bioreactors (MBRs), moving bed biofilm reactors (MBBRs), and nature-based solutions such as constructed wetlands (CWs) (Krzeminski et al., 2019; Nguyen et al., 2021; Pazda et al., 2019; Rizzo et al., 2013; Xue et al., 2019). The CAS process has been the most investigated; however, the conventional layout (i.e., aerobic process) is usually ineffective for

removing these contaminants (Krzeminski et al., 2019). Furthermore, it has been suggested that the removal of ARGs is affected by various operational and environmental factors during treatment.

1.4.1 Operational factors

The influence of hydraulic retention time (HRT) on ARGs has rarely been studied. Ding et al. (2020) explored the presence of ARGs in three WWTPs - two for municipal wastewater and one for industrial wastewater. Their findings demonstrated a correlation between HRT and ARGs removal; notably, the WWTP with a 24-hour HRT exhibited greater ARGs reduction compared to that with an HRT of 12-14 hours. A lower reduction in ARGs was observed in an industrial WWTP with an HRT of 240-320 minutes, a lower reduction of ARGs was observed. Similarly, Li et al. (2020) investigated sulfonamide antibiotic resistance genes in Microbial Fuel Cell Constructed Wetlands (CW-MFCs) under varying circuit operation conditions and HRT. Low HRT led to an increase in the absolute copies of the target ARGs, suggesting a significant influence on ARGs accumulation. According to Ding et al. (2020) and Li et al. (2020), prolonged HRT may enhance ARGs removal.

Despite the recognized importance of the solid retention time (SRT), few studies have directly assessed its relationship with ARG prevalence. A lab-scale A_2O-MBR (anaerobic-anoxic–oxic membrane reactor) revealed that a longer SRT (50 days) reduced total ARGs gene copies by 0.33 logs compared to a shorter SRT (25 days) (Zhang et al., 2018). Li et al. (2019) studied full-scale WWTPs and found that SRT significantly influenced ARG removal, with the highest SRT (27 days) achieving 2∼4 orders of magnitude higher log removal of certain ARGs compared to lower SRT. In contrast, Sui et al. (2018) reported that gene removal rates decreased as SRT increased, attributing it to frequent HGT in reactors with an SRT of 30 d. Overall, parameters such as HRT and SRT play crucial roles in bacterial selection and genetic recombination through HGT (Manaia et al., 2018). However, there is a lack of research on the influence of SRT and HRT on ARG elimination in WWTPs, emphasizing the need for future investigations in these areas.

1.4.2 Environmental factors

In addition, some environmental factors such as temperature have been correlated with the fate of ARGs. For example, Schages et al. (2020) monitored a WWTP in Germany for 1 year to quantify the ARGs corresponding to beta-lactamase, *mcr,* and *intl1* genes; ARGs which were

reported to be significantly higher in cold than hot months in influent and biosolids, resulting in a negative correlation between temperature and ARGs abundance. According to the authors Schages et al. (2020), this may be due to the fact that in cold months, there are higher rates of prescription and use of antibiotics from clinical establishments that are connected to the WWTP. Another study indicated that temperature is an important factor that affects the distribution of ARGs in the influent and effluent of WWTPs by affecting the expression of functional genes in microorganisms, especially in sensitive species (Jiao et al., 2018).

1.4.3 Microbiological factors

Microbial communities are considered important drivers of ARG dissemination in environmental compartments (Chen et al., 2019). Yu et al. (2020) reported that *Arcobacter, Cloacibacterium, Cyanobacteria, Acinetobacter, Flavobacterium, and Dechloromonas* were the most abundant genera in a wastewater reclamation system (WWRS) and exhibited significant correlations with ARGs ($p < 0.05$). In addition, another study found that co-occurrence network analysis explained that the dominant pathogens (*Pseudomonas*, *Neisseria*, and *Streptococcus*) were supposed to carry multiple ARG (Huang et al., 2023). The microbial community is not the only contributor to changes in ARGs, and other studies have found that mobile genetic elements are an important factor that also significantly contribute to changes in ARGs (Yin et al., 2021).

1.4.4 Latin American perspective of WWTPs

Although conventional biological WWTPs can remove ARB and ARGs from wastewater, they have never been designed for this purpose. However, if WWTPs are well designed and operated, they are a critical barrier against broader spread resistance, especially relative wastewater release without effective treatment (WHO et al., 2020). For example, WWTPs can reduce ARB and ARGs by up to two logs, and tertiary treatment techniques such as ozonation, activated carbon adsorption, coagulation, and nanofiltration/reverse (NF/RO) osmosis have been reported to be more effective (Wang et al., 2020; Michael-Kordatou et al., 2018). However, the capital and operational costs of advanced treatments increase the costs of conventional processes (Barancheshme and Munir, 2018), making them the only option in high-income settings. In low-to middle-income countries (LMICs), less expensive, more self-sustaining, and energy-efficient treatment technologies are needed because LMICs often lack

well-developed community health surveillance programs and wastewater coverage is often low.

Furthermore, in Latin America, only 50% of the human population is connected to contained sewer drains, and only 30% receive wastewater treatment (Tabla-Vázquez et al., 2020). In Mexico, national coverage of domestic wastewater treatment in 2020 was 67.2%, and the most common process was activated sludge (52.9%) (CONAGUA, 2021). Despite the national coverage being higher than other countries in Latin America, some studies based on the current state of wastewater treatment plants in Mexico highlight that 50% have a global rating from poor to very poor, mainly because WWTPs are not 100% functional for many reasons, including that ozone, UV, and membrane equipment are often malfunctioning (Morgan & Benítez, 2016). Furthermore, it is crucial to note the inadequate wastewater sanitation coverage and the lack of proper operation of the WWTPs installed in Mexico potentiates the spread of antibiotic resistance in the environment.

1.5 The potential of microalgae-bacteria systems for the removal of antibiotic resistance genes

Ecological and efficient alternative treatments have been studied as substitutes for the conventional sewage treatment processes. An example of a technology that has gained relevance in recent years is the microalgal–bacteria system, which is an efficient alternative to conventional wastewater treatment plants (Quijano et al., 2017). A raceway system in which microalgae and bacterial species are grown symbiotically is usually referred to as a high-rate algal pond (HRAP), and its first application was carried out over 60 years ago (Oswald et al., 1953). In these systems, the photosynthesis process developed by microalgae produces the required oxygen that allows aerobic bacteria that, in turn, can degrade the organic pollutants, generating the CO_2 needed by microalgae (Sial et al., 2020). Therefore, costly aeration, which is necessary to support aerobic growth, should be avoided. Several literature reviews are available on microalgae-based wastewater treatment for carbon, nitrogen, and phosphorus removal and biofuel production (Quijano et al., 2017; Sial et al., 2020; Zhang et al., 2020). Recently, microalgae-based wastewater systems for treating real wastewater worldwide have been reviewed (Inuwa et al., 2023). This study highlighted the ability of these biological systems to deal with the resistome (antibiotic-resistant bacteria and antibiotic resistance genes) as emerging contaminants.

On the other hand, there is little evidence of the use of high-rate algal ponds (HRAPs) for real wastewater treatment in outdoor conditions (Hom-Diaz et al., 2017; Norvill et al., 2017). Hom-Diaz et al. (2017) used an HRAP to remove ciprofloxacin (2 mg L^{-1}). Photodegradation was the main mechanism for the removal of ciprofloxacin during the day, whereas at night, the substrate gathered on the surface of the biomass (sorption). Together with changes in temperature and solar irradiance, seasonal changes in the effluent properties may affect the photochemistry and magnitude of ciprofloxacin removal. In another study, the fate of tetracycline during outdoor continuous operation of a pilot HRAP treating real domestic wastewater was studied; daily changes in pH, dissolved oxygen (DO), and redox conditions in these systems could also enhance the removal mechanism; these mechanisms mainly via indirect photodegradation and sorption (Norvill et al., 2017). In these microalgae-based systems, irradiance is critical because it can cause direct photodegradation and enhanced microalgae growth, resulting in greater bioabsorption and biodegradation (Vassalle et al., 2020).

A few studies have been reviewed for ARB and ARG. Kumar et al. (2021) reported that in microalgae *Pseudochlorella pringsheimii* was assessed in a pilot-scale for phyco-mitigation of various pollutants in the raw urban wastewater, were 7 of 10 ARB isolates were removed and total coliform bacteria (6×10^3 CFU mL^{-1}) were detected in the raw wastewater but after the 5th day of treatment, the total coliforms dropped to non-detectable counts. Nõlvak et al. (2018) examined the fate of the antibiotic resistome and class 1-3 integron-integrase genes in photobioreactors treated with diluted municipal wastewater (70/30) with lake or tap water for algal biomass production. They reported that photobioreactors proved to be at least as effective, or in the case of lake water reactors even more efficient, in controlling antibiotic resistance dissemination through treated municipal wastewater to natural environments as compared to conventional WWTPs. The reduction of the resistome was strongly related to changes in the bacterial community composition during the wastewater treatment process, responding to rising pH levels caused by intense algal growth. In addition, Cheng et al. (2020) compared the relative abundance of different ARGs and an integron in the primary effluent against those in the effluents of conventional wastewater treatment and algal wastewater treatment, where activated sludge can promote the spread of antibiotic resistance. In contrast, the algal-based wastewater treatment system utilizing *Galdieria sulphuraria* could reduce not only the abundance of ARB, but also the relative abundance of ARGs (qnrA, qnrS, tetW). The authors mentioned that this high reduction could be attributed to two reasons.

First, the unique conditions in algal reactors, including low pH (4.0) and high temperature (40–46 °C), suppress gene transfer via transduction. Other study, showed that tetO, tetW, tetX and ermB were decreased beyond detection within the first 4 days of treatment using a wastewater microalgae consortium (Inuwa et al., 2022)

1.5.1 Microalgae-bacteria interactions

The signaling network in the algal-bacterial system has been previously reviewed by You et al. (2021) and includes bacterial intercellular communication, algal intercellular communication, and cross-kingdom communication. However, it has been assumed that HGT is less common in eukaryotes, and the mechanisms of HGT in eukaryotes remain unclear (Suzuki et al., 2015). Recently, HGT between cyanobacteria and bacteria has been demonstrated. Wang et al. (2020) demonstrated that cyanobacteria could serve as an important reservoir and source for ARGs maintenance and propagation in aquatic environments; they also discovered that plasmids containing ARGs were more easily conjugated from *E. coli* (donor strain) to cyanobacteria when the temperature was high, and the cyanobacterial concentration was high (recipient strains) (Wang et al., 2020). Regarding the HGT of ARGs between prokaryotic microalgae and bacteria, no evidence has been found; there is only evidence of HGT of other types of genes. For example, Schönknecht et al. (2013) it reported that the microalgae *Galdieria sulphuraria* lives in hot, acidic environments rich in heavy metals; this adaptation to extreme conditions is due to HGT since phylogenetic analyses demonstrated the acquisition of 75 genes from bacteria and archaea. Matsuzaki et al. (2004) analyzed the genome of the red algae *Cyanidioschyzon merolae* and found genes derived from bacteria. Finally, the greatest transfer of genes comes mainly from bacteria since they have an extensive metabolic diversity that they can exchange (Keeling, 2009). Interactions between algae and bacteria have recently been studied (You et al., 2021), revealing that pollutants influence algae and bacteria in their coexisting systems (including pesticides, metals, engineered nanomaterials, pharmaceutical and personal care products, and aromatic pollutants). However, to the best of our knowledge, very little is known about microalgae-bacterial interactions with respect to ARB and ARGs.

1.6 Antibiotic resistance in biosolids

After wastewater treatment, another concern that arises is that the concentration of ARGs and ARB in the sludge generated from a wastewater treatment plant is significantly higher ($p < 0.05$) than the effluent (Munir et al., 2011). Therefore, control, monitoring and alternatives that remove antibiotic resistance present in the sludge or biosolids generated are also important. In this context, anaerobic digestion is widely applied as a sludge treatment due to its capacity for volume reduction, stabilization, and energy recovery in the form of biogas (Youngquist et al., 2016), and it is also considered a promising way to reduce ARG (Tian et al., 2016).

By a literature review in SCOPUS with the following search parameters: TITLE-ABS-KEY ("antibiotic resistance genes" AND "anaerobic digestion" AND "sludge" OR “biosolids”) AND (LIMIT-TO (DOCTYPE, "ar") AND (LIMIT-TO (LANGUAGE, "English"), 61 results were found. After a detailed revision of resulted manuscripts, only 26 articles performed the AD of sludge from WWTP and analyzed some determination of ARG. Most of these studies have been conducted at a laboratory scale. However, 3 of the 26 articles have explored the fate of ARGs in industrial-scale anaerobic digestion reactors (Ghosh et al., 2009; Luo et al., 2017; Tong et al., 2019). Different temperature ranges studied are shown, from 22 to 60 °C, SRT ranges from 4 to 44 days; with respect to the origin of the sludge, either primary sludge, secondary sludge or a mixture of both were used. The most commonly studied ARG is tetracycline. A detailed revision of scientific manuscripts aimed at evaluating antibiotic resistance in biosolids anaerobic digestion is presented below, focusing on the main operational factors: pretreatment, solids retention time, and temperature.

1.6.1 Pretreatment effect

Several authors have investigated the effect of pretreatment before anaerobic digestion to increase ARG reduction, such as ozone pretreatment, thermal hydrolysis, microwave, ultrasonic and alkaline pretreatment (Pei et al., 2016; Sun et al., 2019; Tong et al., 2017, 2016; Wang et al., 2019; Zhang et al., 2017). Sludge pretreatment can increase up to 24% ARG removal after AD compared to AD with sludge without pretreatment (Wang et al., 2019), also methane production increases up to 5.7 times compared to sludge without pretreatment (Pei et al., 2016). Tong et al. (2016) performed AD by testing 3 different pretreatments: microwave, microwave-acid, and microwave-H_2O_2-alkali. The total relative abundance of ARGs was observed to be reduced by 42.7% employing microwave-acid pretreatment; however, microwave and microwave-H_2O_2-alkali pretreatment increased ARGs by 45.0% and

17.7%, respectively. The relative abundances of tetC, tetM, and tetX decreased in the range of 25.3-92.1%, but the relative abundance of tetA increased in the range of 234.4-945.9% for all pretreatments. Wang et al. (2019) studied the distribution and removal of ARG in three different pretreatments prior to sludge AD, which were thermal hydrolysis, alkaline, and ultrasonic. The results showed that the relative abundance of ARG after AD was reduced by 52.2, 66.4 and 75.1%, respectively; moreover, the intl1 gene significantly decreased only in thermal hydrolysis. However, information regarding the effect of different pretreatments on the fate of ARGs and their discrepancies between removal efficiencies is still limited.

1.6.2 Solids retention time effect

The SRT is an operational parameter that should also be considered important because it defines the time at which the microbial biomass resides in the system; however, there are very few studies with information on this matter (Ma et al., 2011). Zhang et al. (2019) studied mesophilic AD by evaluating two different SRT, 15 and 20 days. They reported that decreasing the SRT from 20 to 15 days achieved higher removal of ARGs, ermB, ermF, mefA, sulII, tetG, and tetX, whereas ereA, sulI, and tetM subtypes increased. Additionally, the reduction of the intl1 gene was studied, and was reduced by up to 18.7% when the SRT was decreased from 20 to 15 days. Ma et al. (2011) reported a significantly greater reduction of sulI, sulII, and tetG achieved with an SRT of 20 days than employing 10 days. In contrast, tetO was reduced with a 20-day TRS but increased with a 10-day TRS. The authors indicated that a higher SRT significantly increased communication between microorganisms, and thus, the efficiency of HGT may increase (Zhang et al., 2019). However, a better understanding of the effect of this variable on HGT is still lacking, and it is hoped that future studies will evaluate the effect of SRT.

1.6.3 Temperature effect

Temperature is a critical environmental variable for reducing ARG, during anaerobic digestion of sludge generated in WWTP (Luo et al., 2017). In general, several studies have conducted anaerobic digestion at both mesophilic and thermophilic temperatures; however, a greater decrease of certain types of ARGs has been observed in thermophilic (Table 1.1). This is because, at thermophilic temperatures, there is an increase in microbial metabolic activities (substrate degradation and microbial proliferation), which has an impact on the microbial community and, subsequently, can directly influence vertical gene transfer, which is related to high removals of the intl1 gene (>80%) (Diehl and Lapara, 2010; Xu et al., 2018).

However, the same ARGs subtypes had higher removal at mesophilic temperatures (Zhang et al., 2015). These differences may be due to the influence of different operational parameters, such as SRT or the type of feed sludge, suggesting that the effects of sludge AD is not universal for all types of ARGs (Zhang et al., 2015).

Table 1.1. The temperature regime in which the highest removal efficiency was obtained for various ARG subtypes during AD.

ARG subtype	(Diehl and Lapara, 2010)	(Ghosh et al., 2009)	(Xu et al., 2018)	(Ma et al., 2011)	(Tian et al., 2016)	(Zhang et al., 2015)
*erm*B	-	-	-	T	T	T
*erm*F	-	-	-	T	T	-
*tet*A	T	T	T	-	T	-
*tet*C	-	-	-	M	T	-
*tet*G	-	-	-	M	T	M
*tet*L	T	-	-	-	T	-
*tet*O	T	T	T	M	T	M
*tet*W	T	-	-	M	B	M
*tet*X	T	T	T	-	T	-
*sul*1	-	-	T	B	-	-
*sul*2	-	-	-	B	-	T
*intI*1	T	T	T	M	T	-

T: Thermophilic; M: Mesophilic; B: Both

All of these studies indicate that several factors are involved in the breakdown of ARGs during anerobic digestion. However, these studies are mainly on primary sludge from conventional wastewater systems. Therefore, in the case of biosolids generated in emerging systems, such as microalgae-bacteria systems, no information is available.

1.6.4 Anaerobic digestion of microalgae-bacteria aggregates

Microalgae have been widely studied as substrates for methane production via anaerobic digestion, with Chlorella, Spirulina, and *Scenedesmus* being the most studied genera (Ganesh Saratale et al., 2018). However, for microalgae-bacteria aggregates (MABA), few papers are published in the literature. Table 1.2 shows the conditions and methane yields for MABA. Studies have shown that factors such as the S/I ratio (Hernández et al., 2013; Wieczorek et al., 2015), HRT (Carrillo-Reyes et al., 2021), MABA composition (Arcila and Buitrón, 2016; Bohutskyi et al. 2018; Hernández et al., 2016; Wieczorek et al. 2015), temperature (Carrillo-

Reyes et al., 2021; Wieczorek et al., 2015) and co-digestion (Cea-Barcia et al., 2018) have significant effects on methane production.

Most of these studies performed batch experiments that did not demonstrate long-term stability and performance. Nevertheless, the stability of methane production in continuous systems has also been demonstrated. For example, Cea-Barcia et al. (2018) evaluated the co-digestion of MABA with papaya waste for 95 days and found that the methane yield increased by 59. 8% compared to MABA; therefore, these authors not only demonstrated the stability of anaerobic digestion but also the positive effect of co-digestion in MABA aggregates. Carrillo-Reyes et al. (2021) evaluated the effect of HRT (15 and 30 days) and temperature (35 and 55 °C), from which they obtained the best methane yield (410 mL CH_4 g VS^{-1}) operating with an HRT of 30 days and at thermophilic temperature of 55°C. It is likely that the high methane yield is attributed to the high removal of biochemical components, such as lipids, since these authors reported a removal of up to 96%, as opposed to the other operating conditions, where lipid removal was in the range of 1-30%. It should be noted that no studies have been conducted on the persistence of ARGs or ARB during the anerobic digestion of MABA so far.

Table 1.2. Methane yields from anaerobic digestion of MABA.

Operation type	Temperature, °C	HRT, d	Operation time, d	Substrate/inoculum ratio	Methane yield, mL CH_4 g VS^{-1}	Reference
Batch	37	-	54	0.5	518[1]	(Hernández et al., 2013)
	37	-	29	0.2	206.9 ± 7.8	(Wieczorek et al., 2015)
	55	-	29	0.2	~100	
	35	-	20	0.5	347.9 ± 3.2	(Arcila and Buitrón, 2016)
	38	-	42	0.5	195	(Hernández et al., 2016)
	37	-	20	0.5	308	(Van Den Hende et al., 2016)
	35	-	60	-	340	(Bohutskyi et al., 2018)
Continuous	35	31	95	-	550 ± 70	(Cea-Barcia et al., 2018)
	35	30	276	-	260 ± 40	(Carrillo-Reyes et al., 2021)
	55			-	410 ± 80	

[1]mL CH_4 g COD_{added}^{-1}

1.7 EPS as a reservoir of ARGs

ARGs can exist either intracellularly (iARGs) or extracellularly (eARGs), with the latter originating from the secretion of living cells or released during cell lysis (Dong et al., 2019). A recent review revealed that most previous studies on ARGs in various environments have primarily focused on iARGs, primarily as a result of conventional DNA extraction methods with poor yields of extracellular DNA (eDNA) (Zarei-Baygi & Smith, 2021). Recently, new novel methods have been proven to enhance eDNA recovery, such as the use of magnetic beads, which efficiently extracted eDNA (> 85.3%) with higher recovery than current methods, such as alcohol precipitation, CTAB-based extraction, and DNA extraction kits (< 10%) (Yuan et al., 2019).

Detection of eARGs is important because they can be acquired by bacterial cells and facilitate HGT through the transformation pathway (Zarei-Baygi & Smith, 2021). Moreover, eARGs persist for extended periods in environments such as river sediments and are present at higher concentrations than iARGs, serving as a significant reservoir for ARG propagation (Mao et al., 2014). However, ARGs associated with extracellular polymeric substances (EPS) have been investigated, as extracellular ARGs can be absorbed or mobilized by EPS (Wang et al., 2022). A higher abundance of ARGs in the EPS matrix of activated sludge flocs from a wastewater treatment plant (WWTP) has also been reported and EPS-associated ARGs had higher transformation potential compared to cell-free ARGs, with transformation abilities ranging from 3.3-236.3 times higher (Wang et al., 2021). The formation of EPS is a relevant factor in wastewater treatment in microalgae-bacteria systems, as it promotes the formation of flocs and granules (Arcila & Buitrón, 2017). Consequently, it is possible that ARGs exist within the matrix of microalgae-bacteria aggregates. However, to the best of our knowledge, there are no reports on ARGs associated with EPS in microalgae-bacteria systems.

1.8 Scope and structure of the thesis

In Mexico, there is little evidence of the fate of ARB and ARGs in wastewater, and the lack of wastewater treatment coverage and the current state of the WWTPs suggests a potential risk for the spread of ARB and ARGs into the environment. Therefore, it is important to develop treatment alternatives that are promising for reducing antibiotic resistance and are, at the same time, less expensive than conventional systems or advanced processes that are only feasible in high-income countries. This thesis will elucidate the fate of ARGs in a microalgae-bacteria system for wastewater treatment, as well as the use of anaerobic reduction of antibiotic resistance that still remains in the microalgae-bacteria aggregates generated in these systems. This will allow the use of an integrated system to further reduce antibiotic resistance during the wastewater treatment train and potentially save energy costs through biogas production. In addition, the spatial distribution of ARGs and microbial communities present in the different EPS fractions in the MABA aggregates will be elucidated. Finally, the knowledge generated in this work will allow a better understanding of ARG removal in MABA systems and contribute to an effective solution for ARG mitigation in wastewater treatment for low-to-middle-income countries. For this purpose, this thesis is structured in one literature revision section, including the hypothesis and objectives, and three experimental sections that describe the results that respond to the scientific hypothesis. Finally, a general discussion section integrates results and final remarks and identifies the study perspectives. The specific content of the following sections is presented below.

In **Section 2**, we investigate the fate of ARGs in microalgae-bacteria system employed for the treatment of domestic wastewater. This was conducted using two parallel, outdoor HRAPs. The effects of granulation and microbial community conditions on resistomes were assessed using a combination of metagenomic and quantitative qPCR approaches. There are no studies based on microalgae-bacteria that evaluate the robustness of ARG removal over time in pilot-scale wastewater treatment systems, as the few existing studies have focused on batch operation or using monoalgal cultures (Cheng et al., 2021, Cheng et al., 2020; Kumar et al., 2021; Nõlvak et al., 2018). Therefore, this study provides knowledge on the efficient reduction of ARGs using microalgae-bacteria systems as an alternative treatment for LMICs, exemplified by a case study in Mexico.

Section 3 is conduct with the understanding that anaerobic digestion can be coupled to wastewater treatment not only for biogas generation (Carrillo-Reyes et al., 2021), but also for further reduction of antibiotic resistance. This follows our observations from the previous section, where certain ARGs were significantly more concentrated in the MABA than in the effluent (from the HRAPs). Therefore, in this section, we assess the influence of temperature (under both mesophilic and thermophilic conditions) during the anaerobic digestion of MABA on biogas production and the elimination of ARB and ARGs. Noting that studies of ARGs in anaerobic digestion are primarily derived from biosolids originating from activated sludge systems, our research addresses the knowledge gap regarding the fate of ARGs in biosolids produced from emerging systems, such as HRAPs.

In **Section 4**, we investigate the distribution of ARGs in wastewater and the effectiveness of microalgae-bacterial systems for ARB and ARGs reduction. This section examines how eARGs interact with EPS in microbial aggregates and distinguishes between the roles of bound and free EPS in ARGs dynamics. This study aimed to map the spatial distribution of ARGs by focusing on both the intracellular and EPS-associated extracellular fractions.

Section 5 provides a comprehensive discussion of this thesis's key findings, placing them within the broader context of current trends in antibiotic resistance in microalgae-bacteria systems. It also discusses the challenges and future perspectives to be considered.

1.9 Hypothesis

- The ecological exclusion of microorganisms and the spatial distribution of antibiotic resistance genes in the extracellular polymeric substances fractions have a significant effect on the fate of antibiotic resistance during the treatment of domestic wastewater using a microalgae-bacteria system.
- Thermophilic conditions during anaerobic digestion of microalgae-bacteria aggregates, significantly enhances the removal of antibiotic resistance genes and antibiotic-resistant bacteria compared to mesophilic conditions. This improvement is due to reduced microbial diversity, decreased mobilome, and enhanced microbial functionality.

1.10 Thesis objectives

General objective

To evaluate the fate of antibiotic resistance genes during domestic wastewater treatment using a microalgae-bacteria system and the persistence of antibiotic resistance genes and resistance bacteria during anaerobic digestion of microalgae-bacteria aggregates.

Specific objectives

- To determine the fate of antibiotic resistance genes during the treatment of domestic wastewater using a microalgae-bacteria system.
- To evaluate the effect of temperature (mesophilic and thermophilic) on the removal of antibiotic resistance genes and antibiotic-resistant bacteria during the anaerobic digestion of microalga-bacteria aggregates.
- To assess the impact of ecological exclusion of microorganisms and the spatial distribution of antibiotic resistance genes in the extracellular polymeric substances fractions on the fate of antibiotic resistance during the treatment of domestic wastewater using a microalgae-bacteria system.

1.11 Experimental strategy

To answer the proposed hypotheses, the general objective and the specific objectives, the thesis was structured into three main experiments (Figure 1.4):

Experiment I: Exploring resistomes and microbiomes in pilot-scale microalgae-bacteria wastewater treatment systems for use in low-resource setting

For the first experiment, two HRAPs reactors were operated for replication purposes, fed continuously with domestic wastewater and with an established HRT of 6 d. They were operated for 140 days. Physical and chemical parameters were evaluated to determine the performance of the reactors and their settling capacities. Finally, for antibiotic resistance, metagenomic analysis was used to identify the resistome and qPCR was used to evaluate some ARGs.

Experiment II: Antibiotic resistance reduction during anaerobic digestion of microalgae-bacteria aggregates: temperature effect

Building on the results from the HRAPs, the second phase focused on anaerobic digestion of MABA. Two different temperatures in anaerobic digestion were tested, thermophilic and mesophilic. In this study, we evaluated antibiotic-resistant bacteria using selective agar plates.

Experiment III: Extracellular polymeric substances associated to antibiotic resistance genes in microalgae-bacteria systems

In this experiment, plate photobioreactors were used to study the ARGs present in the EPS fractions. In this study, analyses of ARB and ARGs using metagenomics were included. In addition, metagenomic sequencing was carried out on selective agar plates to construct MAGs and have a more specific identification of bacteria and resistance genes.

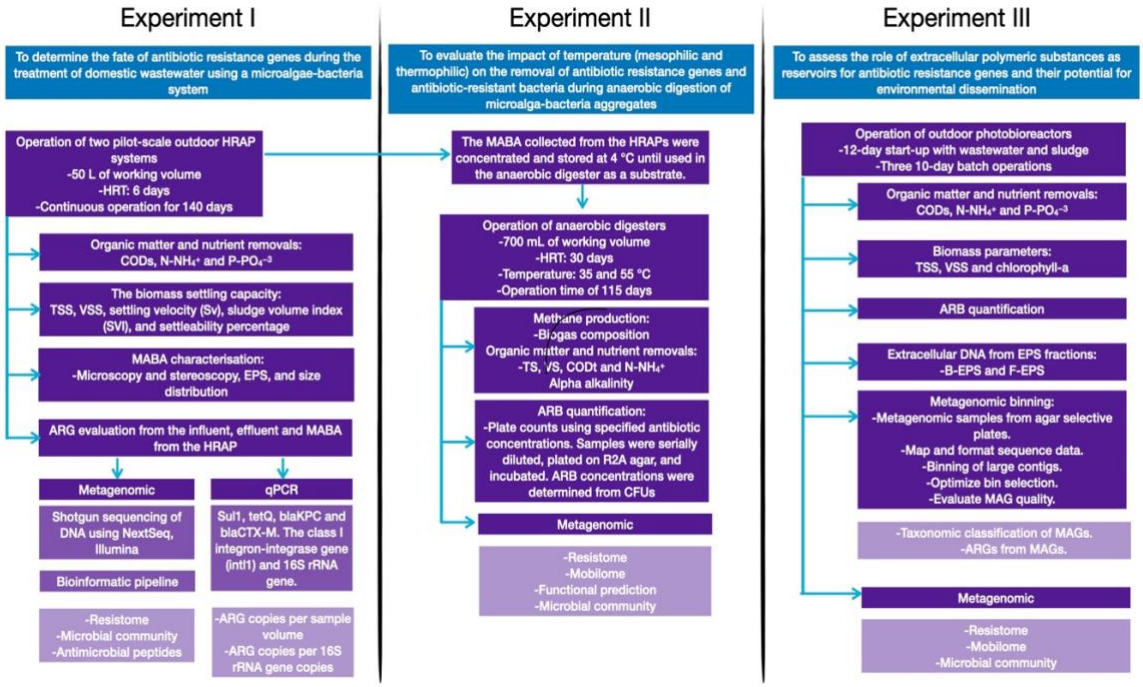

Figure 1.4. Experimental strategy.

2. Exploring resistomes and microbiomes in pilot-scale microalgae-bacteria wastewater treatment systems for use in low-resource setting

Abstract:

Antibiotic resistance genes (ARGs) released into the environment are an emerging human and environmental health concern, including ARGs spread in wastewater treatment effluents. In low-to-middle income countries (LMICs), an al- ternate wastewater treatment option instead of conventional systems are low-energy, high-rate algal ponds (HRAP) that use microalgae-bacteria aggregates (MABA) for waste degradation. Here we studied the robustness of ARG removal in MABA-based pilot-scale outdoor systems for 140 days of continuous operation. The HRAP system successfully removed 73 to 88 % chemical oxygen demand and up to 97.4 % ammonia, with aggregate size increasing over operating time. Fourteen ARG classes were identified in the HRAP influent, MABA, and effluent using metagenomics, with the HRAP process reducing total ARG abundances by up to 5-fold from influent to effluent. Parallel qPCR analyses showed the HRAP system significantly reduced exemplar ARGs ($p < 0.05$), with 1.2 to 4.9, 2.7 to 6.3, 0 to 1.5, and 1.2 to 4.8 log-removals for *sul1*, *tetQ*, bla_{KPC}, and *intl1* genes, respectively. Sequencing of influent, effluent and MABAs samples showed associated microbial communities differed significantly, with influent communities by Enterobacteriales (clinically relevant ARGs carrying bacteria), which were less evident in MABA and effluent. In this sense, such bacteria might be excluded from MABA due to their good settling properties and the presence of antimicrobial peptides. Microalgae-bacteria treatment systems steadily reduced ARGs from wastewater during operation time, using sunlight as the energetic driver, making them ideal for use in LMIC wastewater treatment applications.

Reference to published article: Ovis-Sánchez, J. O., Perera-Pérez, V. D., Buitrón, G., Quintela-Baluja, M., Graham, D. W., Morales-Espinosa, R., & Carrillo-Reyes, J. (2023). Exploring resistomes and microbiomes in pilot-scale microalgae-bacteria wastewater treatment systems for use in low-resource settings. *Science of The Total Environment*, 882, 163545. https://doi.org/10.1016/j.scitotenv.2023.163545

Abbreviations

AMP	Antimicrobial peptides
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance genes
CAS	Conventional activated sludge
CODs	Soluble chemical oxygen demand
EPS	Extracellular polymeric substance
ESKAPEE group	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.* and *Escherichia coli*
DO	Dissolved oxygen
LDA	Linear discriminant analysis
LMIC	Low-to-middle income countries
HRAP	High-rate algal ponds
HRT	Hydraulic retention time
intl1	Class I integron-integrase gene
MABA	Microalgae-bacteria aggregates
NMDS	Non-metric multidimensional scaling analysis
q	Diversity Hill numbers
SRT	Solid retention time
Sv	Settling velocity
SVI	Sludge volume index
TSS	Total suspended solids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plants

2.1 Introduction

The continued use of antibiotics in human and animal health care and agriculture, and wider pollution as has led to development, transmission and spread of antibiotic resistant bacteria (ARB) and genes (ARG) across many environments (Laxminarayan and Heymann, 2012). Consequently, wastewater treatment plants (WWTPs) receive wastes containing ARB and ARGs from multiple sources and are key foci for their treatment (Barancheshme and Munir, 2018). Biological WWTPs often have high microbial cell densities and diversity and, if poorly maintained and operated, can provide selective conditions for ARB and ARGs. Still, if WWTPs are well-designed and operated are a critical barrier against the broader spread resistance, especially relative wastewater releases without effective treatment (WHO et al., 2020).

Although conventional biological WWTPs can remove ARB and ARGs from wastewater, WWTPs were never designed for this purpose. Therefore, much work is focused on alternate treatment technologies to improve ARG removals. As advanced processes like chlorination, ultraviolet radiation, ozone disinfection, or chemical oxidation are being promoted (Michael-kordatou et al., 2018), but such processes are costly and require high maintenance, making them only options in high-income settings. In low-to- middle income countries (LMICs), less expensive, and more self-sustaining and energy efficient treatment technologies are needed because LMICs often lack well-developed community health surveillance programs and have poor quality antibiotics, but also easy access to last-resort antibiotics, making inappropriate use common (Chokshi et al., 2019; Michael et al., 2013). Further, in Latin America, only 50 % of human populations are connected to contained sewer drains and only 30 % receive any waste- water treatment. In Mexico, national coverage by domestic wastewater treatment in 2017 was 63 % (Tabla-Vázquez et al., 2020). Therefore, improved civil infrastructure and new WWTPs are needed. However, new technologies must more sustainable than traditional options, such as conventional activated sludge (CAS), especially options that effectively remove organic carbon and nutrients, but also reduce the release of ARB and ARGs to the environment.

Among sustainable emerging technologies, microalgae-bacteria treatment systems that reply on symbiotic consortia including microalgae and heterotrophic bacteria, show particular promise. Microalgae-bacteria systems are low energy due to reduced oxygen demand, allow nutrient recycling during wastewater treatment, and generate levels of O_2 while fixing CO_2, unlike CAS systems. Moreover, they are efficient at pollutant removal, and produce biomass, achieving a positive energy balance when coupled with anaerobic digestion (Carrillo-Reyes

et al., 2021). High-rate algal ponds (HRAP) for wastewater treatment are particularly economical and sustainable, and as such, are a particularly promising alternative for LMIC applications. In continuous operations, HRAP systems propitiates the formation of microalgae-bacteria aggregates (MABA), such as flocs and granules (dos Santos Neto et al., 2021; Nwoba et al., 2020), which have already shown to effectively remove antibiotics from wastewater, including 100 % removal of sulfamethazine, tetracycline, ciprofloxacin, and others (Leng et al., 2020). There is evidence that MABA chemistry and ecology drive antibiotics removal because granules appear to contain elevated extracellular polymeric substance (EPS) levels when exposed to tetracycline (Wang et al., 2020a; Wang et al., 2021). Therefore, if HRAP systems also re- move ARGs and ARB, they become an ideal and economic option for reducing the spread of both antibiotics and antibiotic resistance via wastewater, which are amenable to LMIC conditions.

Up to now, few studies have evaluated the potential of monoalgal cultures to remove ARB or ARG (Cheng et al., 2021, Cheng et al., 2020; Kumar et al., 2021; Nõlvak et al., 2018). For instance, microalgae *Pseudochlorella pringsheimii* removed 7 of 10 ARB isolates (Kumar et al., 2021). Nõlvak et al. (2018) reported a decrease in the antibiotic resistome by 3.4 times using municipal wastewater and lake water for algal biomass production. Further, Cheng et al. (2020) compared ARG and one integron gene fate between CAS and a monoalgal systems of *Galderia sulphuraria*, and the algae system reduced ARB and ARG levels by 1 to 4 and 0.6 to 3 log, respectively, which greater than the CAS system. Using metagenomic profiling, they also found that the monoalgal system eliminated 7 of 10 ARG classes in wastewater and 4 of 19 virulence genes. Some studies have assessed antibiotics, ARG, and ARB removals in algae systems, but they have not used continuous-flow systems (Cheng et al., 2021, Cheng et al., 2020; Kumar et al., 2021; Nõlvak et al., 2018). Therefore, evaluating the microalgae-bacteria treatment options under more realistic conditions is urgently needed to verify antibiotic resistance are sustainable reductions over time (González-Camejo et al., 2021). To our knowledge, there are no studies based on MABA that evaluate the robustness of ARG removal over time in pilot-scale wastewater treatment systems. Therefore, here we studied the fate of ARG in microalgae-bacteria systems for domestic wastewater treatment in two parallel, outdoor HRAP systems. The effect of granulation and microbial community conditions on resistomes, were assessed using a combination of metagenomic and qPCR approaches. This study provides scientific support to validate the removal of ARGs from wastewater with a robust and sustainable emerging technology based on microalgae-bacteria.

2. 2 Materials and methods

2.2.1 Wastewater

The domestic wastewater was collected weekly from a WWTP (30 L s^{-1} treatment capacity for 13,535 inhabitants) in Santiago de Queretaro, Mexico (20.7349 N 100.4517 W), from June to November 2020 (16 samples total to continuous feeding of the HRAPs). Wastewater was collected after primary treatment (coarse and fine screening and primary sedimentation). Before use, large residual solids were removed from the wastewater through a sieve (Tyler No. 65) and the wastewater was stored at 4 °C for maximum of seven days. The average pH value of the wastewater over operations was 7.5 ± 0.3, total and soluble chemical oxygen demand (CODt and CODs, respectively) were 673 ± 376.6 and 328 ± 155.2 mg L^{-1}, volatile suspended solids (VSS) was 139 ± 110 mg L^{-1}, and ammonium ($N\text{-}NH_4^+$) and phosphate ($P\text{-}PO_4^{3-}$) levels were 45.6 ± 21.9 and 21.3 ± 7.6 mg L^{-1}, respectively.

2.2.2 Experimental set-up

All experiments were conducted using two pilot-scale outdoor HRAP systems (Fig. S1) that were operated as duplicates at Querétaro, México (20.7034 N 100.4459 W, 1900 m above sea level), from June to November 2020. The HRAPs had 80 L and 50 L total and working volumes, respectively, a surface area of 0.26 m^2, and mixing consistent with a liquid velocity of 0.2 m s^{-1}, as described previously (Arcila and Buitrón, 2016). The effluent was settled using a 2 L settler prior to sample collection.

Initially, both HRAPs were inoculated with 10 L of activated sludge and 40 L of a secondary effluent to propitiate the growth of the microalgae-bacteria consortium and were run in batch mode for 32 days. After acclimation, each HRAP was continuously fed with wastewater for 140 days, at a constant hydraulic retention time (HRT) of 6 d, as recommended by Arcila and Buitrón, (2016), using different solid retention times (SRT) to prompt the formation of the MABA (Table 2.1). Organic matter and nutrient removals were monitored over time using CODs, $N\text{-}NH_4^+$ and $P\text{-}PO_4^{-3}$ in influent and effluent samples. The biomass settling capacity was followed by total suspended solids (TSS), VSS, settling velocity (Sv), sludge volume index (SVI), and settleability percentage. The presence of microalgae, and the formation of aggregates during experimentation, were followed by microscopy and stereoscopy. Extracellular polymeric substances (EPS), and their protein and carbohydrate content were analysed to characterize the MABA.

Physicochemical determinations were performed once per week in duplicate in each reactor. The dissolved oxygen (DO), pH, temperature and irradiation were measured online using calibrated probes every 30 min; using a plug-and-play LabQuest® card (Vernier, Oregon, USA), and the Logger Pro® 3 software (Vernier, Oregon, USA). Influent, effluent, and MABA samples were collected from each HRAP replicate during the last week of each operational stage (Table 1). These samples were collected for DNA extraction to characterize the microbial communities and ARG persistence (Fig. S2).

2.2.3 Analytical procedures

TSS, VSS, Sv, and SVI of the HRAP were analysed according to standard APHA methods (APHA, 2005). The settleability percentage was calculated using Eq. (1).

$$\text{Settleability percentage } (100\%) = 100 * \left(1 - \frac{X_e}{X}\right) \quad (1)$$

Xe is the TSS in the effluent, and X is the TSS in HRAP. From the soluble fraction (samples filtered through 0.45 µm pore size membranes), N-NH_4^+, CODs, and P-PO_4^{3-} were analysed using the 10,031, 8000, and 10,127 Hach colourimetric methods, respectively.

Two types of EPS were evaluated from HRAP mixed liquor, firmly bound to the cells and weakly bound to the cells or present in solution, i.e., bound EPS (B-EPS) and free EPS (F-EPS), respectively (Arcila and Buitrón, 2017). A Leica DM500 microscope with an image acquisition system (Leica ICC50 HD) and a stereoscopic lens (Zeiss Stemi DV4) were used to follow the formation of aggregates. The size distribution of the MABA was determined according to Alves et al. (2018), by capturing images of the granules (digital scanner, G3110, Hewlett-Packard Company, USA), and measuring their dimensions computationally. Images were analysed using the ImageJ software (version 1.52a), sizing the Feret diameter as previously reported for microalgae floccules (Vandamme et al., 2014).

2.2.4 DNA based analysis

2.2.4.1 DNA extraction

Fifteen samples were processed in total for DNA extraction, 3 samples from influent (one sample per stage), 6 from MABA (2 replicates for each stage), and 6 from effluent (2 replicates for each stage). 60 mL for influent, 45 mL for MABA and 200 mL for effluent samples were concentrated via vacuum filtration using sterile 0.22-µm membrane disc filters (Millipore,

USA). These volumes were selected to avoid the filter clogging, but recovering enough DNA for downstream analysis. The membrane was then stored at −20 °C until the DNA extraction using the DNeasy PowerWater kit (Qiagen, Germany) according to manufacturer's instructions. The quality and concentration of DNA samples were verified by spectrophotometry using a NANODrop 2000c (Thermo Scientific, USA).

2.2.4.2 Resistome analyses by metagenomic approach

Shotgun metagenomic sequencing of DNA samples from influent (n = 3), effluent (n = 6), and MABA (n = 6) were sequenced using NextSeq, Illumina (2× - ~8 million PE 150 + 150 bp reads) at the Integrated Microbiome Resource Lab (IMR) at Dalhousie University (Halifax, Canada). After sequencing, one sample failed (effluent replicate of stage 1), so we didn't consider it for further analysis. The metagenomic sequences processing pipeline is described in Supplementary Information. Raw metagenomic sequences were deposited into the NCBI sequence read archive (SRA) database under the project number PRJNA807808.

The processed sequences were uploaded to the Metagenomics Rapid Annotation using Subsystems Technology server (MG-RAST) (Meyer et al., 2008). The taxonomic profiles were generated using the RefSeq data-base with 95 % of sequence identity, 50 of minimum query coverage and 1 of minimum abundance.

2.2.4.3 Antimicrobial peptide screening

The assembled contigs were screened using the Macrel pipeline to determine the antimicrobial peptides (Santos-Júnior et al., 2020). The potential inhibitory effect on E. coli of such peptide sequences was predicted using the Database of Antimicrobial Activity and Structure of Peptides (DBAASP)(https://dbaasp.org/home).

2.2.4.4 Antibiotic resistance genes quantification

Exemplar genes for different types of antibiotic resistance were selected to follow their persistence during the various operational stages. Based on influent sequencing data, the following genes were selected: *sul1*, *tetQ*, bla_{KPC} and bla_{CTX-M}. The class I integron-integrase gene (*intl1*) was monitored as an indicator of putative multiple antibiotic resistance (Zhang et al., 2018). *16S rRNA* was quantified to estimate the total bacterial concentration and normalise ARG abundances to cell density in the samples. Hence, those six genes were quantified for all 15 DNA samples, according to the replicates recommendations for

reproducible qPCR experiments (Taylor et al., 2019), and using qPCR (Applied Biosystems, StepOne Real-Time PCR System) and SYBR Green chemistry (Power SYBR Green PCR Master Mix). Standard curves were constructed in each PCR run, and the copied numbers of the genes in each sample were interpolated using these standard curves. Standards were quantified in triplicate, and replicated samples and negative control (sterile water) were quantified in duplicate. The following requirements were satisfied to obtain reliable quantification: R^2 higher than 0.98 for standard curves over five orders of magnitude and amplification efficiencies based on slopes between 85 % and 106 % (Table S1). The Supporting Information (Table S1) describes the primers, annealing temperatures, and calibration curves data. The specificities of the used primer sets (Table S1) have been validated in recently published papers (Tian et al., 2016; Nõlvak et al., 2018) and verified in this study by gel electrophoresis and melting curves. qPCR data are reported as absolute (ARG copies per sample volume) and relative abundances (ARG copies per *16S rRNA* gene copies) (Liu et al., 2014).

Table 2.1. Operation strategy of the HRAP.

Stage	Batch	I	II	III
Period of days	0-32	32-63	64-106	107-140
HRT (d)	-	6	6	6
STR (d)	-	12 ± 0.0	6.5 ± 0.5	9.1 ± 2.0

2.2.5 Statistical analysis

Data normality was assessed using a Shapiro–Wilk test. In the case of parametric data, significant differences were determined by a parametric One-way ANOVA test and a Tukey post-hoc test. Otherwise, Kruskal–Wallis and post-hoc pairwise multiple comparisons (Pairwise Wilcoxon test with Benjamini & Hochberg adjustment) for non-parametric data. A significance level $\alpha = 0.05$ was used for all the tests. For shotgun metagenomic sequencing, results represent a mean of n = 2 biological replicates, and for qPCR there were n = 2 biological replicates and two technical replicates, for a total of n = 4, as suggested previously (Ju et al., 2016; Flores-Orozco et al., 2020; Wang et al., 2020b; Cheng et al., 2021). The statistical analysis was performed using RStudio v.3.4.1 (RStudio Team, 2016).

To determine bacterial taxa with significantly different abundance among sampling sites, the linear discriminant analysis (LDA) effect size (LEfSe) method (http://huttenhower.sph.harvard.edu/lefse/) was used based on a normalized relative

abundance matrix. The LEfSe method uses the Kruskal-Wallis test to identify significant differences and performs a LDA to evaluate the effect of taxa group size. A threshold score of 2 and a significance α of 0.05 were used to detect differences. Sites (influent, MABA and effluent) were considered as classes and different stages (Stage I, II and III) as subclasses.

Alpha-diversity was calculated using Hill numbers (q) using hilldive package, with q from 0 to 3 and were expressed in units of "effective" number of species. In this method, as q increases, lower abundance OTUs are assigned less weight, while higher abundance OTUs are assigned more weight. This permits investigation of diversity at different scales. At q = 0, is simply the number of observed OTUs (richness). At q = 1, exp. (Shannon), and at q = 2, is equal to inverse Simpson's index.

A correlation matrix was developed for the ARG network by calculating all possible pairwise Spearman's rank correlations among bacterial orders and ARG types. Only the item with a correlation index higher than 0.7 ($p < 0.05$) was considered. The co-occurrence networks were visualised by Gephi (version 0.9.5). The Heatmap of microbial community composition dissimilarity was estimated by the Bray Curtis index using the heatmap.2 function of the gplots package. Spearman's correlation was calculated using Hmisc package. The Venn diagram was plotted using InteractiVenn (http://www.interactivenn.net).

2. 3 Results and discussion

2.3.1 Wastewater treatment efficiency and MABA characterisation

Both HRAP systems achieved acceptable CODs (i.e., 73.3 to 88.4 %) and N-NH_4^+ (93.3 to 98.2 %) removal rates (Table 2.2), which is consistent with other studies with similar systems (Arcila and Buitrón, 2017, Arcila and Buitrón, 2016). Detectable P-PO_4^{3-} removal only occurred in stage I (51.8 ± 23.4 %), probably from chemical precipitation because the pH approached 10.9 at this early stage due to abundant photosynthetic activity (Table 2.2). At stages II and III, the pH was controlled at 7.5 with HCl 10 N additions to allow better production of biomass (Eze et al., 2018). At stages II and III, P-PO_4^{3-} removal was negligible, possibly due to cell rupture, releasing the intercellular phosphate content (Martínez et al., 2000).

The two HRAP systems performed as replicates with similar relative to CODs and N-NH_4^+ removal rates, Sv, SVI, TSS in the effluent and settleability percentage during the entire experimentation ($p > 0.05$, ANOVA and Kruskal-Wallis test). For CODs and N- N-NH_4^+ removals, rates were similar between summer to autumn sampling despite differences rates differences in wider environmental conditions season (Table 2.2), suggesting these systems

are potentially resilient technologies, in the Mexican climate, as has been observed under similar conditions such as continuous operation in outdoor (dos Santos Neto et al., 2021) and also in different cli- mate like Sweden (Ferro et al., 2020).

Sv values, settleability percentages, and SVI values indicate good settleability in the biomass (Table 2.2) (Jin et al., 2003). Clear aggregate formation was observed in the HRAP systems, where MABA diameter distribution consequentially increasing from stage I to III (Figure 2.1), with mean particle diameters (< 2 mm) positive correlating with settleability ($p < 0.05$). Only the concentration of the bound fraction of EPS was higher through the three stages (Table S2). Previously, Trebuch et al. (2020) found that aggregation was favoured by increasing EPS protein:carbohydrate (P:C) ratios within the range of 1.2 to 6.9. Here was found P:C ratios in microbial aggregation averaged about 1.3 (Fig. S3).

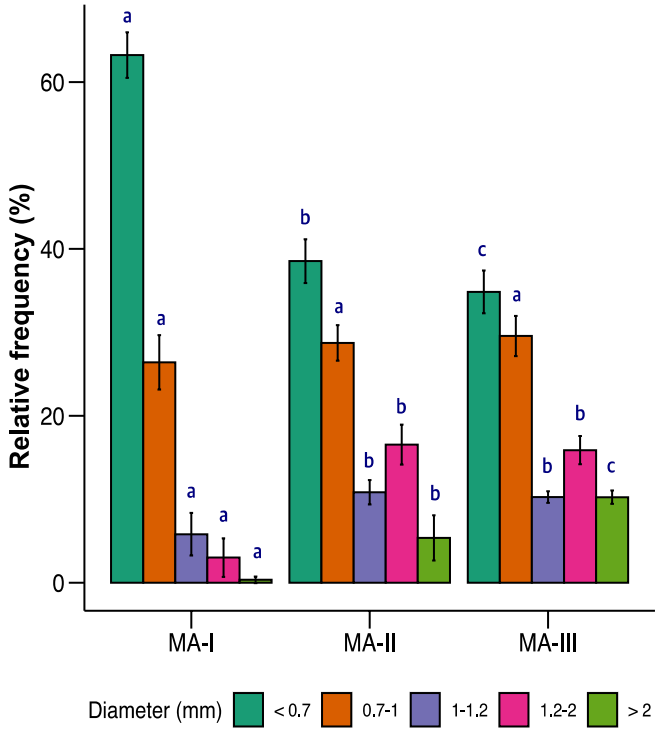

Figure 2.1. Relative frequency of particle diameter distribution of microalga-bacteria aggregates (MA) at operational stages I, II and III. Lowercase letters from post hoc comparison indicated significant differences ($p < 0.05$).

Table 2.2. Performance parameters, environmental conditions, settling capacity parameters and EPS production through the three operation stages.

Parameter	Stage I		Stage II		Stage III	
Performance parameter[a]	Mean ± SD[b]	%[c]	Mean ± SD	%[b]	Mean ± SD	%[b]
CODs[d] (mg L^{-1})	50 ± 26	73.3 ± 10.2	61 ± 11	76 ± 7.6	62 ± 23	88.4 ± 4.6
N-NH_4^+ (mg L^{-1})	1 ± 0.7	98.2 ± 1.4	4 ± 6.4	93.3 ± 8.3	2 ± 2.7	97.4 ± 3.5
P-PO_4^{3-} (mg L^{-1})	9.3 ± 3.6	51.8 ± 23.4	17.5 ± 3.7	0	34 ± 2.8	0
Environmental conditions	Mean ± SD	Min/Max	Mean ± SD	Min/Max	Mean ± SD	Min/Max
pH	8.7 ± 1.1	7.4/10.9	7.5 ± 0.4	6.9/8.6	7.4 ± 0.3	6.9/8.4
DO[e] (mg L^{-1})	6.5 ± 4.2	0/20.1	6.1 ± 2.0	1.3/11.9	5.4 ± 1.7	1.4/9.5
Temperature (°C)	19.5 ± 3.5	12.4/29.6	16.7 ± 4.2	5.4/29.4	14.6 ± 4.1	5.1/24.0
Irradiance[f] (μmol m^2 s^{-1})	713.6 ± 294.5		647.5 ± 383.8		800.6 ± 287.9	
Settling capacity parameter	Mean ± SD		Mean ± SD		Mean ± SD	
TSS in effluent (mg L^{-1})	21.4 ± 2.4		24.8 ± 11.5		16.2 ± 5.9	
Settleability (%)	95.6 ± 1.1		97.3 ± 1		97.6 ± 1.1	
SVI (mL g VSS^{-1})	47 ± 12.4		35.9 ± 4.3		31.7 ± 3.7	
Sv (m h^{-1})	ND[g]		0.7 ± 0.1		0.8 ± 0.1	
EPS[h] fraction	Mean ± SD		Mean ± SD		Mean ± SD	
	Free	Bound	Free	Bound	Free	Bound
Proteins (mg g VSS^{-1})	15.4 ± 7.7	31.0 ± 10.0	38.3 ± 6.7	39.4 ± 8.9	10.1 ± 0.8	59.9 ± 21.1
Carbohydrates (mg g VSS^{-1})	20.1 ± 5.8	17.8 ± 2.8	34.7 ± 3.1	31.4 ± 10.1	20.9 ± 1.4	32.3 ± 5.6

[a] effluent concentration; [b] SD = standard deviation; [c] % = percentage of removal, considering effluent/influent concentration ratio; [d] CODs = soluble chemical oxygen demand; [e] DO = dissolved oxygen; [f] Maximum solar irradiance during the day; [g] ND = no determinate; [h] extracellular polymeric substances.

2.3.2 Resistome changes after HRAP wastewater treatment

Metagenomic analysis associated with the structured CARD database found 209 to 238, 8 to 31 and 5 to 40 ARG subtypes in influent, MABA and effluent samples, respectively. Although many ARG subtypes decreased from the influent, 15 ARG subtypes were shared across all samples, and nine ARG subtypes below detection in the influent emerged in the effluent and MABA (Fig. S4). Resistome richness was reduced by treatment with 3 of 14 ARG types (aminocoumarin, fosfomycin and peptide) not detected in effluent and MABA samples. However, various types of ARG were still detected in the MABA and effluent, with aminoglycoside, beta-lactam, macrolide, sulfonamide and tetracycline ARGs being most abundant (Figure 2.2). This is similar to Cheng et al. (2021) who re- ported that macrolide, beta-lactam, and aminoglycoside ARG types remained in algae treatment effluents. HRAP consistently reduced total ARG abundances (resistome) by up to 5-fold when comparing ARGs in the effluent to the influent, which is greater than the 3.4-fold reduction reported previously using a monoalgal photobioreactor (Nõlvak et al., 2018), and also higher than the 2.1-fold reduction reported from CAS WWTPs (Cheng et al., 2021).

The most abundant ARG types in the influent ranked by concentration were multidrug, tetracycline, aminoglycoside, and then beta-lactam genes (Figure 2.2). Multidrug ARGs here refers to genes that confer resistance by more generic cell defense mechanisms, such as for efflux pumps that facilitate defense against different type of substances, such as antibiotics and heavy metals (Huang et al., 2022). It does not refer ARGs, such as bla_{NMD-1} or bla_{KPC}, which provide extended spectrum multi-resistance (Nordmann and Poirel, 2019).

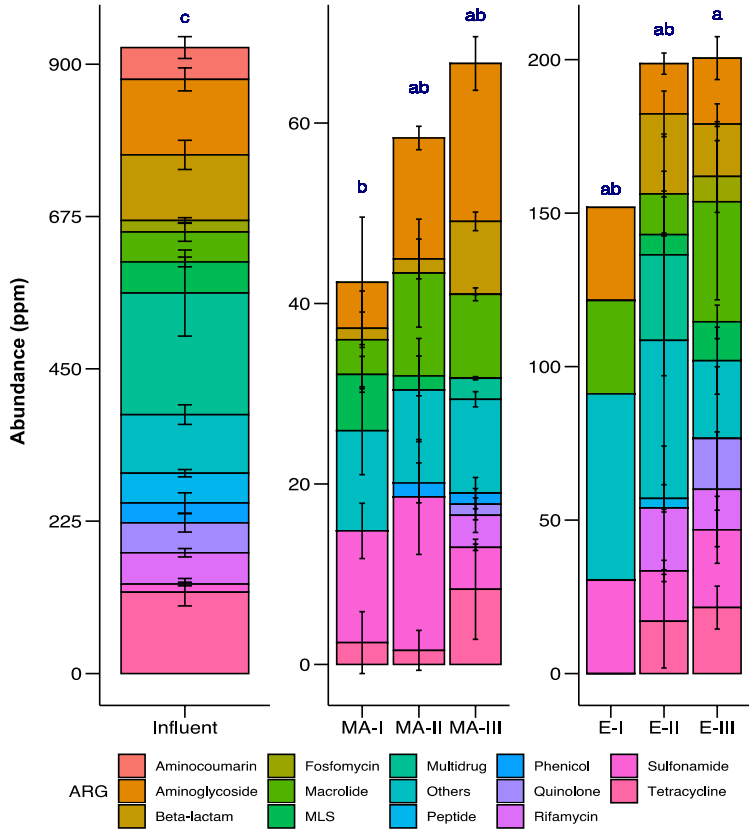

Figure 2.2. Abundance (ppm) of ARG types in influent, MABA (MA) and effluent (E) from the different operational stages I, II and III. Lowercase letters from post-hoc comparison indicated significant differences ($p < 0.05$).

2.3.3 qPCR quantification of specific ARG and intl1 in HRAP

The copy number and abundance of exemplar ARGs and intl1 in the in- fluent, effluent and MABA also were quantified using qPCR (Figure 2.3). Log- reductions of *sul1*, *tetQ*, bla_{KPC} and *intl1* from influent to effluent were 1.2 to 4.9, 2.7 to 6.3, 0 to 1.5 and 1.2 to 4.8, respectively, were higher removals that observed by Nõlvak et al. (2018). Conversely, levels of $bla_{CTX\text{-}M}$ increased in the HRAP systems, which was unexpected (Figure 2.3), which has sometimes been seen in other wastewater treatment studies (Pazda et al., 2019). Increases in *sul1* were previously observed by Cheng et al. (2020) in their monoalgal system. Although the exemplar ARGs and *intl1* were reduced through treatment, their concentration was higher in MABA granules than in the effluent, which is explained by efficient clarification in HRAP processes. These results are consistent with Quintela-Baluja et al. (2019), who showed that microorganisms in activated sludge floc in a CAS WWTP may be a selective microecosystem

with different ARGs, including lower diversity, compared with the liquid effluent fraction exiting the final clarifier.

In the case of HRAP systems, if ARG concentrations in MABA are potentially greater than the effluent, it is essential to manage aggregate disposal to reduce potential spread to the wider environment from the resultant biosolids, which has been suggested elsewhere (Xue et al., 2019). Influent *tetQ*, *sul1*, *16S rRNA* and *intl1* absolute abundances observed here are consistent with other reported raw wastewater (Li et al., 2017; Narciso-da-Rocha et al., 2018). However, influent bla_{KPC} and $bla_{CTX\text{-}M}$ observed here lower than the other ARG detected (Log 3.7 ± 0.0 and 2.6 ± 0.6, respectively), which may partially explain why $bla_{CTX\text{-}M}$ increased in our systems (i.e., influent levels were low).

Significantly higher relative ARG levels (as gene copy per *16S rRNA* gene) were seen in the influent compared with MABA and the effluent (Figure 2.4; $p < 0.05$), except for $bla_{CTX\text{-}M}$. Decreasing relative abundances of most ARGs is consistent with Cheng et al. (2020) in their monoalgal system. However, work here shows ARG removal in a continuous-flow outdoor pilot system, more typical of future applications. These results imply that HRAP systems promote ecological exclusion, which reduces ARG's presence. Thus, although the bacterial load is not significantly different from the effluent (stage III) and the influent (Figure 2.3), HRAP treatment changes the microbial composition (Fig. S6). Similar consequential differences in microbial communities and resistomes between different compartments has been seen in CAS WWTPs (Quintela-Baluja et al., 2019, 2021). Specific observations comparing different ARGs detected by qPCR and *intl1* (Figure 2.3; Figure 2.4) between the effluent and MABA are discussed in the following sections.

The ARGs and *intl1* identified and removed in this system are relevant considering the potential dissemination from effluent or MABA. The sulfonamide-resistant gene *sulI* is commonly present in WWTPs as it is part of class 1 integrons (Gillings et al., 2008; Li et al., 2017). *TetQ* confers resistance against tetracycline via ribosomal protection protein (Xiong et al., 2018). Plasmid-encoded $bla_{CTX\text{-}M}$ enzymes represent an important subgroup of class A b-lactamases which can hydrolyse expanded-spectrum cephalosporins preferentially cefotaxime in Enterobacteriaceae (Monstein et al., 2007). Bla_{KPC} is one of the leading causes of resistance to carbapenems in *K. pneumoniae*, and it can widely spread due to its location on various plasmids (Ghasemnejad et al., 2019). Besides ARG, another genetic element, the integrase gene (*intl1*) of class I integrons is believed to be involved with the evolution and proliferation of multiple antibiotic-resistant bacteria, determining the success of horizontal

genes transfer through of competent cell (Zhang et al., 2018). Furthermore, according to post-hoc comparison, significant differences were observed in the concentrations of ARGs and *intl1* (Figures 2.3, 2.4) in the different stages of effluent and MABA, which are discussed the following sections.

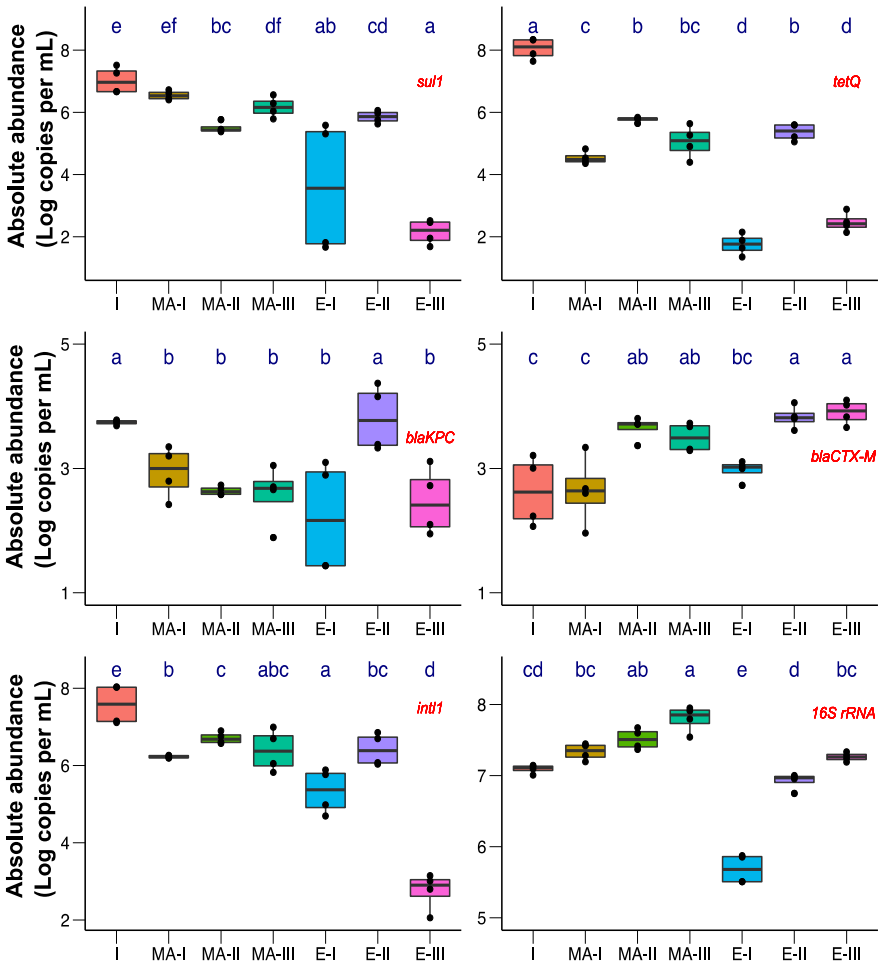

Figure 2.3. The absolute abundance of antibiotic resistant genes: *sul1*, *tetQ*, bla_{KPC}, $bla_{CTX\text{-}M}$, *intl1* and *16S rRNA* gene in influent (I), MABA (MA) and effluent (E) from the different operational stages I, II and III. Lowercase letters from post-hoc comparison indicated significant differences ($p < 0.05$).

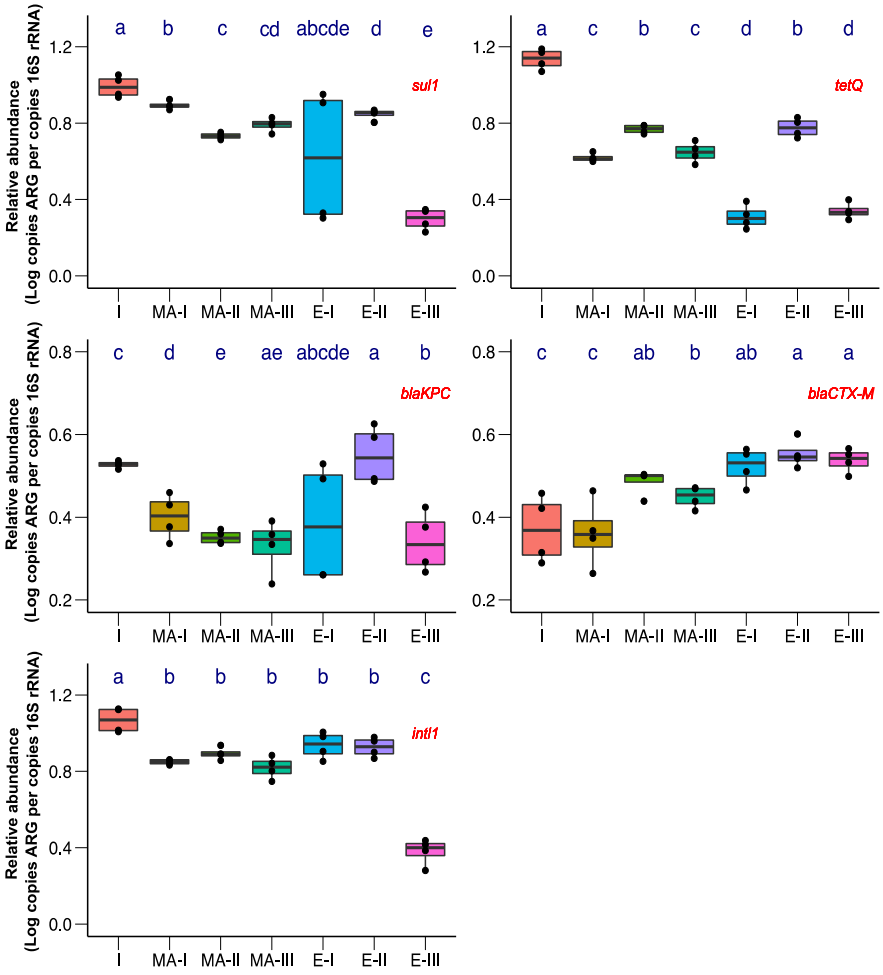

Figure 2.4. The relative abundance of antibiotic resistant genes: *sul1*, *tetQ*, bla_{KPC}, $bla_{CTX\text{-}M}$ and *intl1* in influent (I), MABA (MA) and effluent (E) from different operational stages I, II and III. Lowercase letters indicate significant differences ($p < 0.05$).

2.3.4 Factors influencing spatial differences in detected ARGs and intl1

To assess possible interconnections between absolute abundances of ARGs and *intl1* in the effluent and MABA with EPS, SVI and diameter distributions (<2 mm), Spearman's rank correlations were determined (Fig. S5). Improved settling properties (i.e., SVI) only significantly correlated with lower bla_{KPC} in MABA and greater $bla_{CTX\text{-}M}$ abundances in effluent samples ($p < 0.05$). Also, greater aggregate concentrations (i.e., more <2 mm particles) correlated with lower *intl1* abundances in the effluent. Conversely, greater EPS significantly correlated with lower *sul1* abundances in the MABA, whereas positive correlation between EPS levels with *tetQ* and $bla_{CTX\text{-}M}$ in MABA, suggests the generation of EPS may contribute to

the accumulation of these genes in the granules, which has been suggested previously (Wang et al., 2022).

Based on these observations, results here imply the generation of EPS in microalgae-bacteria consortia is important to aggregate formation and may play a role ARGs retained or released in the MABA. Antibiotics can be accumulated and degraded intracellularly in microalgal cells, while extracellular degradation depends on the excretion of EPS by microalgae acting as an external digestive system (Xiao and Zheng, 2016). Further studies are needed to elucidate the role of the specific ARG prevalence associated with the different EPS fractions.

2.3.5 Microbial community composition across compartments

Recent studies have shown that microbial community composition can help explain ARG demographics in wastewater treatment systems (Yu et al., 2020). Taxonomic profiles from our metagenomic analysis showed that bacteria were dominant influent samples, representing 98.6 % of sequences, with archaea and virus sequences reflecting 1.3 % and 0.02 % of the metagenomes, respectively. This taxonomic profile is slightly similar from WWTP influent, where around 94 % of the bacterial domain was covered (Reddington et al., 2020). In effluent and MABA samples, sequences corresponding to viruses and/or archaea were not detected, whereas eukaryotes ranged from 3 to 14 %.

All genera belonging to the ESKAPEE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, and *Escherichia coli)* were detected in the influent (Ngoi et al., 2021), with *Escherichia* being the predominant detected genus. However, most of the ESKAPEE strains were significantly reduced in the effluent and MABA compared with the influent (Table S3). It is possible that the significant removals of ARGs and *intl1* in the HRAP system is due to reductions in ESKAPEE bacteria since they are often reservoirs of ARGs, including multidrug strains (Ngoi et al., 2021). Using two-dimensional hierarchical clustering in conjunction with a heatmap of relative abundances (Fig. S6), influent samples cluster together in terms of microbial genera (bacteria, eukaryote, archaea and virus), including effluent and MABA data are not biased by changes in influent communities.

In contrast, the microbial communities in MABA and effluent cluster away from influent samples, except for E-I-R1. For MABA samples, the most representative microalgae were *Chlorella*, which had highest abundances in stage II (0.9–1.4 %). Diatoms like *Thalassiosira* and *Phaeodactylum* and cyanobacteria like *Synechocystis* also were observed. Chlorellales

(order pertaining to *Chlorella* genus) have been reported previously in HRAP systems for domestic wastewater treatment, with diatom abundances often related to improved granule sedimentation (Buitrón and Coronado-Apodaca, 2022).

The microbial community composition varied according to the sampling site and the operational stage, as shown by non-metric multidimensional scaling analysis (NMDS) (Fig. S7). Briefly, the microbial communities from influent samples grouped, suggesting that communities were similar during the experimental period. The MABA and effluent communities varied based on operational stage. It has been previously reported that microalgae-bacteria communities in HRAP systems that were operated outdoors varied according to season and wider environmental conditions (Ferro et al., 2020). Here we observe the same behavior, this implies that the microalgae-bacteria community also varies according to environmental conditions, however, although these conditions can disturb the system, the microalgae-bacteria community maintains stability regarding the reduction of ARGs.

When comparing richness and diversity across sampling sites, bacterial species richness (q = 0) was highest in influent samples, but bacterial diversity (q = 1 and q = 2) was lower in the influent than the MABA (Table S4), implying the influent bacteria community had the lowest evenness. Linear discriminant analysis showed that Enterobacteriales and Verrucomicrobia were the dominant groups in the influent (Fig. S8). Enterobacteriales has been found to correlate with clinically relevant ARGs in the WWTP influents in past studies (Rodríguez et al., 2021). In contrast, the MABA was dominated by the Rhizobiales, Rhodobacterales, Rhodospirillales and Rhodocyclales groups, which has been observed previously in microalgae-bacterial systems, where Rhodobacterales was the predominant order (Sánchez Zurano et al., 2020). Furthermore, it has been seen that Rhodobacterales appear to promote microalgal growth, and Rhodospirillales are active in ammonium degradation (Ferro et al., 2020). Although it is speculation, Rhodospirillales may partially explain high $N\text{-}NH_4^+$ removals observed herein. Finally, the effluent had relatively elevated Campylobacterales, which has been previously insinuated as host of ARGs, such as *tetM*, *intl1*, *qacEΔ1* and $bla_{OXA\text{-}58}$ (Hultman et al., 2018).

2.3.6 Evidence of antibacterial peptides in the HRAP systems

Kumar et al. (2021) showed that HRAP systems can significantly reduce pathogenic bacteria, potentially more so than CAS WWTPs (Alexander et al., 2020), and bactericidal metabolites produced by the microalgae have been suggested as an explanatory factor. Based on sequence contigs from MABA, 177 were related to antimicrobial peptides (AMP), where 59 peptides predicted inhibitory activities against *E. coli* (ATCC 25922) (Table S5). Escherichia were reduced significantly reduced in our HRAP systems, which might be explained by potential elevated AMPs, although this must be proven. However, recent work reported the antimicrobial effect of AMPs extracted from microalgae, removing up to 96 % of *E. coli* (Guzmán et eal., 2019).

2.3.7 Co-occurrence patterns among ARGs types and microbial taxa

Through network analysis, it is possible to predict possible hosts for ARGs (Ju et al., 2016). However, here only *sul1*, *tetQ* and $bla_{CTX\text{-}M}$ abundances correlated with bacteria (Figure 2.5). For example, Fusobacteriales and Erysipelotrichales had more positive correlations with *sul1* and *tetQ*. Previously, Fusobacteriales has been related to sulfonamide and tetracycline ARGs subtypes (Jia et al., 2020), and Erysipelothrix bacteria belonging to the order Erysipelotrichales, was found to co-occur with some ARGs (Tong et al., 2022). Correlations of Methanobacteriales with *sul1* and *tetQ* were also observed, which agrees with previously reported Archaea as potential hosts of 24 subtypes of ARGs (Flores-Orozco et al., 2020). The *intl1* abundances correlated with *sul1*, *tetQ* and bla_{KPC}. Correlations of *intl1* with these and other ARGs have been previously reported (Quintela- Baluja et al., 2019). It is possible that abundances of *sul1*, *tetQ* and bla_{KPC} are related to *intl1* as there is evidence that *sul1* are part of class 1 integrons (Gillings et al., 2008). Alternately, $bla_{CTX\text{-}M}$ were correlated with two eukaryotic microalgae (Sphaeropleales and Chlorellales), however, these microalgae are not ARG reservoirs, as are non-target microorganisms for antibiotics and their disturbance by antibiotics is limited (Li et al., 2022). Regarding prokaryotic microalgae, in this study Cyanobacteria were the most abundant and were not correlated with any ARGs (Figure 2.5); contradictory to previous works where Cyanobacteria was related to ARGs (Qixin et al., 2022) and demonstrated as ARGs reservoirs (Wang et al., 2020b). This co-occurrence analysis suggests that in the continuous-flow outdoor HRAP, only bacteria play a role as ARG reservoirs.

Overall, these analyses suggest changes in ARGs across the compartments relates changes in local microbial communities. As Cheng et al. (2020) has suggested, lower antibiotic resistance released from algal-bacterial systems may be due to different bacterial sub-populations, possibly with lower diversity, that are selected in association with algal sub-population. Although it is speculation, such bacterial selection and the presence of algal AMPs might partially explain why HRAPs systems reduce antibiotic resistance, both excluding and killing potential possible ARG hosts.

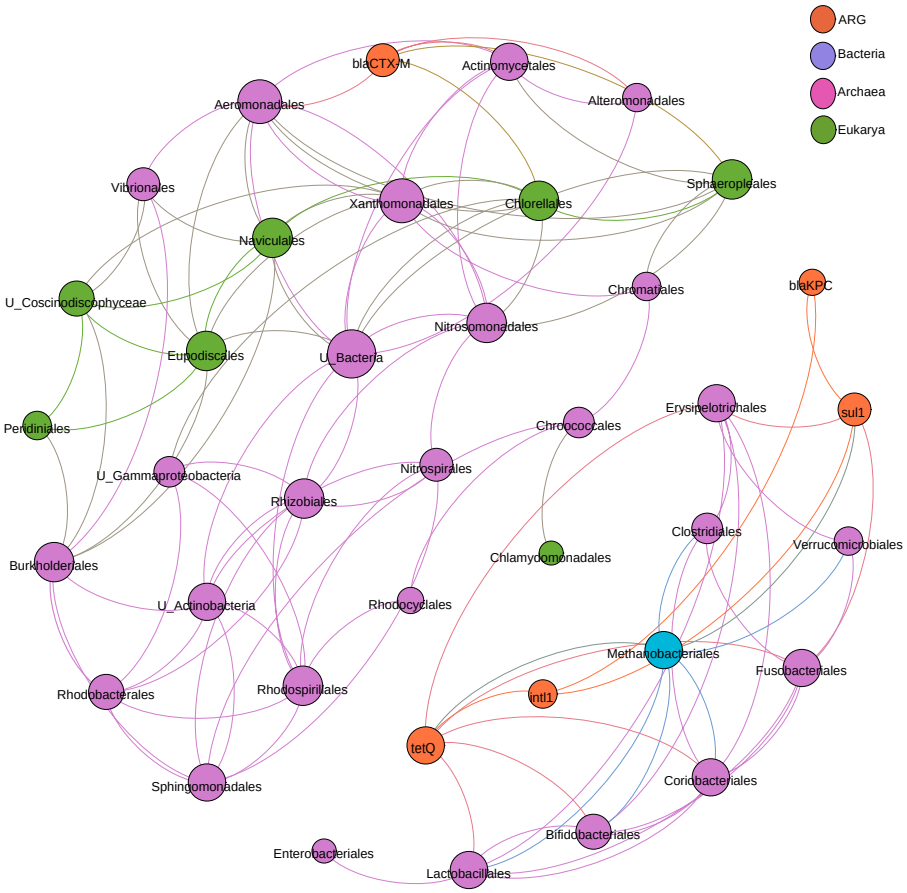

Figure 2.5. Network analysis revealing the co-occurrence patterns between ARG and *intl1* (relative abundance) and microbial taxa (order-level) during the transition period. A connection represents a strong (Spearman's correlation coefficient $\rho > 0.7$) and significant (p value <0.05) correlation. The size of each node is proportional to the number of connections.

2.4 Conclusions

- As far as the authors know, this is the first systematic study to demonstrate the potential of a microalgae-bacteria-based system to remove ARG from wastewater in HRAP.
- Here we show that pilot-scale outdoor HRAP system continuous treating domestic wastewater were resilient across and consequentially reduced the resistome (total ARG abundance), and the absolute and relative abundance of *tetQ*, *sul1,* bla_{KPC} and *intl1*. Further, reduction of ESKAPEE bacteria very evident (e.g., *Escherichia* genus), which may be related AMPs that appears to be elevated in MABA based on metagenomic data.
- Conditions within compartments in HRAP systems, especially micro- environments in MABA, their efficient settling properties, and the presence of antimicrobial peptides might exclude influent ARG-carrying strains, reducing antibiotic resistome in the reactor effluents.
- Results here are hugely promising for LMIC applications, especially in warmer and sunnier climates, because microalgae-bacteria-based treatment systems are very effective at reducing the resistome from influent to effluent. This technology might be especially valuable for low-resource settings because the energy demands, and their operation is comparatively simple. Hopefully, our results will encourage further study on these promising systems, particularly in regions with a dire shortage of sustainable wastewater treatment options like Latin America.

3. Antibiotic resistance reduction during anaerobic digestion of microalgae-bacteria aggregates: temperature effect

Abstract

Wastewater treatment plants (WWTPs) are reservoirs of antibiotic-resistance genes (ARGs), with treatments, such as chlorination and UV disinfection, partially mitigating this issue. However, concentrations of ARGs and antibiotic-resistant bacteria (ARB) in the activated sludge are significantly higher ($p < 0.05$) than in the effluent. This study investigated the influence of temperature regimes (thermophilic vs. mesophilic) on the anaerobic digestion (AD) of microalgae-bacteria aggregates (MABA) for biogas production and the elimination of ARB and ARGs. MABA were treated in continuous stirred tank reactors at 35°C (mesophilic) and 55°C (thermophilic) with a hydraulic retention time of 30 days. Thermophilic conditions yielded ARB log reductions of 1.4, 1.7, 1.1, 1.1, and 1.6 for ampicillin, ciprofloxacin, erythromycin, sulfamethoxazole, and tetracycline. Anaerobic digestion significantly reduced the total abundance of ARGs ($p < 0.05$), with higher reductions in thermophilic conditions (19.5 ± 0.8 ppm) compared to mesophilic conditions (36.9 ± 2 ppm). The findings highlight the critical role of temperature in the AD process, where the microbial community, the mobilome, and functionality were significantly responsible for resistome reduction ($p < 0.05$). Thermophilic AD emerges as a promising method for treating MABA, achieving efficient waste degradation, energy recovery, and serving as a significant barrier against antibiotic resistance. This research provides insights into the mechanisms behind ARG mitigation and suggests thermophilic AD as beneficial for environmental health and resource recovery in WWTPs.

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3.1 Introduction

Wastewater treatment plants (WWTPs) receive discharges from multiple sources for their treatment, which is why they are considered reservoirs of antibiotic-resistance genes (ARG) and antibiotic-resistant bacteria (ARB) (Barancheshme & Munir, 2018). Conventional WWTPs remove ARG from water to a certain extent, and this removal is significantly enhanced by advanced processes such as chlorination, ultraviolet, and ozone disinfection or by chemical oxidation (Michael-Kordatou et al., 2018). Algal-based systems have also been evaluated for reducing ARGs in wastewater treatment, being even more effective than conventional activated sludge (Cheng et al., 2020) and potentially more sustainable in low-to middle-income countries (Ovis-Sanchez et al., 2023).

However, after wastewater treatment, another concern that arises is that the concentrations of ARG and ARB in the activated sludge generated in a WWTP are significantly higher ($p < 0.05$) than in the effluent (Munir et al., 2011). Limited information is available on sludge or biosolids generated in developing or emerging treatment systems, such as high-rate algal ponds (HRAPs). In these systems, the promotion of microalgae-bacteria aggregates (MABA), such as flocs and granules, is encouraged, resulting in good settling properties (dos Santos Neto et al., 2021; Nwoba et al., 2020). This implies a higher precipitation of resistant cells and, therefore, higher ARGs concentrations in the biosolid phase (Wang et al., 2023a). We found previously that certain ARGs exhibit higher concentrations in biosolids (MABA) than in the effluent (Ovis-Sanchez et al., 2023). Therefore, the use or subsequent disposal of sludge resources can result in the spread of ARGs retained in the sludge to the environment through food chains or bioaerosols, thereby increasing risks (Cui et al., 2020). It is also important to control, monitor, and look for alternatives that remove ARB and ARG that persist in the sludge generated from a WWTP.

The potential of anaerobic digestion (AD) as a promising pathway for reducing antibiotic resistance in sludge has recently been extensively demonstrated (Syafiuddin & Boopathy, 2021). Temperature has proven to be a critical variable for reducing ARGs during the AD of sludge generated in WWTP (Luo et al., 2017). In addition, various studies have reported a greater decrease in ARG and ARB under thermophilic conditions than under mesophilic conditions (Miller et al., 2016; Tian et al., 2016). Several mechanisms for ARGs removal under thermophilic conditions have been suggested, such as reduction in microbial community diversity and reduction of either vertical gene transfer (VGT) or horizontal gene transfer

(HGT), resulting in decreased ARG abundance (Miller et al., 2016; Shin et al., 2020); reduction of mobilome, including plasmids, insertion sequences, and integrons, suggesting a lower HGT potential of ARGs under thermophilic conditions (Tian et al., 2016); and distinctive microbial composition characterized by higher archaeal abundance and lower bacterial abundance in thermophilic digesters compared to their mesophilic counterparts, further contributing to the pronounced reduction of ARGs (Zhang et al., 2021). These combined mechanisms highlight the efficacy of thermophilic conditions in anaerobic digestion for ARG mitigation.

However, these studies have mainly focused on secondary sludge from conventional activated sludge (CAS). To the best of our knowledge, this is the first study to analyze the potential removal of ARB and ARGs from MABA via anaerobic digestion. Therefore, the subsequent treatment of these aggregates is necessary to ensure the reduction of antibiotic resistance in the environment. Studies on MABA in anaerobic digestion have mainly focused on methane production; for example, a previous study showed that these aggregates can be used for energy generation (methane) through the AD process, achieving a maximum net energy ratio of 1.5 under mesophilic conditions (Carrillo-Reyes et al., 2021). Hence, this study aimed to investigate the influence of different temperature regimes (thermophilic vs. mesophilic) on the anaerobic digestion of MABA for biogas production and the elimination of ARB and ARG. Functional annotation and mobilome analysis will be used to gain a better understanding of the underlying mechanisms.

3.2 Materials and methods

3.2.1 MABA production

The MABA were produced in two parallel outdoor HRAPs of 50 L (working volume), treating the primary effluent from a domestic wastewater treatment plant (Santa Rosa Jauregui, Queretaro, Mexico). The MABA collected had good settling capacity, a sludge volume index of 31.7 ± 3.7 (mL g VSS^{-1}) and settling velocity of 0.8 ± 0.1 (m h^{-1}). Concentrated MABA was stored at 4 °C until use in an anaerobic digester as a substrate. The details of the HRAP operation have been reported previously (Section 2).

3.2.2 Experimental set-up

MABA were digested in two 700 mL (working volume) anaerobic continuous stirred tank reactors (AnCSTR) and inoculated with 10 g VS L^{-1} of granular mesophilic anaerobic sludge from a brewery. The AnCSTRs were operated in parallel, with an HRT of 30 days and different

temperatures, mesophilic and thermophilic, 35 and 55 °C, respectively, during a total operation time of 115 days, which is enough time to obtain acclimatization in thermophilic conditions (Carrillo-Reyes et al., 2021). Biogas production was measured daily through the liquid displacement method using a saturated acid solution to avoid CO_2 dilution. Methane production results were reported under standard conditions (0 °C, 1 atm). Steady state was defined by a stability criterion derived by obtaining a methane productivity with a coefficient of variation < 20% (Vital-Jácome et al., 2022).

Determinations of total solids (ST), volatile solids (SV), total chemical oxygen demand (CODt), $N\text{-}NH_4^+$, and alpha alkalinity were performed every two weeks. Over the last two weeks, samples of each temperature condition were collected for DNA extraction to characterize the microbial communities and ARG persistence. The ARB in MABA and digestates (mesophilic and thermophilic) were determined were determined in the last week of reactors operation.

3.2.3 Analytical procedures

Biogas composition was determined three times per week using gas chromatography with a thermal conductivity detector (Arcila & Buitrón, 2016). TS and VS were analyzed according to standard APHA methods (APHA, 2005). COD_t and $N\text{-}NH_4^+$ were analyzed using the 10031 y 8000 Hach colorimetric method. Alpha alkalinity was determined according to the method of Vital-Jacome and Buitrón (2021).

3.2.4 Antibiotic resistance bacteria quantification

The plate count method was used to quantify ARB in each sample. Antibiotics and concentrations were considered according to the algae-based system reported by Cheng et al. (2020). The following concentrations were used (mg L^{-1}): tetracycline (16), erythromycin (8), sulfamethoxazole (50.4), ampicillin (32), and ciprofloxacin (4). R2A agar (BD, USA) was used as the culture medium, and 50 mg L^{-1} nystatin was added to prevent fungal growth. Samples were serially diluted 10-fold and 0.1 mL of the dilution was used for spread plating. All analyses were performed in duplicates. The plates were then incubated for a maximum period of 48 h at 37°C. Colony forming units (CFU mL^{-1}) were recorded on each plate to determine the ARB concentration (Cheng et al., 2020). The concentrations were reported in log10, and the removal was determined between the ARB counts obtained in the MABA (before the AD treatment) and the digestate under mesophilic and thermophilic conditions. A sample of all colonies from each agar plate (MABA sample) was recovered in a single Eppendorf tube with

20% (w/v) glycerol for further microbial characterization by 16S rRNA sequencing. Total heterotrophic bacteria were determined by plating the samples on media without antibiotics.

3.2.5 DNA extraction

Microbial characterization samples were stored at −20 °C before use. MABA and thermophilic and mesophilic digestate samples were processed using the DNeasy PowerWater Kit (Qiagen, Germany), and samples from MABA agar plates were processed using the DNeasy PowerSoil Kit (Qiagen, Germany), according to the manufacturer's instructions. The quality and concentration of DNA samples were verified by spectrophotometry using a NANODrop 2000c (Thermo Scientific, USA).

3.2.6 16S rRNA sequencing processing

DNA samples from MABA agar plates were submitted to the Integrated Microbiome Resource Lab (IMR) at Dalhousie University (Halifax, Canada) for sequencing on the MiSeq Illumina platform. The 16S rRNA genes were amplified using the primer sets 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). Raw fastq files were processed using the DADA2 pipeline (Callahan et al., 2016) following package guidelines. Sequences were filtered and truncated to avoid low-quality scores: (280 bp for forward and 200 bp for reverse reads), maxN=0, trucQ=2, and maxEE=c(2,2). The error rates were then learned (err) and dereplication was completed (derepFastq). The Dada core sample inference algorithm was then applied to filtered and dereplicated data. The reads were merged (mergePairs) with a minimum overlap of 12. Next, an amplicon sequence variant (ASV) table was created (makeSequenceTable) and chimeras were removed (removeBimeraDenovo). Taxonomic assignments for the 16S ASVs were performed using the Silva database (v138.1).

3.2.7 Resistome, mobilome and functional prediction analyses by metagenomic approach

DNA samples from MABA (n=2), thermophilic digestate (n=2), mesophilic digestate (n=2), and granular anaerobic sludge (n=1) were sequenced using NovaSeq 6000, Illumina (~8 million PE 150+150 bp reads) at the Tec BASE Lab (Laboratorio Nacional de Secuenciación Genómica Tec BASE, Tecnológico de Monterrey, Mexico). The metagenomic sequences processing pipeline is described in Supplementary Information. Raw metagenomic and 16S rRNA sequences were deposited into the NCBI sequence read archive (SRA) database under the project number PRJNA983699.

3.2.8 Statistical analysis

Data normality was assessed using a Shapiro–Wilk test. In the case of parametric data, significant differences were determined by a parametric One-way ANOVA test and a Tukey post-hoc test. Otherwise, Kruskal–Wallis and post-hoc pairwise multiple comparisons (Pairwise Wilcoxon test with Benjamini & Hochberg adjustment) for non-parametric data. A significance level of 0.05 was used for all tests. The identification of KOs with differential abundance was carried out using the DESeq2 (version 1.38.3) R package. The analysis employed a Wald test with a local regression fit type, and p-values below 0.001 exceeding a fold-change of two were considered significant. Heatmaps of the microbial community with dendrograms, principal components analysis (PCA), alpha diversity index, and PERMANOVA analysis were performed using R with packages gplots (version 3.1.3) and vegan (version 2.6-4). Spearman correlations were performed (resistome vs. microbial community, resistome vs. mobilome, and resistome vs. functional prediction) using the R package stats (version 4.3.2), and the Mantel test was performed to determine the relationship between these factors using the mantel function (vegan package) with 999 permutations. The statistical analysis was performed using RStudio v.3.4.1 (RStudio Team, 2016). A Venn diagram was plotted using InteractiVenn (http://www.interactivenn.net).

3.3 Results and discussion

3.3.1 Reactors performance

During the operation of the reactor in a thermophilic regime, it was observed from day 66 onwards that the productivity and methane yield remained stable (CV <20%), with values of 25.9 ± 4.7 NmL CH_4 L^{-1} d^{-1} and 97.0 ± 17.6 NmL CH_4 g SV^{-1}, respectively. While, in the reactor operated in mesophilic regime, the stability period was observed from day 37 (CV <20%), with average productivity and methane yield of 16.4 ± 2.8 NmL CH_4 L^{-1} d^{-1} and 61.4 ± 10.3 NmL CH_4 g SV^{-1}, respectively (Figure S1). Those values are lower than the results obtained by Cea-Barcia et al. (2018) who reported a methane productivity of 300 mL CH_4 L^{-1} d^{-1}, however, they used 2-fold higher organic load rate (OLR) that the used in the present study (0.3 g VS L^{-1} d^{-1}) and also, they used co-digestion with papaya waste, so this can be related with higher methane productivity. In terms of productivity and methane yield, a significantly better performance ($p < 0.05$) was demonstrated in the thermophilic regime, as the methane yield and productivity were 1.6 times higher, compared to mesophilic regime. Similarly, the thermophilic anaerobic digestion of MABA resulted in a 1.6-fold increase in methane yield

(Carrillo-Reyes et al., 2021). Under mesophilic conditions, COD and TS removal rates were 6.9% and 6%, respectively. In contrast, higher removal rates were achieved under thermophilic conditions, reaching 11.6% for COD and 16% for TS, which were lower than those previously reported (Carrillo-Reyes et al., 2021). The average ammonium production from both reactors was 620 ± 106.5, which is below the inhibition values of 1500 - 3000 mg $N\text{-}NH_4^+ L^{-1}$ for anaerobic systems (Rajagopal et al., 2013). The alkalinity index was maintained in both reactors at an average of 0.26 ± 0.02, which is considered optimal for anaerobic digestion (Vital-Jácome & Buitrón, 2021). Furthermore, this implies that the addition of chemicals to adjust the pH was not necessary.

3.3.2 Antibiotic resistance bacteria removal

Significantly different ARB concentrations were obtained between MABA and digestates (mesophilic and thermophilic) (Figure 3.1.A), where the thermophilic regime contributed to significantly higher removal rates than the mesophilic regime. In thermophilic, ARB log reduction of 1.4, 1.7, 1.1, 1.1 and 1.6 were obtained for ampicillin, ciprofloxacin, erythromycin, sulfamethoxazole, and tetracycline, respectively. Ampicillin removal was similar to that reported in AD of sewage sludge (1.31 log removal), previously pre-treated with microwave radiation and hydrogen peroxide (Tong et al., 2016). The concentrations of ARB detected from MABA plates (sulfamethoxazole and tetracycline) in our study were within the range of the previously published concentration of 6-7.5 Log CFU/g in biosolids from WWTPs (Muni et al., 2011). In addition, no increase in ARB was observed, whereas a previous study showed that some ARB increased after anaerobic digestion of swine manure (Liu et al., 2022). These authors suggested that this could be due to free nitrous acid (FNA) pretreatment and consequently occasioned re-growth or horizontal or conjugative transfer of free DNA containing ARGs. Currently, there are no regulations or standards for the concentration of antibiotic resistant bacteria (ARB) in biosolids. Meanwhile, the discharge of ARB after AD is also essential for understanding the fate of antibiotic resistance in biological treatment because of its direct impact on the environment. It has been shown that thermophilic digestion is less susceptible to ARG intrusion than mesophilic digestion because in thermophilic conditions, there is a lower ARB survival rate and/or horizontal gene transfer (Miller et al., 2016). However, to unravel potential mechanisms, microbial community and functional predictions were conducted, and these results will be further discussed in the following sections.

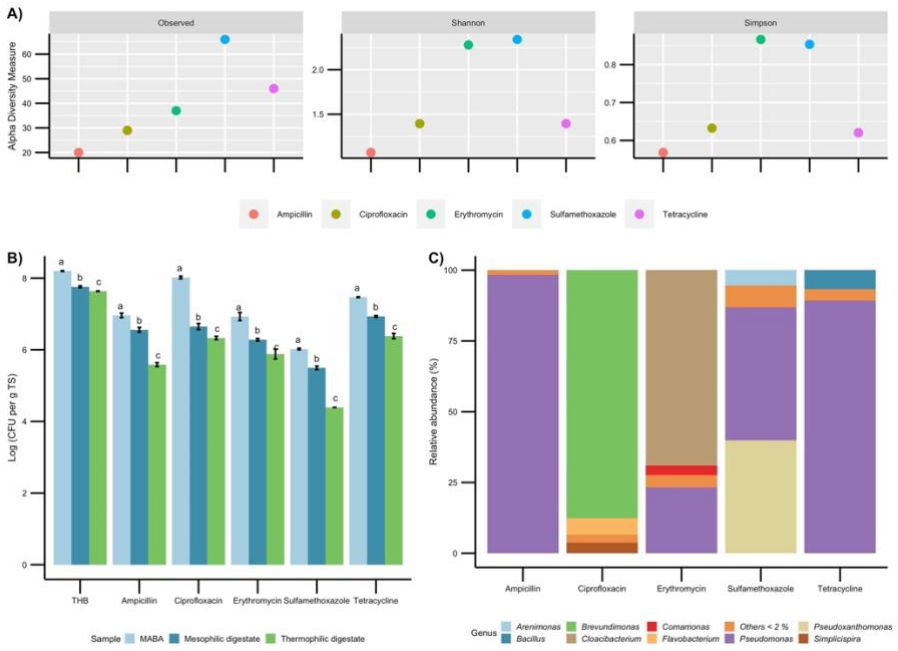

Figure 3.1. A) Alpha diversity of antibiotic-resistant bacteria from MABA samples. B) Log concentrations of antibiotic-resistant bacteria and total heterotrophic bacteria (THB) in MABA and digestates (mesophilic and thermophilic). Lowercase letters from post-hoc comparison indicated significant differences ($p < 0.05$). C) Relative abundance at the genus level in antibiotic-resistant bacteria on MABA agar plates.

3.3.2.1 Antibiotic resistance bacteria identified by 16S rRNA sequencing

The relative abundances of the five different types of antibiotic-resistant bacteria from the MABA samples are shown in Figure 3.1.C. Shannon and Simpson indices showed that bacterial populations recovered on ampicillin-, ciprofloxacin-, and tetracycline-amended agars were less diverse than those resistant to erythromycin and sulfamethoxazole (Figure 3.1.A). *Pseudomonas* was the dominant genus for ampicillin, tetracycline, and sulfamethoxazole, with relative abundances of 98.3, 89.3%, and 47.0%, respectively. This implies that *Pseudomonas* is more resistant to these antibiotics; this bacterium is considered multi-resistant and is commonly found in WWTPs (Li et al., 2022); therefore, its surveillance and control are of clinical and environmental relevance. *Brevundimonas* and *Cloacibacterium* were the dominant genera for ciprofloxacin (87.6%) and erythromycin (69.1%), respectively. It has been reported that bacteria belonging to the *Brevundimonas* genus are resistant to several antibiotics, including ciprofloxacin, and it has also been proposed that they be considered as part of a group of opportunistic pathogens (Ryan & Pembroke, 2018). Very few ASV were

identified at the species level from the taxonomy assignment; however, the most abundant species was *Cloacibacterium normanense* in the erythromycin agar. This species has previously been isolated from WWTP and has shown resistance to erythromycin (Gay et al., 2016). Clinically relevant bacteria were identified in MABA; however, anaerobic digestion resulted in a significant reduction in ARB (Figure 3.1.A), implying a reduced risk of the spread of pathogenic or opportunistic pathogens to the environment.

3.3.3 Identification of resistome and mobilome by metagenomic sequencing

28 different ARG subtypes were identified in MABA, whereas 13 and 9 were identified in the mesophilic digestate and thermophilic digestate, respectively (Figure S2). This implies that the diversity of ARGs was decreased by anaerobic digestion, and no ARGs subtypes were shared simultaneously under both temperature conditions, indicating that the diversity of ARGs was specific for each condition. Additionally, some ARGs were unique to each temperature condition. For instance, a significant proportion of β-lactam resistance-related ARG subtypes were mainly present in thermophilic conditions, consistent with a previous study where in anaerobic digestion of manure, a higher prevalence of these ARGs was reported under thermophilic conditions (Luo et al., 2017). On the other hand, some ARGs were shared between MABA and the digestates (qacEdelta1, tet(C), ANT(3'')-IIa, linG, sul1, and sul2), implying that these genes were not removed through anaerobic digestion either in mesophilic or thermophilic conditions.

The total abundance of ARG in MABA was 58.9 ± 7 ppm, with macrolide, aminoglycoside, and beta-lactam being the most abundant ARG type (Figure 3.2). This total abundance of ARG is slightly similar to that reported in activated sludge from a WWTP (26-54 ppm) (Yang et al., 2013). Resistome richness was reduced by anaerobic digestion with two of the nine ARG types (MLS and Phenicol) that were not detected in either of the digestates. In thermophilic digestates, all ARG types were reduced in comparison with mesophilic digestates; for example, beta-lactams in mesophilic digestates had the highest concentration (8.20 ppm), while in thermophilic digestates, this ARG type was not detected. However, a slight increase in sulfonamide under thermophilic conditions was observed ($p > 0.05$), which is consistent with previous studies where some ARGs increased under thermophilic conditions (Xu et al., 2020), showing that sulfonamide-resistant genes have higher persistency among the detected ARGs types. This result supports the ARB analysis, which found that sulfonamide-resistant bacteria have the highest alpha diversity indices compared to bacteria resistant to other

antibiotics. In general, it was shown that anaerobic digestion significantly reduces the total abundance of ARG ($p < 0.05$), where a higher significant reduction is achieved in thermophilic (19.5 ± 0.8 ppm) compared to mesophilic (36.9 ± 2 ppm). This agrees with previous studies, where thermophilic sludge demonstrated better ARG removal performance in WWTP sludge (Luo et al., 2017; Xu et al., 2020). In this study, anaerobic digestion was proposed as a coupling alternative to reduce antibiotic resistance during wastewater treatment, as shown in Table S1. In the microalgae-bacteria system, characterized by a wastewater resistome concentration of 924.7 ppm, and a residual concentration in MABA was quantified at 58.9. Moreover, anaerobic digestion of MABA further diminished to 19.5-36.9 ppm. Comparatively, activated sludge showed a reduction from 595.3 ppm in the influent to 29.9 ppm in biosolid, however, an increase after anaerobic digestion was obtained (Table S1). Another study did not find any significant reduction in the resistome of biosolids after AD (either mesophilic or thermophilic). The substantial reduction in ARGs, especially during anaerobic digestion, makes the microalgae-bacteria system a promising and efficient approach to biosolid treatment in wastewater management. The MABA samples used in this study exhibited a high abundance of Mobile Genetic Elements (MGEs). The total range of MGE in MABA was 2282-2515 ppm, while that in thermophilic and mesophilic conditions was 1961-2054 and 1440-1767 ppm respectively. As depicted in Figure 3.3, a significant decrease in various MGE modules was observed during anaerobic digestion, regardless of whether it occurred under mesophilic or thermophilic conditions. PCA analysis accounted for 56.8% of the total variation, indicating substantial explanatory differences (Figure S3). The phage MGE module showed significantly lower abundance under thermophilic conditions than under mesophilic conditions. It is widely recognized that phages harboring ARGs could infect new hosts and introduce these ARGs into new cells, thereby conferring them the given resistance (Calero-Caceres et al., 2014). These results suggest that a decrease in the number of phages may result in lower transduction rates, which also plays an important role in the spread of ARGs during and after anaerobic digestion. The Integration/Excision module demonstrated a substantially diminished presence in the thermophilic phase as opposed to MABA. Notably, this module encompasses integrases, which in turn leads us to postulate a potential reduction in ARG dissemination, particularly considering the presence of multiple ARGs within the gene cassettes of integrons (Di Cesare et al., 2016).

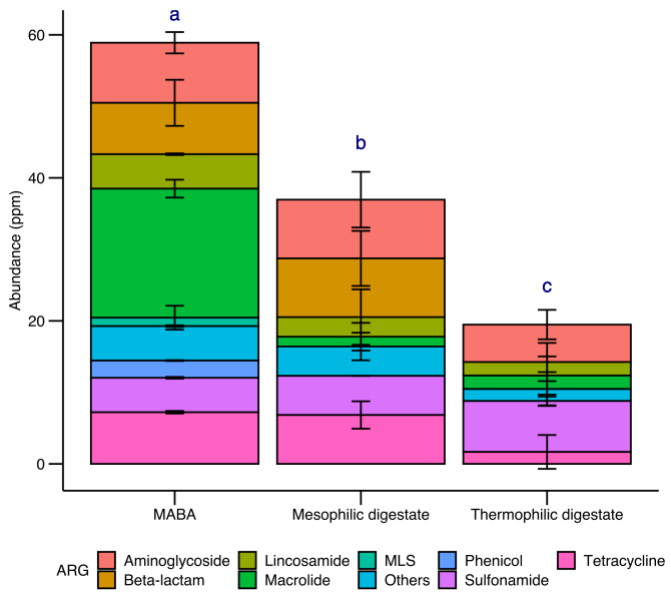

Figure 3.2. Abundance (ppm) of ARG types in MABA, mesophilic digestate and thermophilic digestate. Lowercase letters from post-hoc comparison indicated significant differences ($p < 0.05$). MLS: Macrolides, lincosamides, and streptogramins.

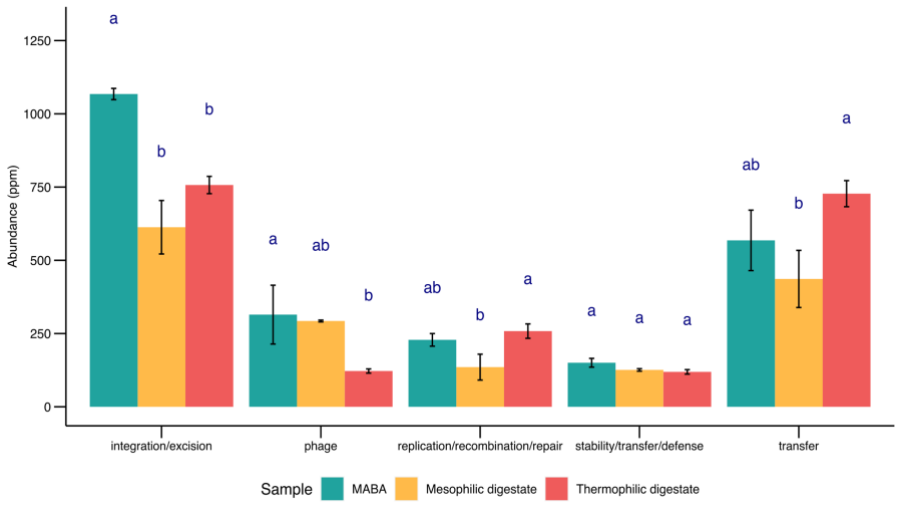

Figure 3.3. Total abundance of different MGE modules. Lowercase letters from post-hoc comparison indicated significant differences ($p < 0.05$).

3.3.4 Microbial community composition

Taxonomic assignment analysis showed that only 0.76 ± 0.03% of the assigned sequences corresponded to archaea in MABA, while a higher proportion (3.70 ± 0.62%) was observed in the digestates. A heatmap was generated to illustrate the differences in archaeal composition

between digestate samples and inoculum. A heatmap (Figure 3.4.A) indicated that the mesophilic samples exhibited a higher similarity to the inoculum, which is consistent with the origin of the inoculum from an anaerobic mesophilic process, whereas the thermophilic samples had a distinct composition, explained by community selection driven by temperature. At the genus level, *Methanosarcina*, *Methanothrix*, and *Methanosphaerula* were the predominant archaea, accounting for 28.4 ± 3.2%, 16.5 ± 9.8%, and 13.3 ± 6.5%, respectively. These archaea have been previously reported under thermophilic conditions and are related to the methane content in biogas (Jiang et al., 2020). Furthermore, *Methanosarcina* has been reported as one of the most predominant archaea during stable methane production under thermophilic conditions (Carrillo-Reyes et al., 2021). In addition, the heatmap highlights the differential abundances of several bacterial classes among the samples (Figure 3.4.B). Under thermophilic conditions, a higher presence of bacteria belonging to the classes *Thermodesulfovibrionia* and *Synergistia* was observed compared to MABA and mesophilic digestate. This finding is consistent with previous studies that have reported bacterial taxa were reported in anaerobic reactors operating under thermophilic conditions (Muñoz-Sierra et al., 2020; Zhang et al., 2021). For instance, the MABA samples had a high abundance of Gammaproteobacteria (17.8 ± 0.4%), which are known to carry most of the ARGs in WWTPs samples (Shin et al., 2022); however, the relative abundance of Gammaproteobacteria decreased in thermophilic to 8.9 ± 1.9%. This suggests that thermophilic conditions may be more effective in reducing part of the bacteria that are ARG reservoirs and, consequently, a lower identification of ARG in digestates. The construction of a heatmap using the Bray-Curtis distance matrix provided insights into the clustering patterns of bacterial communities across the six samples, including the inoculum used in the anaerobic digesters (Figure 3.4.B). Consistent with our expectations, the clustering analysis revealed that the MABA (before anaerobic digestion) samples and the digestates under mesophilic conditions formed a distinct cluster from the digestates under thermophilic conditions, while the inoculum exhibited a different community structure. This observation suggested the presence of dissimilar bacterial compositions in the samples. A PERMANOVA analysis was performed to confirm the observed clustering patterns, revealing a significant difference in community structure due to operational temperature (r^2=0.98; $p < 0.05$).

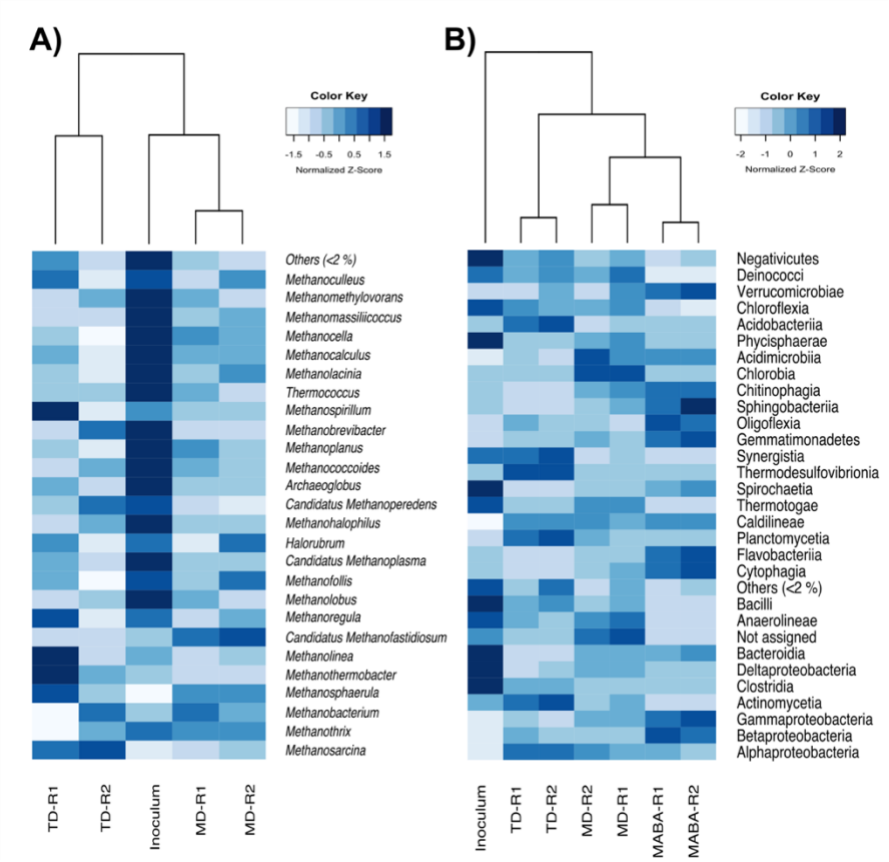

Figure 3.4. Heatmap and cluster analysis (Bray–Curtis) of relative abundance of the different sampling points clustered based on similar abundance and occurrence pattern. A) Archaea at genus level. B) Bacteria taxa at class level. TD: Thermophilic digestate; TM: Mesophilic digestate; R1 and R2: Reactor replicate. Relative abundances are z-score normalized for visualization purposes.

3.3.5 Prokaryotic metabolism functional prediction

Considering the predictive molecular functions represented in terms of functional orthologs by the KEGG Orthology (KO) database, PCA analysis was performed, which demonstrated the presence of differences between the conditions. This was evident from the observation of distinct separations into three clusters corresponding to different temperatures and MABA. The PCA accounted for 92% of the total variation (Figure 3.5.A), which indicates a substantial explanatory difference. Consequently, a DESeq2 analysis was conducted to elucidate the significant KOs differences between mesophilic and thermophilic bacteria.

7 significant KOs ($p < 0.001$) were found according to the DESeq2 analysis, of which 6 were

positive log2FoldChange and 1 negative; the positive values imply that they have a higher abundance in thermophilic bacteria and negative in mesophilic bacteria (Figure 3.5.B). The six KOs with a significantly higher abundance in thermophilic bacteria, K03091, K06295, K19139, K06297, K06385, and K04769, were related to the genes sigE_F_G, gerKA, csm4, gerKC, spoIIP, and spoVT, respectively. The sigE_F_G, gerKA, gerKC, spoIIP, and spoV genes are essential for the formation of the spore cortex, which serves as the outer protective layer of bacterial spores. It provides structural integrity and safeguards spores against various harsh environmental conditions, including high temperature (Abecasis et al., 2013; Fimlaid & Shen, 2015; Hinc et al., 2006). On the other hand, the gene csm4 is involved in the adaptive immune response of the CRISPR-Cas system, which serves as a sequence-specific memory of prior MGE infections (Staals et al., 2014). Only KO K21572 was significantly higher in the mesophilic group, which is related to the gene susD. This gene acts as one of the major starch-binding proteins on the cell surface (Shipman et al., 2000), and recently, Chiang et al. (2022) showed that proteins from the Sus family were the most highly expressed proteins in imipenem-treated (beta-lactam antibiotic) cells, which could be related to the high abundance of β-lactam ARGs detected in mesophilic samples (Figure 3.2). In general, functional prediction indicates that in thermophilic environments, more genes contribute to spore formation and enhanced thermal stress resistance, enabling survival and adaptation in high-temperature environments and defense mechanisms against mobile genetic elements (MGE).

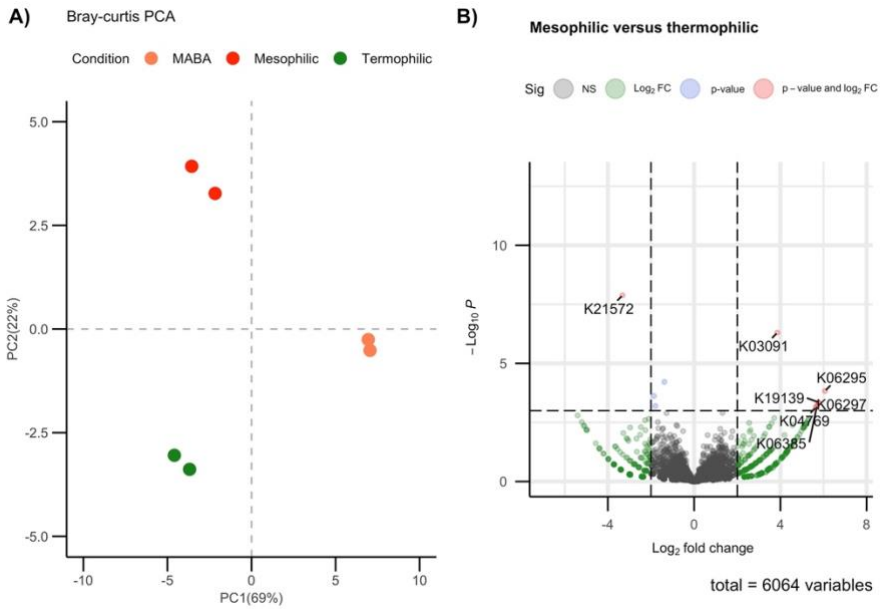

Figure 3.5. A) Principal Component Analysis (PCA) plot generated from DESeq2 showing variation within and between the groups. The groups were differentiated using different colors. B) Volcano plots of DESeq results showing KEGG ortholog (KO) changes under mesophilic and thermophilic conditions.

3.3.6 Factors driving resistome in anaerobic digestion

The significant association between the resistome and the microbial community is shown in Figure 3.6.A. These positive correlations indicate potential bacterial hosts of ARGs (Liu et al., 2022). The results indicated that 25 genera were significantly associated with the 27 ARGs. The class Betaproteobacteria accounted for the majority of these genera (5), followed by Gammaproteobacteria (5), including the clinical pathogen *Pseudomonas*, corroborating the results of ARB growth on agar plates. In this study, a significant reduction in thermophilic classes was observed (Figure 4.B). These classes (Betaproteobacteria and Gammaproteobacteria) have been previously correlated with different types of ARGs (Sun et al., 2021). Furthermore, the ARG subtypes belonging to Aminoglycoside, Beta-lactam and Macrolide had the highest number of correlations with the evaluated genera, partially explaining that the significant reduction of these ARG types in thermophilic bacteria was due to the reduction of these bacteria, including two archaea genera. *Methanosphaerula* and *Methanothrix* were strongly correlated with aadA16 (aminoglycoside ARG). A previous study reported that *Methanosphaerula* has been correlated with several types of ARGs, including

aminoglycosides (Gao et al., 2022). However, the role of archaea as hosts for ARGs remains uncertain, as associations have so far only been inferred from statistical analyses in the literature. Other bacteria that had an evident reduction in thermophilic were the genera *Arenimonas*, *Desulfomicrobium*, *Flavobacterium*, *Gemmatimonas*, *Ilumatobacter*, *Lentimicrobium*, *Pseudomonas*, *Pseudoxanthomonas* and *Thermomonas*, these genera being reported previously as ARGs hosts through positive correlations (Chen et al., 2019; Lu et al., 2019; Wang et al., 2023b; Zhao et al., 2024; Shi et al., 2021).

The presence of MGEs, especially integrons (Ma et al., 2017), is generally favorable for the propagation of ARGs (Wu et al., 2018). The correlation between the resistome and mobilome indicated that 21 ARGs were significantly associated with 12 MGEs (Figure 3.6.B). The MGEs module phage and integration/excision had the highest number of correlations with ARGs 5 and 3, respectively. Phage MGEs were correlated with several types of ARGs. Calero-Caćeres et al. (2014) demonstrated the prevalence of ARGs (blaTEM, blaCTX-M, qnrA, qnrS, and sul1) within phage fractions of anaerobically digested sludge. It has also been demonstrated that phages play a role in the HGT of ARGs; a positive correlation was observed between the absolute abundance of ARGs in bacteriophages and the relative abundance of ARGs in bacteria (Cheng et al., 2020). On the other hand, for the integration/excision category, this includes three MGEs, tnpA (transposase gene), insH6 (insertion element gene), and ORFC (transposase gene), correlated with different ARGs types. TnpA was identified to have the greatest potential for the dissemination of ARGs in the environment (Zhang et al., 2022).

In addition, the correlation between the resistome and functional prediction indicated that 21 ARGs were significantly associated with 18 KOs (Figure 3.6.C). Unclassified: Genetic information processing (KEGG level 2 category) accounted for the majority of this classification (4), followed by carbohydrate metabolism (3). The four KOs from Unclassified: genetic information processing belong to transposases, which is consistent with the correlations obtained from the resistome vs. mobilome, since the integration/excision module also includes transposases, such as ORFC and tnpA (Figure 3.6.B). For Carbohydrate metabolism, the KOs correlated with ARGs, and there has been no evidence in the literature regarding resistance to antibiotics; therefore, its relationship with the resistome is not clear. In the case of protein families, signaling, and cellular processes, K02004 belongs to the ABC transporter family. Interestingly, ABC transporters have been reported to be associated with the spread of ARGs (Li et al., 2022a). As described by Meng et al. (2021), ABC transporters were found to be significantly correlated with the relative abundances of blaTEM, blaOXA-2,

tetG, and ermB genes. In general, the correlations between functionality and resistome may suggest that certain metabolic processes have an effect on the resistome, since these correlations do not imply that the genes related to KOs are hosts of ARGs, as can be inferred in resistome vs. microbial community or vs. mobilome. Therefore, these KOs may lower certain metabolic processes that may potentiate the persistence of certain ARGs or their transfer by HGT.

The Mantel test revealed significant correlations between the resistome and the microbial community ($r = 0.54$), mobilome ($r = 0.47$), and functional predictions ($r = 0.61$), demonstrating that the fate of ARGs is influenced not only by microbial communities and MGEs, as has been extensively supported by previous studies (Flores-Orozco et al., 2023; Zhang et al., 2019; Zhang et al., 2019a), as well as functional capacity. The latter showed a higher correlation, suggesting a substantial impact on the resistome. Recently, correlation analysis showed that changes in the functional module are an important reason for the changes in different types of ARG (Tang et al., 2023).

3.4 Conclusions

This study revealed that operating the reactor in a thermophilic regime led to stable productivity, higher methane yield, and removal of antibiotic-resistant bacteria and antibiotic resistance genes compared with the mesophilic regime ($p < 0.05$). The greater efficiency in reducing antibiotic resistance under thermophilic conditions is likely due to the microbial community, mobilome, and functional genes significantly ($p < 0.05$) contributing to the resistance during anaerobic digestion of the microalgae-bacteria aggregates. In thermophilic conditions, there is a decrease in potential bacteria hosts of ARGs, a decrease in MGEs such phages, an increase in spore formation, and heightened thermal stress resistance, which in turn reduces the presence of antibiotic resistance and pathogenicity. These results promote the use of anaerobic digestion as a microalgae-bacteria aggregate treatment, where methane is obtained to reduce operating costs during wastewater treatment and to limit the spread of antibiotic resistance to the environment.

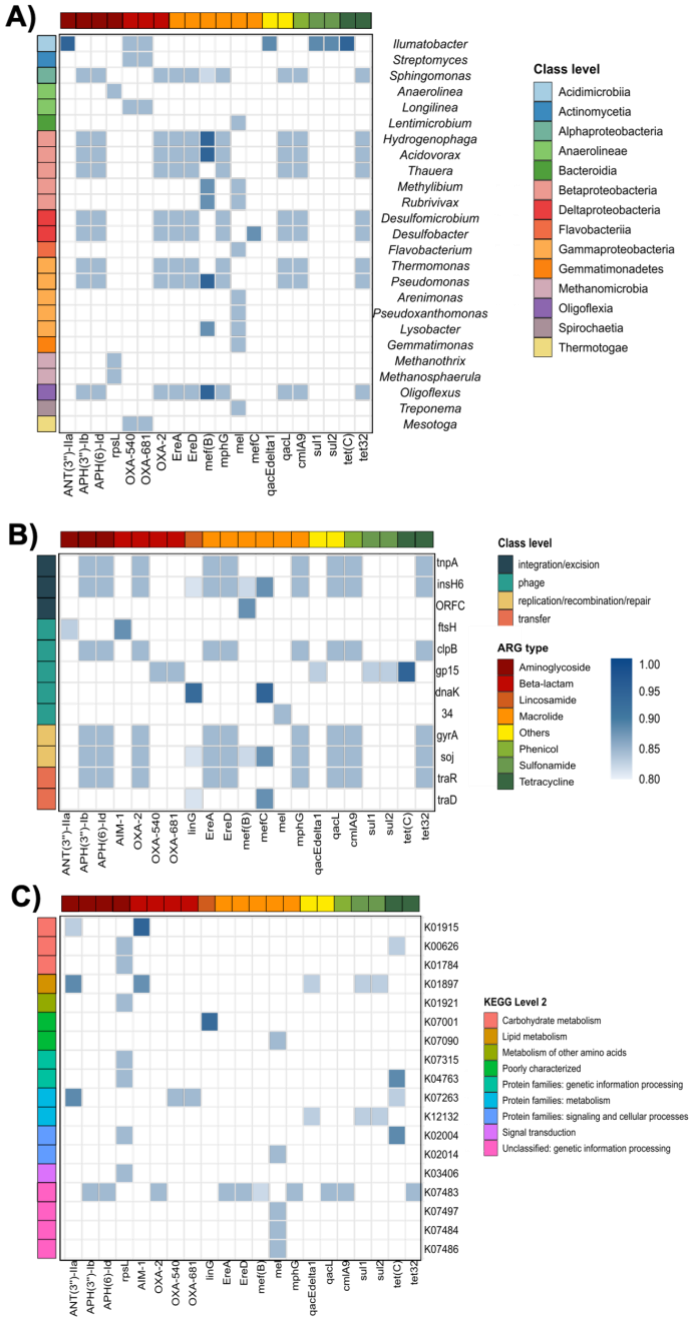

Figure 3.6. Significant positive correlations ($R > 0.7$, $p < 0.05$) associated ARG with the microbial community at the genus level (A), mobile genetic elements (MGEs) (B), and functional prediction (KEGG KOs) (C). Different blue scales show the Spearman correlation coefficients. The colors at the top of the columns indicate the ARG types. The row colors in each figure represent the class of the microbial genera, MGE modules, and KO classification at Level 2, for panels A, B, and C, respectively.

4. Extracellular polymeric substances associated to antibiotic resistance genes in microalgae-bacteria systems

Abstract:

Microalgae-bacteria based wastewater treatment systems are a reliable alternative, showing the capability of managing antimicrobial resistance associated with sewage, including both bacteria and genes. However, the mechanism behind this control has not been fully explained. A key feature of this system is the formation of microalga-bacteria aggregates (MABA), which are favored by extracellular polymeric substances (EPS) and improve biomass settleability; however, EPS can be associated with the persistence of extracellular antibiotic resistance genes (eARGs). This study aims to determine the spatial distribution of ARGs in different fractions, intracellular and extracellular-EPS related, of the MABA in outdoor flat-plate photobioreactors, for the sewage treatment. Using culture techniques and DNA sequencing, metagenomic assembled genomes (MAGs) were recovered from resistant bacteria, and ARGs were identified and quantified. Most strikingly, resistome analysis elucidated a substantial reduction in ARGs across intracellular and EPS-related fractions. Notably, no ARGs were detected in the final F-EPS samples, and a significant decrease in the final B-EPS samples was observed. This decrease is consistent with a significant decrease in various mobile genetic element (MGE) modules, including phages, indicating a decrease in horizontal gene transfer by transformation, implicating a decreased risk of ARGs dissemination post-treatment. These results underscore the importance of EPS in microalgal-bacteria systems, playing a critical role not only in aggregate formation, but also in maintaining ARGs within these aggregates. ARGs were predominantly maintained within the intracellular fraction, thereby reducing the potential spread of ARGs in clarified water and reducing the dissemination of antibiotic resistance in the environment.

Reference to article in preparation: Ovis-Sánchez, J. O., Graham, D. W., Carrillo-Reyes, J. (2024). Extracellular polymeric substances associated to antibiotic resistance genes in microalgae-bacteria systems. To be submitted to Algal Research.

4.1 Introduction

In addition to contributing to environmental pollution, the inappropriate global use of antibiotics in recent years has unavoidably accelerated the emergence and subsequent spread of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) (Andremont and Walsh, 2015). Conventional WWTPs remove ARG from water to a certain extent, and this removal is significantly enhanced by post-treatment of water using advanced processes such as chlorination, ultraviolet, and ozone disinfection, or by chemical oxidation (Michael-Kordatou et al., 2018). Algal-based systems have also been evaluated for the reduction of ARGs in wastewater treatment, and are even more effective than conventional activated sludge (Cheng et al., 2020). Although some mechanisms involved in the reduction of ARGs, such as transduction, have been suggested (Cheng et al., 2020), more studies are needed to support these suggestions and to elucidate other mechanisms involved in ARG reduction. ARGs can exist either intracellularly (iARGs) or extracellularly (eARGs), with the latter originating from the secretion of living cells or the release during cell lysis (Dong et al., 2019). A recent review revealed that most previous studies on ARGs in various environments primarily focused on iARGs (Zarei-Baygi & Smith, 2021). eARGs can be acquired by bacterial cells and facilitate horizontal gene transfer (HGT) through the transformation pathway (Zarei-Baygi & Smith, 2021). Moreover, eARGs persist for extended periods in environments such as river sediments and are present in higher concentrations than iARGs, serving as a significant reservoir for ARG propagation (Mao et al., 2014).

On the other hand, ARGs associated with extracellular polymeric substances (EPS) have started to be investigated, as extracellular ARGs can be absorbed or mobilized by EPS (Wang et al., 2022). A higher abundance of ARGs in the EPS matrix of activated sludge flocs from wastewater treatment plants (WWTP) has also been reported (Wang et al., 2021). Guo et al. (2022a) showed that changes in EPS could influence the conjugal transfer of ARG. EPS can be categorized into bound fractions and slime EPS. Bound EPS are generally represented by a two-layer model, where the inner layer is tightly bound EPS (TB-EPS) and the outer layer is loosely bound EPS (LB-EPS). Because of their various compositions, the adsorption behaviors of TB-EPS and LB-EPS towards bacteria are different (Li et al., 2024)

Different antibiotic resistance genes in sewage sludge have varying spatial profiles, which affect their mobility and transfer potential (He et al., 2019). In a previous study, metagenomic analysis revealed lower abundances of ARGs and mobile genetic elements (MGEs) in algal-bacterial granular sludge than in bacterial granular sludge (Liu et al., 2022a). The formation of EPS is a relevant factor in wastewater treatment in microalga-bacteria systems, as it promotes the formation of flocs and granules (Arcila & Buitrón, 2017). Consequently, it is possible that ARGs exist within the matrix of microalga-bacteria aggregates. However, to the best of our knowledge, there are no reports on ARGs associated with EPS in microalga-bacteria systems. From an engineering perspective, if EPS facilitates the formation of aggregates, it could inherently enhance the adsorption of ARGs within the biomass, effectively removing them during the sedimentation phase of wastewater treatment. Therefore, this study aimed to determine the spatial distribution of ARGs in different factions (intracellular and extracellular EPS-related) and the potential mechanisms involved in ARGs reduction.

4.2 Materials and methods

4.2.1 Wastewater

Domestic wastewater was collected from the influent of the Corregidora WWTP located in Santiago de Queretaro, Mexico (20.5569 N 100.4296 W). Wastewater was collected after primary treatment (coarse and fine screening and primary sedimentation). Before use, large residual solids were removed from the wastewater through a sieve (Tyler No. 80) and the wastewater was stored at 4 °C for maximum of seven days. Also from the same plant, activated sludge was collected from the secondary settler.

4.2.2 Experimental set-up

All experiments were conducted using two outdoor flat-plate photobioreactor that were operated as duplicates at Queretaro, Mexico (20.7034 N 100.4459 W, 1900 m above sea level). The reactors total and working volume were 6 L and 5 L, respectively. First, a start-up stage was carried out, which lasted 12 days and consisted of adding 4 L of wastewater and 1 L of activated sludge, in order to promote growth of microalgae (inoculum). At the end of the start-up stage, the reactors were fully emptied, and the obtained inoculum was stored at 4 °C. Subsequently, reactors were operated in batch mode in three sequential operations, which lasted 10 days each. Wastewater and inoculum were added to each batch in such a way that an initial total suspended solids (TSS) concentration of 500 mg L^{-1} was obtained. In both

reactors, the pH level was adjusted daily to 7.5 with 10 N HCl. Dissolved oxygen (DO), temperature, and irradiance were measured online using calibrated probes every 1 hour, using a LabQuest® plug and play card (Vernier, USA) and Logger Pro® 3 software (Vernier, USA). Liquid samples were collected at the beginning and end of each batch to determine Chemical Oxygen Demand (COD), N-NH_4^+, and P-PO_4^{3-}, chlorophyll-a, TSS, volatile suspended solids (VSS) and microalgae observation. Additionally, during the third batch the removal of ARB was analyzed, and samples were taken for extraction of extracellular DNA in EPS fractions and intracellular DNA from MABA. Two types of EPS were evaluated, namely, bound EPS (B-EPS) and free EPS (F-EPS).

4.2.3 Analytical Methods

TSS and VSS were analyzed following the standard methods of the APHA (APHA, 2005). From the soluble fraction (samples filtered through 1.6 μm pore size membranes), N-NH4+, Chemical Oxygen Demand (COD), and P-PO43− were analyzed using colorimetric methods Hach 10031, 8000, and 10127, respectively. For the observation of microalgae, a Leica DM500 microscope with an image acquisition system (Leica ICC50 HD) and stereoscopic lens (Zeiss Stemi DV4) were used. The chlorophyll-a concentration was measured according to the methodology reported by Tang et al. (2022).

4.2.4 Antibiotic resistance bacteria quantification

The plate count method was used to quantify the presence of ARB in each sample. Antibiotics and concentrations were determined according to the reported by Cheng et al. (2020) in algae-based system. The following concentrations were used (mg L^{-1}): tetracycline (16), erythromycin (8), sulfamethoxazole (50.4), ampicillin (32), and ciprofloxacin (4). R2A agar (BD, USA) was used as a culture medium, in addition, 50 mg L^{-1} of nystatin was added to prevent fungal growth. Samples were serially diluted 10-fold and 0.1 mL of the dilution was used for spread plating. All analyses were performed in duplicate. The plates were incubated for a maximum period of 48 h at 37°C. Colony forming units (CFU mL^{-1}) were recorded on each plate to determine the ARB concentration (Cheng et al., 2020). The concentrations were reported in log10, and the removal was determined between the ARB counts obtained at the beginning and end of batch III. All colonies from each agar plate were recovered in 20% (w/v) glycerol for microbial characterization by metagenomic sequencing. Total heterotrophic

bacteria (THB) were determined by plating samples on media without antibiotics and were used to calculate the relative concentration (ARB concentration/THB).

4.2.5 Extraction of extracellular DNA in EPS fractions

Four hundred mL was taken from the reactor, and EPS extraction was performed (Figure S1). In brief, the process involves an initial centrifugation step to separate the solid and liquid phases, followed by vacuum filtration (0.22 µm) to extract F-EPS from the supernatant. The pellet was then resuspended and heat-treated before undergoing a second centrifugation, resulting in separation that allowed for the collection of B-EPS through further filtration and the retrieval of intracellular DNA from the final pellet. Samples from B-EPS and F-EPS filtrates were processed through precipitation with PEG 8000 and NaCl, after which the mixture was centrifuged to form a pellet, which was then washed and concentrated in the final centrifugation step. The concentrated EPS was stored at -20°C (Figure S2) (Arcila & Buitrón, 2017; Nuramkhaan et al., 2019). Part of the collected filtrate was used for the concentration analysis of the F-EPS and B-EPS fractions. The protein and carbohydrate contents were determined using the phenol-sulfuric acid method (Dubois et al., 1956) and Lowry-Folin method (Lowry et al., 1951), with glucose and bovine serum albumin (BSA) as the standards, respectively.

4.2.6 DNA extraction

Microbial characterization samples were stored at −20 °C before use. Extracellular samples were processed using a Water DNA/RNA Magnetic Bead Kit (IDEXX, USA). Intracellular samples were processed using the DNeasy PowerWater Kit (Qiagen, Germany), and samples from agar plates were processed using the DNeasy PowerSoil Kit (Qiagen, Germany), according to the manufacturer's instructions. The quality and concentration of DNA samples were verified by spectrophotometry using a NANODrop 2000c (Thermo Scientific, USA).

4.2.7 Resistome, mobilome and functional prediction analyses by metagenomic approach

DNA samples (Table S1) were sequenced using NovaSeq 6000, Illumina (~8 million PE 150+150 bp reads) at the Tec BASE Lab (Laboratorio Nacional de Secuenciación Genómica Tec BASE, Tecnológico de Monterrey, Mexico). The metagenomic sequences processing pipeline is described in Supplementary Information. Raw metagenomic sequences were

deposited into NCBI Sequence Read Archive (SRA) database under the project number (In process).

4.2.8 Metagenomic binning

Metagenomic assembled genomes (MAGs) from each sample were obtained from agar plates. Reads were mapped to the assembled contigs using Samtools (v1.8) and converted to BAM format (Li et al., 2009). Binnig was performed using MetaBAT (v2.12.1) (Kang et al., 2015) from contigs longer than 2000 bp. DAS Tool (v1.1.7) was used to compare multiple binning results and select the best binning result. Completeness and contamination of the resulting MAGs were estimated using CheckM (v1.2.1) (Parks et al., 2015). MAGs with a quality score of ≥ 50% (completeness, 5 × contamination) were considered for further analysis. Taxonomic classification of dereplicated MAGs was performed using GTDB-Tk (v2.1.1) based on the Genome Taxonomy Database (Release 207) (Chaumeil et al., 2020). ARGs carried by MAGs were identified against the CARD database using BLAST with an e-value cutoff of 10^{-5} (McArthur et al., 2013). A read was considered an ARG sequence if the result showed an identity ≥ 80% and a bit score ≥ 50.

4.3 Results and discussion

4.3.1 Performance of flat plate photobioreactors

The removal of N-NH_4^+ remained unchanged among the batches evaluated, whereas from batch I to batch III, the removal of P-PO_4^{3-} and DQOs increased 1.56 and 2.93 times, respectively (Table 4.1). The N-NH_4^+ and P-PO_4^{3-} removal results were similar to those reported for raceway reactors (Buitrón & Coronado-Apodaca, 2022). In contrast, the COD removal in stage III was 1.5 times lower than that reported in batch experiments (Arango et al., 2016). An increase in chlorophyll-a was observed at the end of each batch, which suggests an increase in microalgal biomass, as a linear relationship between chlorophyll-a and the VSS and TSS data was observed. Dos Santo Neto et al. (2020) also observed continuous growth of chlorophyll-a in a batch regime with aggregates of microalgae and bacteria, reaching a maximum value of 7.8 mg L^{-1}.

Table 4.1. Performance parameters, environmental conditions, and biomass parameter production in the three batches were evaluated.

Parameter	Batch I		Batch II		Batch III	
Performance parameter[a]	Mean ± SD[b]	%[c]	Mean ± SD	%	Mean ± SD	%
CODs[d] (mg L^{-1})	197 ± 36.8	14.9 ± 2.4	223.5 ± 12.0	27.7 ± 0.2	157 ± 4.2	43.7 ± 1.2
N-NH_4^+ (mg L^{-1})	0.3 ± 0.0	99.5 ± 0.0	0.8 ± 1.1	96.0 ± 5.7	1.2 ± 0.9	99.0 ± 0.8
P-PO_4^{3-} (mg L^{-1})	10.1 ± 1.9	49.5 ± 10.0	9.6 ± 0.9	40.9 ± 0.3	4.8 ± 0.8	77.7 ± 3.4
Operational condition	Mean ± SD	Min/Max	Mean ± SD	Min/Max	Mean ± SD	Min/Max
pH	7.8 ± 0.4	7.2/8.4	7.9 ± 0.5	7.0/8.8	7.7 ± 0.5	6.8/8.5
DO[e] (mg L^{-1})	7.3 ± 1.2	3.5 /10.2	6.9 ± 1.5	0.8/9.2	6.7 ± 1.1	3.7/9.3
Temperature (°C)	23.5 ± 9.2	7.8/38.8	24.5 ± 8.2	11.2/37.7	26.8 ± 7.8	14.0/39.8
Irradiance[f] (µmol m^2 s^{-1})	642.3 ± 389.3		631.1 ± 403.0		676.3 ± 370.1	
Biomass parameter	Initial Mean ± SD	Final Mean ± SD	Initial Mean ± SD	Final Mean ± SD	Initial Mean ± SD	Final Mean ± SD
VSS (mg L^{-1})	447.8 ± 11.6	658 ± 77.5	450 ± 19.7	655 ± 28.5	437.3 ± 23.3	667 ± 15.4
TSS (mg L^{-1})	546.8 ± 11.6	762 ± 65.5	481 ± 24.5	727 ± 23.4	495.8 ± 15.2	713 ± 18.6
chlorophyll-a (mg L^{-1})	0.7 ± 0.0	1.8 ± 0.1	1 ± 0.1	1.4 ± 0.1	0.7 ± 0.1	1.5 ± 0.0

[a] final concentration; [b] SD = standard deviation; [c] % = percentage of removal, considering initial/final concentration ratio; [d] CODs = soluble chemical oxygen demand; [e] DO = dissolved oxygen; [f] Maximum solar irradiance during the day.

4.3.2 Extracellular DNA from EPS fractions

Statistical analyses showed that there was no significant difference ($p > 0.05$) between the data obtained in reactors 1 and 2; therefore, the data from both reactors were considered replicates. No significant differences were found between the free and bound fraction of carbohydrates between the beginning and end of the batch III, while for the free and bound fraction of proteins a significant decrease was found from the beginning to the end of the batch (Table S2). Previously, it has been reported that protein content in EPS plays a key role in microalgae-bacteria granulation (Zhang et al., 2020; Arcila & Buitrón, 2017). In this study,

the P/C ratio was 0.9, which increased to 3.5, implying that the formation of aggregates was favored (Trebuch et al., 2020).

4.3.3 Antibiotic resistance removal

The photobioreactors significantly removed ($p < 0.05$) ARB and THB (Figure 4.1.A). In general, some similarities were observed in the removal of ARB and THB to what was reported in the monoalgal system, with the exception of Ciprofloxacin, Tetracycline and Erythromycin, as these ARB were removed to a greater extent in the microalgae-bacteria system (Table S3). Furthermore, it should be noted that removals similar to those of an activated sludge system with a disinfection process were obtained (Gao et al., 2012), which is of interest and potentiates the use of microalgae-bacteria systems for the effective reduction of ARB. The low removal of THB in this study (0.8) is also highlighted, and it is worth mentioning that the sample was collected from the mixed liquor rather than the supernatant owing to the absence of a settling tank; however, the concentrations of ARB normalized with the total heterotrophic bacteria count showed significant reductions ($p < 0.05$) in the antibiotics evaluated throughout the treatment (Figure 4.1.B).

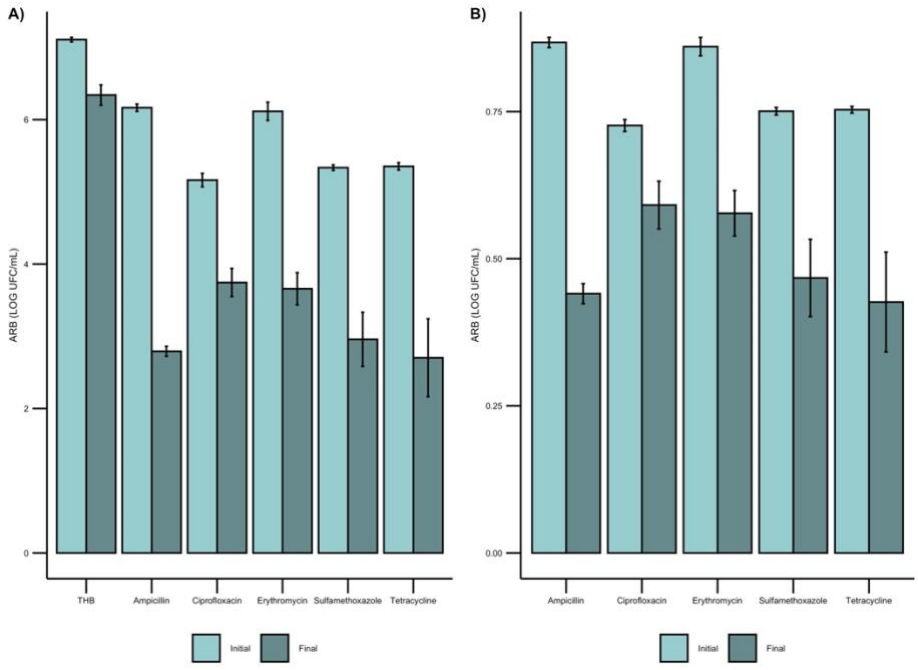

Figure 4.1. A) Absolute ARB removal; B) Relative removal of AB during the microalgae-bacteria system in batch III; THB: total heterotrophic bacteria.

4.3.4 MAGs from selective agar plates

The MAG-based method is a reliable tool to identify and quantify health-related strains for microbial risk assessment in the environment (Liang et al. 2020). From 95 MAGs recovered from the assembly of contigs, only 46 passed quality evaluations. The recovered MAGs were classified and annotated into nine different bacterial orders: Actinomycetales, Bacteroidales, Burkholderiales, Enterobacterales, Pseudomonadales, Rhizobiales, Rhodobacterales, Sphingomonadales, and Xanthomonadales. Entorobacterales and Pseudomonadales were the most abundant orders. Several significant patterns and trends in ARGs in MAGs were identified. First, the initial samples exhibited notable diversity of resistance genes. In contrast, in the final samples, there was a discernible trend towards reduced diversity in the resistance genes. *Escherichia coli* was only detected in the majority of the wastewater and initial samples, carrying a high number of subtypes of ARGs, including multi-antibiotic-resistant hosts. According to a previous report, *E. coli* is a significant source of ARGs in wastewater and horizontal gene transfer may be the means by which many ARGs are acquired (Poirel et al., 2018). *E. coli* is a pathogenic bacterium that poses a greater danger to human and animal health due to its ability to produce serious infections and its resistance to multiple antibiotics. However, *E. coli* was not detected in the MAGs from the final samples, but other bacteria were detected in the final samples, such as *Enterobacter kobei*, which cause*s* nosocomial urosepsis (Hoffmann et al., 2005), and non-pathogenic bacteria, such as *Thauera humireducens* and *Pseudomonas fluvialis*. This implies that although pathogenic bacteria, such as *E. coli*, are evidently reduced in microalgae-bacteria systems, some non-pathogenic bacteria persist at the end of the treatment and are hosts of ARGs; therefore, these bacteria must also be monitored to reduce the risk of ARGs spreading in the environment.

4.3.5 Resistome changes after photobioreactor wastewater treatment

In the intracellular wastewater fraction, a total of ARGs of 257.1 ppm were obtained, with the most abundant types being Aminoglycoside, Beta-lactam and Multidrug (Figure 4.2.A). Furthermore, an abundance of ARGs was detected in the extracellular fraction of the wastewater (146.9 ppm). At the beginning of batch III, a slightly higher concentration of ARGs was obtained in the B-EPS fraction than in the intracellular fraction, while in the F-EPS fraction, only two tetracycline ARGs genes were detected, tet(Q) and tet(W). At the end of the 10 days of operation, significant reductions in ARGs were observed in the different fractions. For example, if we consider the sum of the three fractions as a resistome, this was reduced by

up to 6.3 times, which is greater than the 3.4-fold reduction reported previously using a monoalgal photobioreactor (Nõlvak et al., 2018). Furthermore, no ARGs types for Fosfomycin, Peptide, Phenicol, Quinolone and Tetracycline were detected at the end of batch III in any of the three fractions. This is similar to what was previously reported, where Fosfomycin and Peptide were not detected in the effluent and MABA samples from the HRAP system (Ovis-Sánchez et al., 2023). The composition of ARGs in both types (Figure 4.2.B) were distinct between the initial and final fractions. These results revealed higher diversity in the wastewater samples and initial fractions; however, lower diversity was observed in the final intracellular and final B-EPS samples. Moreover, the absence of ARGs in the final F-EPS and an evident reduction in ARGs in the final B-EPS samples might reduce the possibility that extracellular ARGs can reenter cells through spontaneous transformation under ideal circumstances and provide new host resistance features (Wang et al., 2021).

4.3.6 Mobilome identification

As show in Figure 4.3.A, a significant decrease in the various MGE modules was observed in the final fractions. Mobilome reduction was reduced by up to 6.8 times. The phage MGE module showed a significantly higher abundance in extracellular fractions, such as WW extracellular and Initial F-EPS (Figure 4.3.A), This was also confirmed by the percentage, where the WW extracellular, Initial F-EPS and Final F-EPS samples had a proportion of Phages between a range of 35-67% (Figure 4.3.B). It is widely recognized that phages harboring ARGs could infect new hosts and introduce these ARGs into new cells, thereby conferring them the given resistance (Calero-Caceres et al., 2014). These results suggest that a decrease in the number of phages may result in lower transduction rates, which also plays an important role in the spread of ARGs during wastewater treatment (Cheng et al., 2020). PCA analysis accounted for 92.66% of the total variation, indicating substantial explanatory differences (Figure 4.3.C), the only samples that had notable differences in the mobilome where the initial and final F-EPS were mainly due to the higher presence of phage-related genes such as *gp26* and *gp45* and the replication/recombination/repair-related gene *polA_2*.

4.3.7 Microbiome profiling from extracellular and intracellular fractions

The diversity assessed according to Shannon's index demonstrated that all samples yielded ranges between 4.8-5.5, with the Final B-EPS sample being the most diverse and the initial intracellular sample being the least diverse (Figure S3.A). By contrast, the observed domains

(Figure S3.B), a higher proportion of Eukaryota was only obtained in the intracellular samples of the photosynthetic system (initial or final) and in the final B-EPS sample, with a relative abundance in the range of 14.2-24.1%. In the WW Intracellular sample, 99.4% of the domains were represented by bacteria. The virus domain was mainly represented in extracellular samples such as WW Extracellular, Initial F-EPS, and Final F-EPS. The microbial community composition varied according to the different fractions or samples, as shown by the PCoA analysis (Figure S3.C).

The comparison of community diversity at the class level was more informative, indicating a distinctive pattern for each sample. A total of 17 bacterial classes were identified in this study (excluding others < 2% and NA), with Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria being the most abundant (Figure 4.4). These were distributed differently among samples. Alphaproteobacteria was found to be the most abundant class in initial and final intracellular samples. Gammaproteobacteria was decreased in the final intracellular in comparison with the initial intracellular from 10.7 to 4.5%. These results indicate that microalgae-bacteria treatment decreased the abundance of Gammaproteobacteria, a class containing several medically vital groups of bacteria and even pathogens (Narciso-da-Rocha & Manaia, 2017).

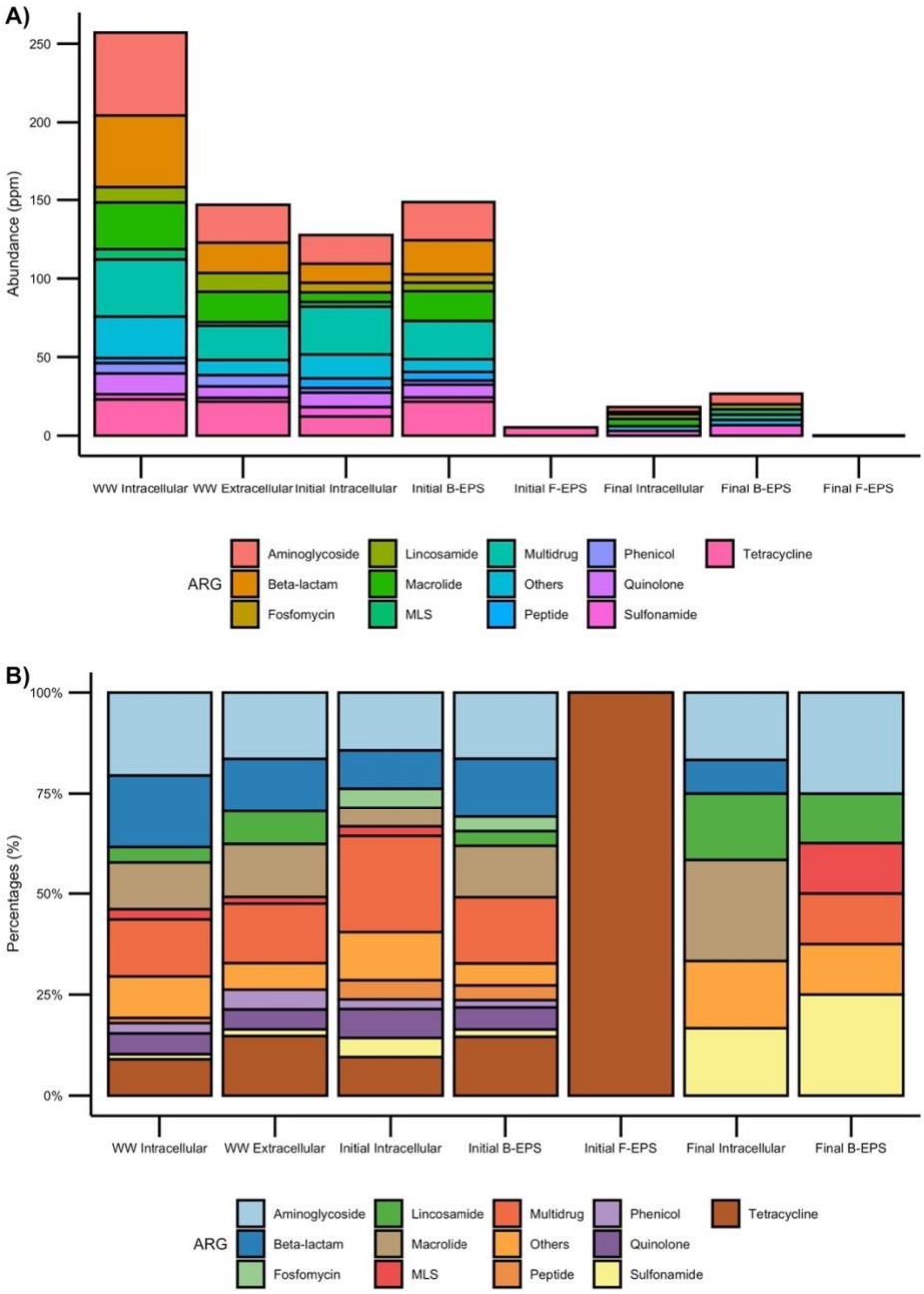

Figure 4.2. ARGs profiles of different EPS and intracellular fractions from batch III. A) Total abundance (ppm) of ARGs. B) Percentage composition at the type level.

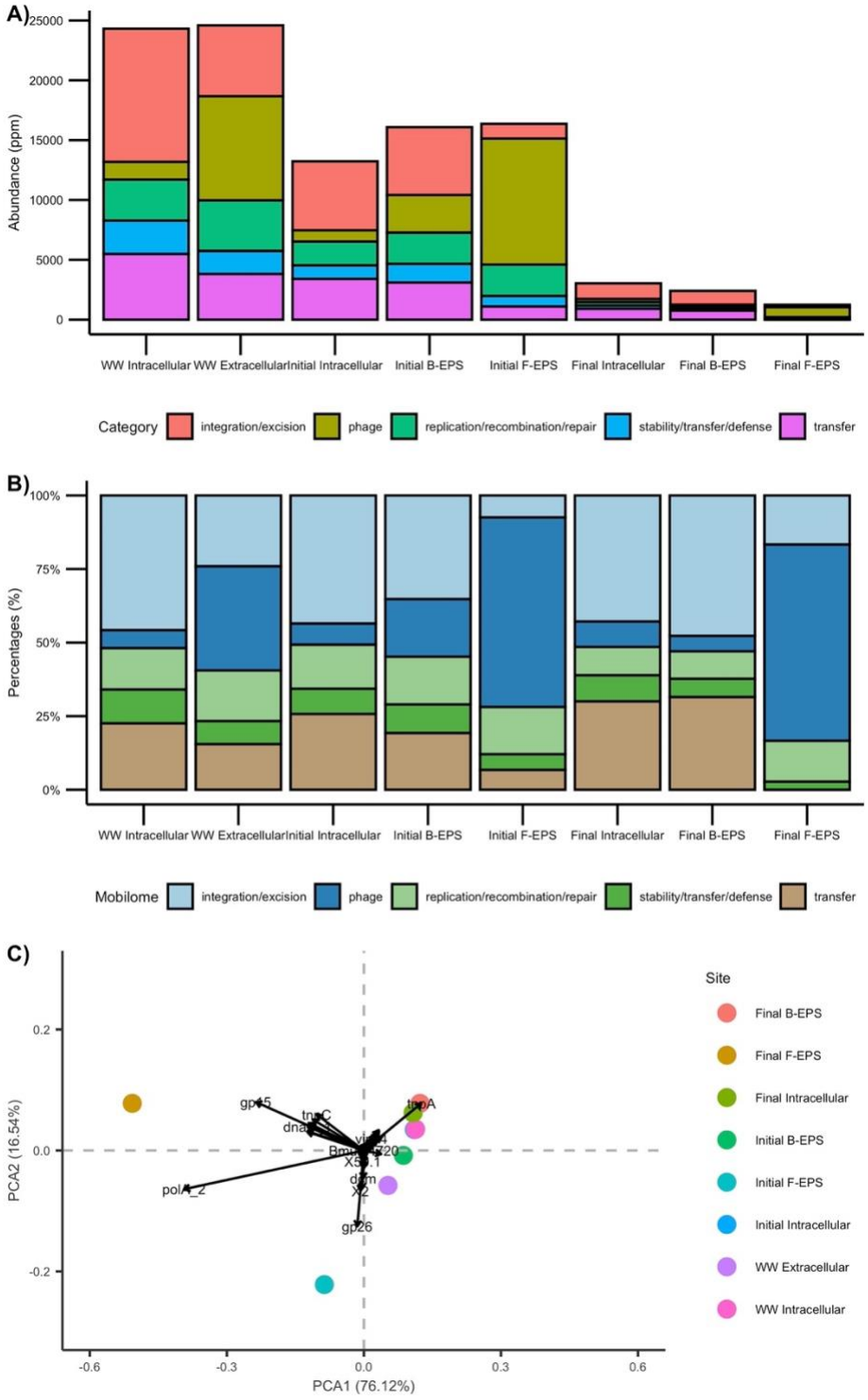

Figure 4.3. A) Total abundance (ppm) of the mobilome in the different categories. B) Percentage composition of categories. C) PCA at the MGEs level.

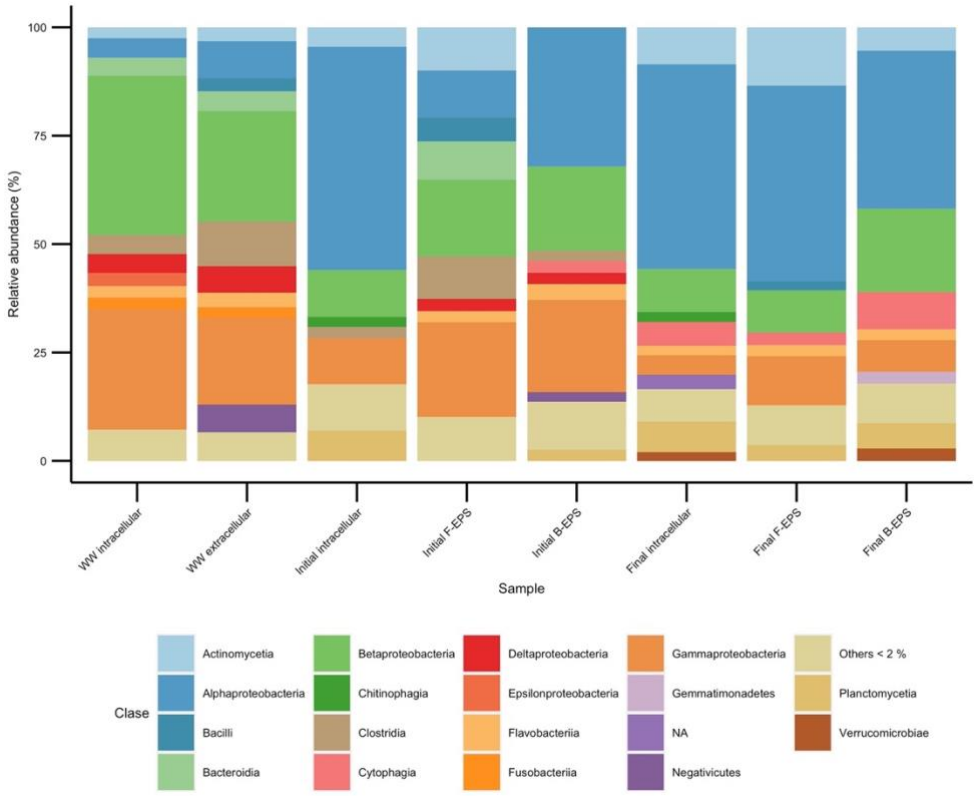

Figure 4.4. Relative abundance at the class level of bacteria from different EPS and intracellular fractions from batch III.

4.4 Conclusions

In this study, the distribution of ARGs associated with EPS was obtained. Notably, the highest concentrations of ARGs were detected within the intracellular fraction; however, they were substantially reduced throughout the treatment process. The absence of ARGs in the final F-EPS fraction and the pronounced decrease in the final B-EPS fraction were particularly noteworthy. These trends point to several underlying mechanisms in ARG reduction: the removal of clinically relevant bacteria known as ARG reservoirs, potentially reducing HGT by transduction due to a significant decrease in various MGE modules, including phages, and potentially reducing HGT by transformation due to the absence or low presence of ARGs in the EPS fractions.

Substantial removal of ARB and THB by the photobioreactors was observed, with potential comparable to activated sludge systems with disinfection processes. This study highlights the efficacy of MAG-based methods in assessing microbial risks in environmental samples. Significant reductions in *Escherichia coli* and diversity of resistance genes observed in the final samples underscore the potential of microalgae-based treatment systems in mitigating antibiotic resistance dissemination. Overall, these results underscore the importance of EPS in microalgae-bacteria systems. EPS plays a critical role not only in aggregate formation and sedimentation properties in treatment sequences, but also in maintaining ARGs within these aggregates, predominantly within the intracellular fraction, thereby reducing the potential spread of ARGs in the clarified water.

5. General discussion and conclusions

5.1 Discussion and perspectives

The resistome analysis allowed us to identify a better view of ARGs in the wastewater, where higher concentrations of ARGs types related to multidrug, tetracycline, aminoglycoside, and beta-lactam genes were highlighted (Section 2). This may imply that antibiotic resistance is present in segments of the population, as a recent study has suggested that the influent wastewater resistome is likely to reflect the clinical use of antimicrobials in the sewershed community (Honda et al., 2023). It has even been suggested that seasonality also influences the concentrations of ARGs from wastewater having a positive correlation between antibiotic use and the abundance of ARGs (Caucci et al., 2016; Harnisz et al., 2020).

This study inferred that the main mechanism of ARGs removal in microalgae-bacteria systems was mainly through the reduction of vertical gene transfer (VGT) due to the ecological exclusion of microorganisms that are potential hosts of ARGs (Nguyen et al., 2021). During the operation of the HRAPs (section 2), we observed a decrease in clinically relevant bacteria, such as ESKAPEE, widely reported in the literature as ARGs hosts (Ngoi et al., 2021). This implies that the microalgae-bacteria systems have conditions that do not favor the reproduction of this type of bacteria, which was also observed in the operation of the flat-plate photobioreactors (Section 4), where bacteria of the order Gammaproteobacteria were evidently reduced. Therefore, this section reaffirms the efficacy of microalgae-bacteria systems in reducing the resistome, whether in HRAP or flat-plate reactors.

On the other hand, the results obtained in Section 4 suggest that the reduction of the ARGs is also due to the reduction of the HGT due to the removal of mobile genetic elements such as plasmids, among others, that are related to the conjugation and also evident reduction of phages related to the transduction (Li & Zhang, 2022). Previously, it was suggested that in an algal system for wastewater treatment, there is a reduction in HGT transfer due to the reduction of phages (Cheng et al., 2020). In addition, intracellular and extracellular fractions related to extracellular polymeric substances (EPS) were studied. This revealed the reduction in ARGs in these fractions, reducing the potential HGT of extracellular genes.

This suggests that microalgae-bacteria systems reduce antibiotic resistance mainly by decreasing VGT and HGT, although it is limited to know which mechanism had the greatest

influence on the reduction of antibiotic resistance. The results obtained in Sections 2 and 4 suggest that different mechanisms are involved (Figure 5.1).

The use of anaerobic digestion was also demonstrated as a coupled system in which the antibiotic resistance that persists in the microalgal biomass generated in HRAP reactors can be reduced. The proposed treatment scheme and reduction in the resistome (ppm of total ARGs) are shown in Figure 5.2. However, comparisons of the resistome with other studies are limited due to differences in the bioinformatics processing of the sequences (programs used, quality criteria, databases, among others). The persistence of the six ARGs subtypes (qacEdelta1, tet(C), ANT(3'')-IIa, linG, sul1, and sul2) highlights the importance of their quantitative evaluation in future studies using techniques such as qPCR. While their functional roles and contributions to overall antibiotic resistance in the MABA microbiome require further investigation, including transcriptomic analyses, these genes merit focused attention for a deeper understanding and targeted mitigation. Additionally, the reduction of ARB during anaerobic digestion was evaluated, yielding significant reductions ($p < 0.05$) under thermophilic conditions. This reduction diminishes the potential spread of pathogenic bacteria, as evidenced by identifying of clinically important bacteria, such as *Pseudomonas,* on various selective (antibiotic) agar plates. It is crucial to mitigate their environmental dissemination to prevent the transfer of ARGs, as *Pseudomonas aeruginosa* is a highly resistant bacterium that poses a significant challenge in eradicating infections (Pang et al., 2019). Despite the reduction in pathogenic bacteria, the MAGs analysis allowed us to observe that the bacteria that grew on the selective plates at the end of the water treatment did not recognize pathogens such as *Thauera humireducens* and *Pseudomonas fluvialis*. This indicates that although the microalgae-bacteria system can ensure the reduction of pathogens that contain a large number of ARGs, commensal bacteria must also be monitored because they contain ARGs that can potentially be transferred.

Therefore, this thesis can encourage and potentiate microalgae-bacteria systems as wastewater treatment alternatives to reduce ARGs and ARB. However, is important to highlight that it is also crucial to expand wastewater treatment coverage across our country and enhance the current state of operation of WWTPs. The contribution of transmission routes to the total number of acquisitions of ARB bacteria remains unknown (Godijk et al., 2022). Ensure wastewater treatment is essential to decreasing exposures and chances of spread (Coque et al.,

2023). Additionally, public policies and preventive measures at the healthcare sector level can significantly mitigate antibiotic resistance.

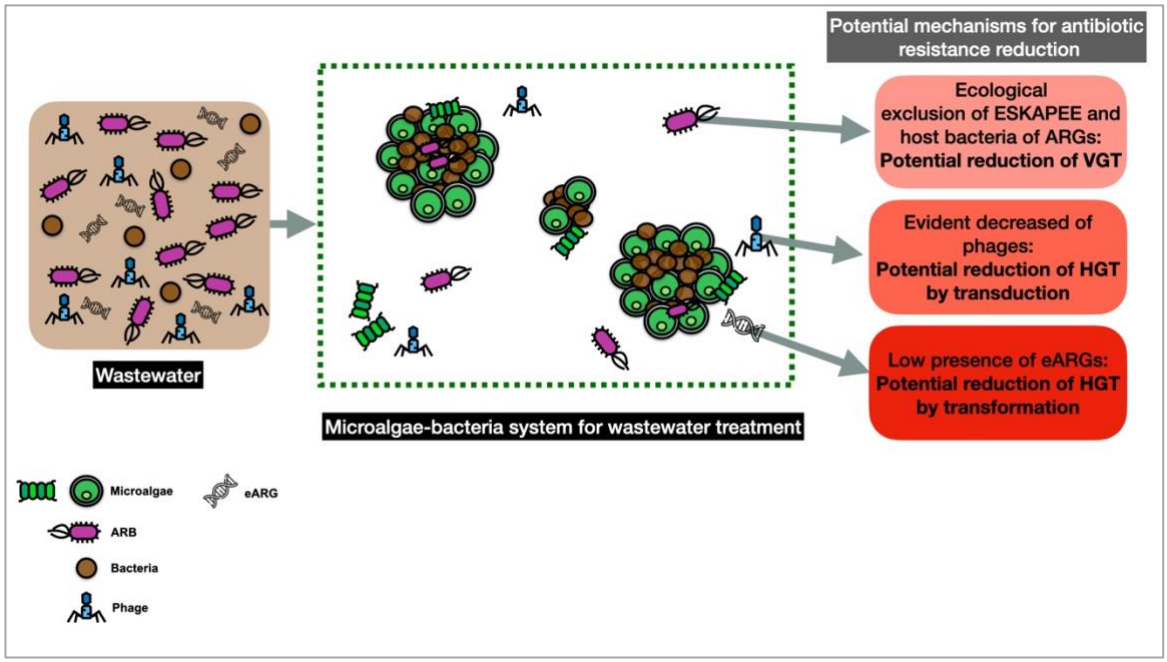

Figure 5.1. Mechanisms involved in the antibiotic resistance reduction in the microalgae-bacteria system.

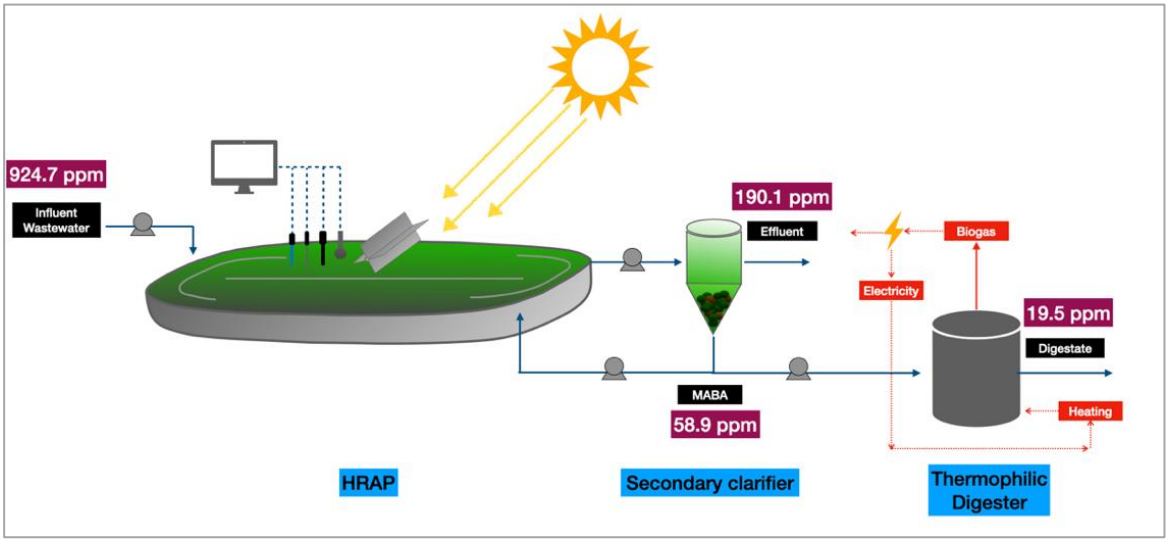

Figure 5.2. Diagram of the microalgae-bacteria system coupled with anaerobic digestion for resistome (ppm) reduction.

5.2 Final conclusions

It has been shown that the ecological exclusion and spatial distribution of ARGs in microalgae-bacteria systems influences the reduction of the resistome. This promotes the potential reduction of the horizontal and vertical transfer of ARGs. The data demonstrated that treatment using the microalgae-bacteria system, particularly the HRAP, resulted in a marked reduction of ARGs by metagenomics and qPCR. The shift in microbial community composition, with a decrease in clinically relevant ARG-carrying bacteria in the effluent, suggests that the system conditions may have favored the ecological exclusion of such organisms. Moreover, the altered spatial distribution of ARGs in the EPS fractions due to the treatment suggests that the microalgae-bacteria system can disrupt the reservoirs of resistance genes. The reduced presence of ARGs in these fractions implies that the treatment process lowered the risk of horizontal gene transfer by transformation.

In the anaerobic digestion of MABA, it was demonstrated that the thermophilic regime was more efficient than the mesophilic regime in terms of methane production and antibiotic resistance reduction. ARB were significantly reduced in the range of 1.1-1.7 log, and resistome was also significantly reduce from 58.9 ± 7 to 36.9 ± 2 ppm. Therefore, anaerobic digestion can be coupled with the microalgae-bacteria system as an alternative for the production of methane and the reduction of antibiotic resistance, although more studies are needed to increase methane yields and economic studies to demonstrate its sustainability.

Although the resistome was significantly reduced in all three experimental stages, some genes persisted after treatment, for example, sul1 and sul2. These genes can be considered for future studies and to expand the understanding of their persistence in these types of systems. In addition, other variables such as solar irradiance should be evaluated in the role of resistome reduction in microalgae-bacterial systems, mainly in the HRAP.

In conclusion, the treatment of domestic wastewater using microalgae-bacteria systems, followed by thermophilic anaerobic digestion of MABA, is a viable approach for reducing ARGs and ARBs. This thesis supports the proposed hypothesis and highlights the potential of this integrated treatment strategy, particularly for application in low-to middle-income countries, to mitigate the public health threats posed by antibiotic resistance. These systems can be used for decentralized water treatment, including the treatment of effluents from the pharmaceutical industry or hospitals. Further research is necessary to fully explore and validate their efficacy in specific applications.

Dissertation achievements

Published articles:

Ovis-Sánchez, J. O., Perera-Pérez, V. D., Buitrón, G., Quintela-Baluja, M., Graham, D. W., Morales-Espinosa, R., & Carrillo-Reyes, J. (2023). Exploring resistomes and microbiomes in pilot-scale microalgae-bacteria wastewater treatment systems for use in low-resource settings. *Science of The Total Environment*, 882, 163545.

In preparation:

Ovis-Sánchez, J. O., Buitrón, G., Vital-Jácome, M., & Carrillo-Reyes, J. (2024). Antibiotic resistance reduction during anaerobic digestion of microalgae-bacteria aggregates: temperature effect. To be submitted in Bioresource Technology

Ovis-Sánchez, J. O., Graham, D. W., & Carrillo-Reyes, J. (2024). Extracellular polymeric substances associated to antibiotic resistance genes in microalgae-bacteria systems. To be submitted in Algal Research.

Pallares-Vega, R., Ovis-Sánchez, J. O., Jobling, K., Kreft, J., Graham, D. W., & Harwood, C. (2024). Assess the spread of Antimicrobial Resistance Plasmids in conditions relevant for Indian rivers.

Ovis-Sánchez, J. O., Romero-Borja, I., & Carrillo-Reyes, J. (2024). Antibiotic resistance bacteria and enteric pathogen removal by microalgae-bacteria system.

Conference participation:

1. **Ovis-Sánchez, J.O.**, Carrillo-Reyes, J. Antibiotic resistance reduction anaerobic digestion of microalgae-bacteria aggregates: temperature effect. XIV Latin America Workshop and Symposium on Anaerobic Digestion. 23-27 October 2023. Oral presentation.

2. **Ovis, J**., Buitrón, G., Carrillo-Reyes, J. Remoción de genes de resistencia a antibióticos en aguas residuales mediante sistemas microalga-bacteria. XIX Congreso Nacional de Biotecnología y Bioingeniería. September 27 to October 1, 2021. E-poster presentation, 2021. E-poster presentation.

3. **Ovis, J**., Perera-Pérez, V., Buitrón, G., Carrillo-Reyes, J. Fate of Antibiotic Resistance Genes in Microalgae-bacteria System for Domestic Wastewater Treatment. World Microbe Forum. 20-24 June 2021. Poster presentation.

4. **Ovis-Sánchez Julián O.**, Perera-Pérez Víctor D., Buitrón Germán, Carrillo-Reyes Julián. Producción de metano a partir de agregados microalga-bacteria. Latin American Meetings on Anaerobic Digestion. November 2020. Oral presentation.

5. **Ovis, J**., Buitrón, G., Carrillo-Reyes, J. Potential of microalgae-bacteria systems for the removal of antibiotic resistance genes in domestic wastewater**.** 2nd Latin American & Caribbean Young Water Professionals Conference. November 2020. Poster presentation.

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Supplementary material

Section 2

Appendix A. Metagenomic sequence processing

Raw sequences were trimmed to remove adapters and low-quality nucleotide stretches using Trim Galore (v0.6.5) (Yoo et al., 2020). MEGAHIT (v1.2.9) was used to assemble clean reads into contigs (Nõlvak et al., 2018). The resulting contigs were aligned against the Comprehensive Antibiotic Resistance Database (CARD) using BLASTP, with an e-value cutoff of 10^{-5} (McArthur et al., 2013). A read was considered as an ARG sequence if the result showed identity ≥ 80% and a bitscore ≥ 50. The levels of ARG or mobile element-like sequences in the present study were described using the unit of "ppm" (one read in one million reads) as recommended by Yang et al. (2013), which was defined as the portion of ARG or mobile element-like sequences in "total metagenome sequences".

Appendix B. Statistical analysis

Nonmetric Multi-Dimensional Scaling (NMDS) based on Bray–Curtis distance was used to compare microbial community structures among samples. NDMS was calculated using the R package vegan (Oksanen et al., 2019).

Figure S1: Outdoor HRAP systems at Querétaro, México.

Figure S2: Sampling points (★) for comparing antibiotic resistance during domestic wastewater treatment using HRAP.

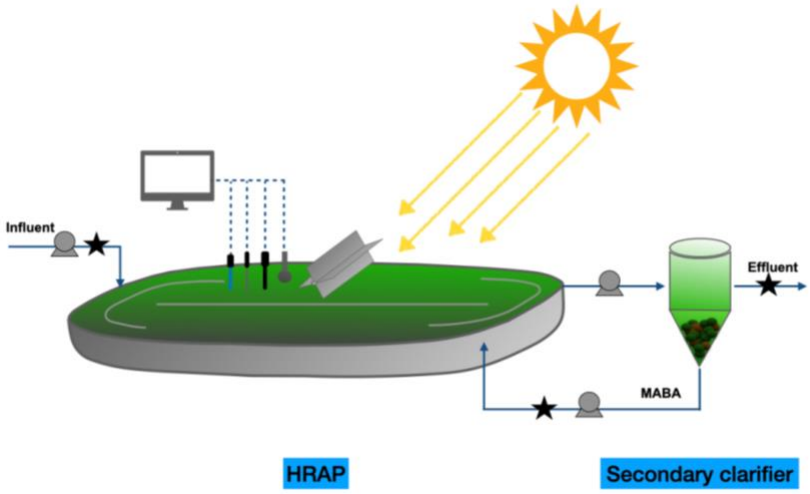

Figure S3: Stereoscopic images of MABA using 10X, representing the changes in MABA observed during different operational stages I (a), II (b) and III (c).

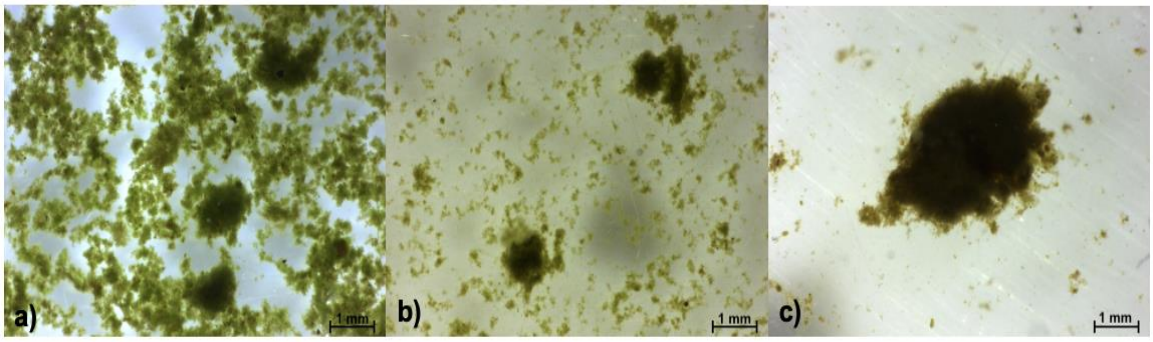

Figure S4: Venn diagram showing the number of shared and unique ARG (at the subtype level) among influent, MABA and effluent. For each site, data from all three stages were considered and duplicate ARG were removed.

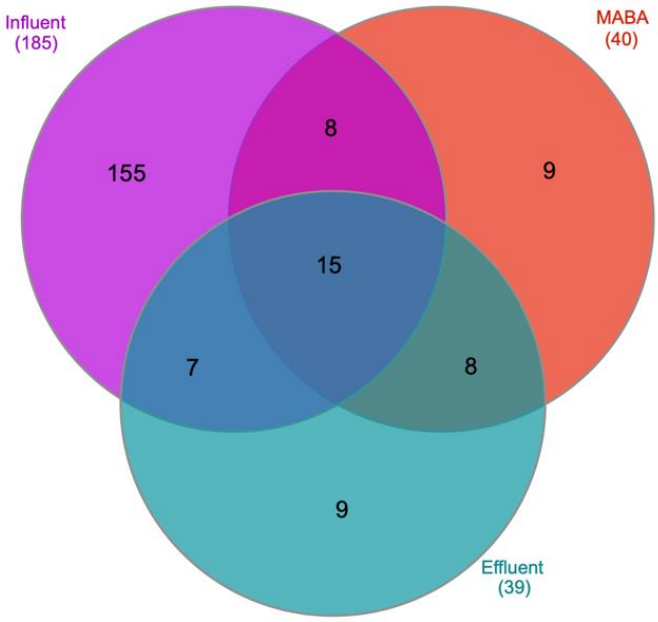

Figure S5: Spearman's rank correlation analysis among absolute abundance of ARG and *intl1* from E(effluent) and MA (MABA), EPS and SVI in MABA trough the different operational stages ($p < 0.05$). * F= Free; B= Bound; C= Carbohydrate; P= Protein. ∗: $P < 0.05$, ∗∗: $P < 0.01$, ∗∗∗: $P < 0.001$.

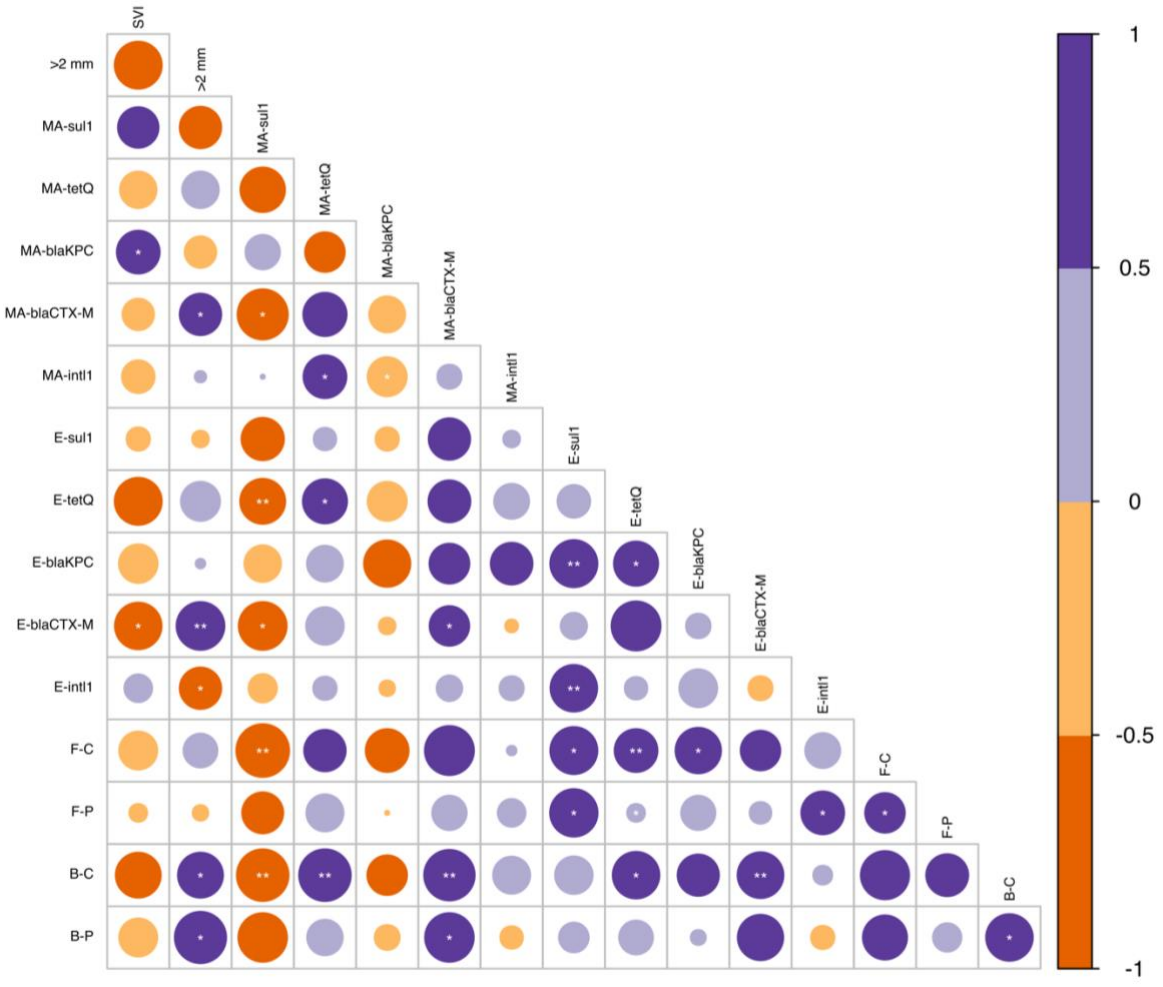

Figure S6: Heatmap and cluster analysis (Bray–Curtis) of relative abundance of the different sampling points; relative abundances are z-score normalized for visualization purposes. Sites (x-axis) and microbial taxa (y-axis) are clustered based on similar abundance and occurrence patterns. Sites codes: IN) influent, E) effluent, MA) MABA, R1) HRAP 1, R2) HRAP 2.

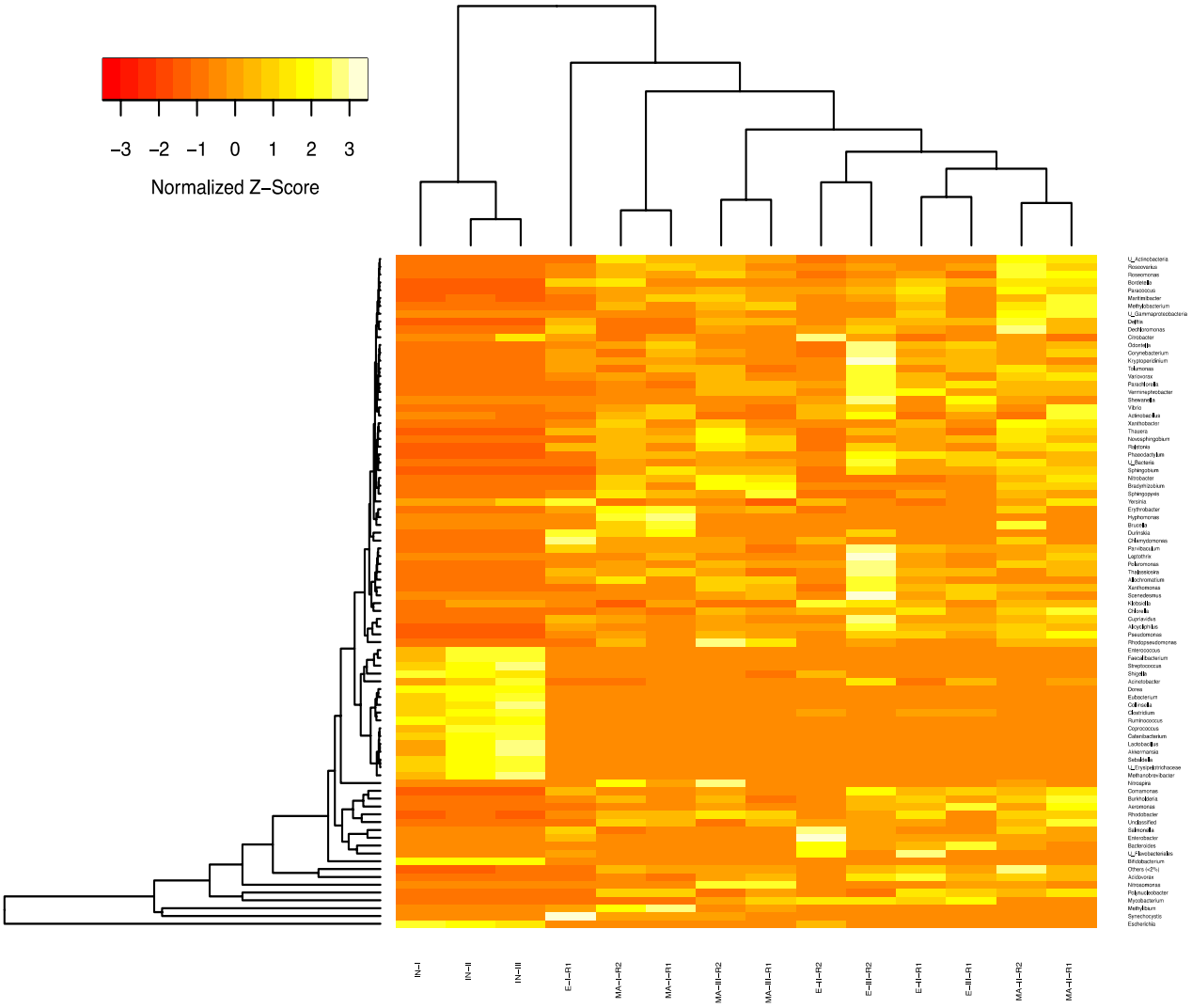

Figure S7: NMDS analysis based on the BrayCurtis distance, which showed the overall distribution of microbial community among different samples from HRAP.

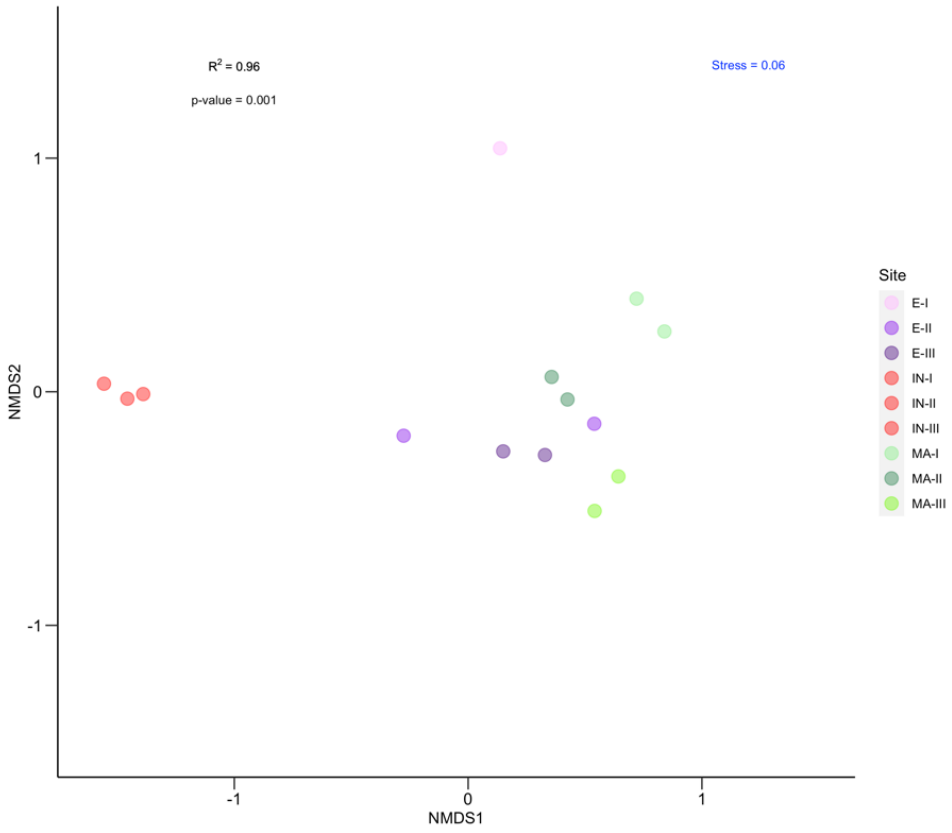

Figure S8: Coloured dots represent the taxa with significantly different abundances among sites, and from the centre outward, they represent the kingdom, phylum, class, and order. The coloured shadows represent trends of the significantly differed taxa. Each coloured dot has an effect size linear discriminant analysis (LDA) score.

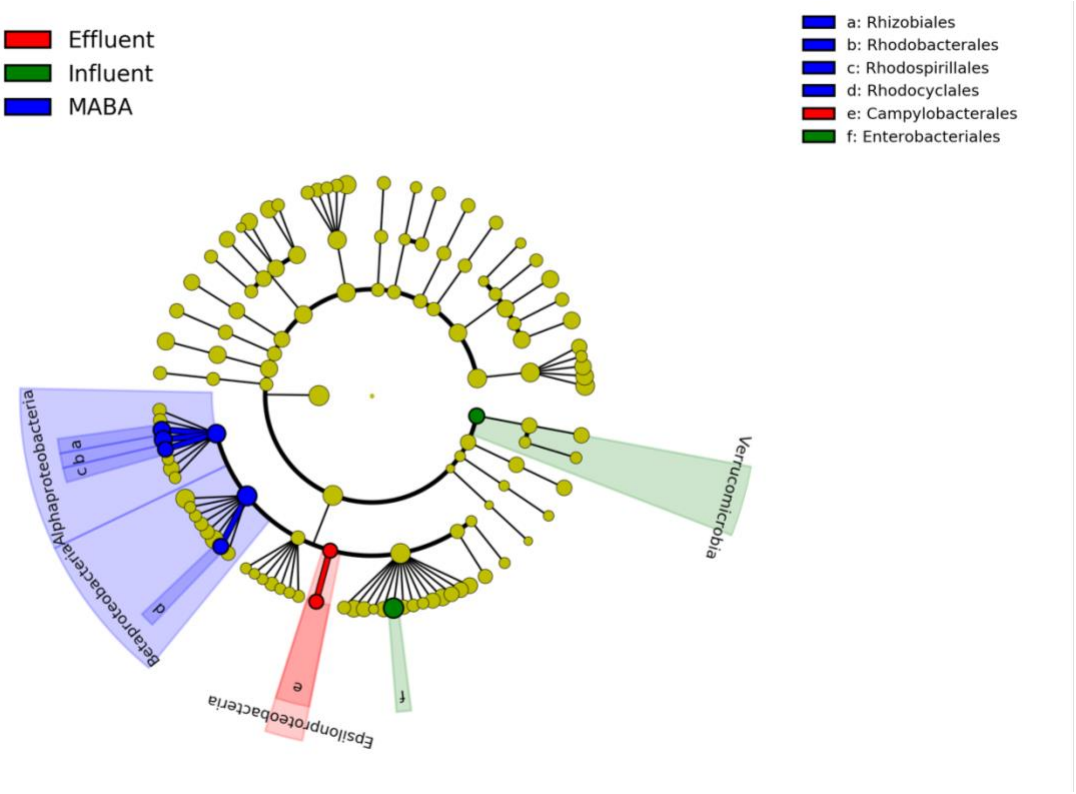

Table S1: The primers, annealing temperatures and calibration curves data of the determined genes.

Target genes	Primer	Sequences	Annealing temp (°C)	Size (bp)	R^2	Amplification efficiency (%)
sul1	*sul1*-F	CACCGGAAACATCGCTGCA	55	158	0.99	90.5
	sul1-R	AAGTTCCGCCGCAAGGCT				
tetQ	*tetQ*-F	GCTCACATTGATGCAGGAAA	58	153	0.99	106
	tetQ-R	CGTAGAAGCCCGRACAGTAA				
blaKPC	*blaKPC*-F	TCGCCGTCTAGTTCTGCTGTCTTG	57	353	0.99	91.6
	blaKPC-R	ACAGCTCCGCCACCGTCAT				
blaCTX-M	*blaCTX-M*-F	ATGTGCAGYACCAGTAARGTKATGGC	60	593	0.99	86
	blaCTX-M-R	TGGGTRAARTARGTSACCAGAAYCAGCGG				
intl1	*intl1*-F	CCTCCCGCACGATGATC	55	280	0.98	87.2
	intl1-R	TCCACGCATCGTCAGGC				
16S rRNA	1055-F	ATGGCTGTCGTCAGCT	52	354	0.99	85
	1392-R	ACGGGCGGTGTGTAC				

Table S2: The p-values of parametric and non-parametric test for environmental conditions and performance parameters follothrough the three operation stages.

		Parametric Data		Non-parametric	
Parameter	Shapiro–Wilk test	One-way ANOVA	Tukey post-hoc	Kruskal-Wallis test*	Pairwise Wilcoxon test post-hoc*
DO (mg L^{-1})	0.00	NA	NA	0.00	I:a; II:b; III:a
Temperature (°C)	0.00	NA	NA	0.00	I:a; II:b; III:c
pH	0.00	NA	NA	0.00	I:a; II:b; III:b
Irradiance (μmol m^2 s^{-1})	0.06	0.01	I:ab; II:b; III:a	NA	NA
CODs removal (%)	0.11	0.01	I:b; II:b; III:a	NA	NA
N-$NH4^+$ removal (%)	0.00	NA	NA	0.25	I:a; II:a; III:a
TSS in effluent (mg L^{-1})	0.21	0.10	I:a; II:a; III:a	NA	NA
Settleability (%)	0.23	0.00	I:b; II:a; III:a	NA	NA
SVI (mL g VSS^{-1})	0.00	NA	NA	0.00	I:a; II:b; III:c
Sv (m h^{-1})	0.04	NA	NA	0.60	I:a; II:a; III:a
Free EPS Carbohydrate (mg g VSS^{-1})	0.15	0.00	I:b ; II:a; III:b	NA	NA
Free EPS Protein (mg g VSS^{-1})	0.15	0.02	I:ab; II:a; III:b	NA	NA
Bound EPS Carbohydrate (mg g VSS^{-1})	0.25	0.89	I:a; II:a; III:a	NA	NA
Bound EPS Protein (mg g VSS^{-1})	0.01	NA	NA	0.39	I:a; II:a; III:a

* Different lowercase letters indicated significant difference ($p < 0.05$) between stages.

Table S3: Hits from ESKAPEE caught at the experimental HRAP operation. Sites codes: IN) influent, E) effluent, MA) MABA, R1) HRAP 1, R2) HRAP 2.

Site	*Enterococcus*	*Staphylococcus*	*Klebsiella*	*Acinetobacter*	*Pseudomonas*	*Enterobacter*	*Escherichia*
IN-I	1991	20	522	2048	139	143	128711
IN-II	6417	97	1748	5303	135	236	110130
IN-III	5814	64	1304	6764	195	299	73875
MA-I-R1	0	0	34	35	43	19	124
MA-I-R2	0	0	10	16	62	25	78
MA-II-R1	0	0	29	30	70	25	115
MA-II-R2	0	0	39	31	71	53	245
MA-III-R1	0	0	35	64	123	23	209
MA-III-R2	0	1	28	63	103	22	269
E-I-R1	0	0	16	7	20	87	169
E-II-R1	0	1	163	93	52	786	1977
E-II-R2	0	0	40	12	73	45	78
E-III-R1	0	1	34	86	64	47	173
E-III-R2	0	1	23	47	32	22	120

Table S4: Hill numbers for the sites caught at the experimental HRAP operation. Sites codes: IN) influent, E) effluent, MA) MABA, R1) HRAP 1, R2) HRAP 2.

Site	q0	q1	q2
IN-I	232	5.1	2.2
IN-II	245	8.5	3.2
IN-III	236	11.2	4.2
MA-I-R1	142	12.1	4.0
MA-I-R2	156	18.3	6.2
MA-II-R1	142	35.4	13.5
MA-II-R2	176	51.4	24.4
MA-III-R1	176	18.0	7.5
MA-III-R2	180	24.4	8.7
E-I-R1	89	8.2	3.1
E-II-R1	162	25.0	10.5
E-II-R2	160	16.4	8.3
E-III-R1	156	21.4	9.1
E-III-R2	102	30.2	13.0

Table S5: Active peptide sequences (MIC<25 µg mL^{-1}) against Escherichia coli ATCC 25922.

Access	Sequence	Predictive value (Type)
smORF_12354	RLPGVARSNSMLVLRTVKKTTALAL	0.66 (PPV)[a]
smORF_15699	SMSLHQKRVALSHFSSLVRYQRVWYQAVKKARPIVSGTNMK	0.58 (PPV)
smORF_16981	GALTSRMLKVSPPPEAMVPEEKRLPMNRLSTIQRISSVVIRK	0.57 (PPV)
smORF_19996	SSLTVGRTGITAHASASSATRITNQATVATWRLTVVKGRTPTASGGAYM	0.56 (PPV)
smORF_20891	VAVMSPWLKTSMAKVTLLWPGTSGLGAKATRFISARSKT	0.53 (PPV)
smORF_23043	AIRKREAAIPLRIPLVPYAQKVKVARIIKKE	0.51 (PPV)
smORF_23811	AGSASGRTILMRMRKGPAPSMRADSNSSFGTVRKKVVRR	0.60 (PPV)
smORF_2746	ALFATMPYGKFAHGIFRTASLLRHAVEKRQPNPIGLGAD	0.52 (PPV)
smORF_29337	NIASIGRFSRSVKTVPFQPISSIVRKGASCPAWIAVSRR	0.58 (PPV)
smORF_32546	VSTALRISLSKLLAWSRQCSSGVVRMKCTARTML	0.71 (PPV)
smORF_35178	GLTLEQLQQQTNLTRKQVVNFLGRGLPGFHKIGRAHYAYTR	0.59 (PPV)
smORF_35235	IVYWKVSRGVRASTVKLSPLATVPLRRASVRPNAPTGKKVGEA	0.68 (PPV)
smORF_36486	PRSFAASAKRPLVGFMNMFFQISAATGGMMKKGLMTRIRTGP	0.58 (PPV)
smORF_39676	RKNNSGGIPSTAVTTSIISAPCSGKKASASAPSGAAAMVRTPCIAWFIPATRVSHSGGARFG VLASAAGP	0.60 (PPV)
smORF_39687	AALIKMLTAWKIAKTSVQKWREKQISTAVLTKMKMAFLTKMMSV	0.64 (PPV)
smORF_41742	IGSALRPELGSGRACSGCCLRKSIRKVDSALVKVVLIAWLVSPTRTQLPRRAVNMRNMRS WPALESCASSSST	0.50 (PPV)
smORF_43528	AAKLEAGLLAVAQGVRRVRIGDLAALVRGDAGTTLVPHR	0.59 (PPV)
smORF_51233	RSMRAAVATRASISALGKRRFLSAKLMFSATVMCGYRA	0.65 (PPV)

smORF_52945	QLGLVGLGKMGFNMRSRLRAAGAYLGQSTVMRSKGRKAP	0.59 (PPV)
smORF_55383	ALLGGQIRVRRDKLRAGFGAKARGGVLAKGAEHVVIAGNGIEA	0.53 (PPV)
smORF_62853	AARDRADKKALAVLRKQAKLEKKIKRKAAAQVGAEVILLMQGWRAHTRF	0.53 (PPV)
smORF_64971	WLFCAAVSSTTRVTRVSTFSAETPGHGVTALATRTGISGSLRLGMVW	0.50 (PPV)
smORF_73603	QEVNRLLNQFEQTQKMMKQFGSMFKGKGGMMKMMRGAKSMFGGKLPF	0.54 (PPV)
smORF_77497	AMMGMTKSVPMRMMKRLSGAEAASAMVRLSGTMKG	0.58 (PPV)
smORF_80548	AALSGPHRVAAPPISAMSTVWKPMKGLNTVVGSM	0.54 (PPV)
smORF_82449	IAGEAAVKFSGRLYRRSRLKVLKTCDCSMNGRTIVKALW	0.58 (PPV)
smORF_9510	VSTVTTATPAWVRMGFICKWVRKSGSKVSRPAAHRRW	0.62 (PPV)

[a] positive predictive value (PPV)

Section 3

Appendix A. Metagenomics workflow

Raw shotgun sequences were trimmed to remove adapters and low-quality nucleotide stretches using Trim Galore (v0.6.5) (Krueger, 2015). MEGAHIT (v1.2.9) (Li et al., 2015) was used to assemble the clean reads into contigs. The resulting contigs were aligned against the Comprehensive Antibiotic Resistance Database (CARD) using BLAST with an e-value cutoff of 10^{-5} (McArthur et al., 2013). A read was considered as an ARG sequence if the result showed identity ≥ 80% and a bitscore ≥ 50. Mobile genetic elements (MGEs) were identified using mobileOG-db (Data Version: Beatrix 1.6 v1) (Brown et al., 2022) and were divided into five major mobileOG categories that represent the key groups of MGE-associated molecular mechanisms: replication/recombination/repair (RRR), integration/excision (IE), stability/transfer/defense (STD), Inter-Organism Transfer (T), and phage-related biological processes (P). The levels of ARG or MGEs sequences in the present study were described using the unit of "ppm" (one read in one million reads) as recommended by Yang et al. (2013), which was defined as the portion of ARG or MEGs sequences in "total metagenome sequences."

For taxonomic classification of contigs, Kaiju (v 1.9), with the NCBI nr + euk database (2022-03-10) as a reference, was used, applying the default greedy run mode (Menzel et al., 2016). For functional prediction, contigs were annotated using Prokka v.1.14.6, default settings, and mincontiglen 500 (Seemann, 2014). The amino acid sequences were annotated to the KEGG database by GhostKOALA using the genus_prokaryotes datasets (https://www.kegg.jp/ghostkoala/).

Figure S1: Methane productivity in the anaerobic digester over 115 d of operation.

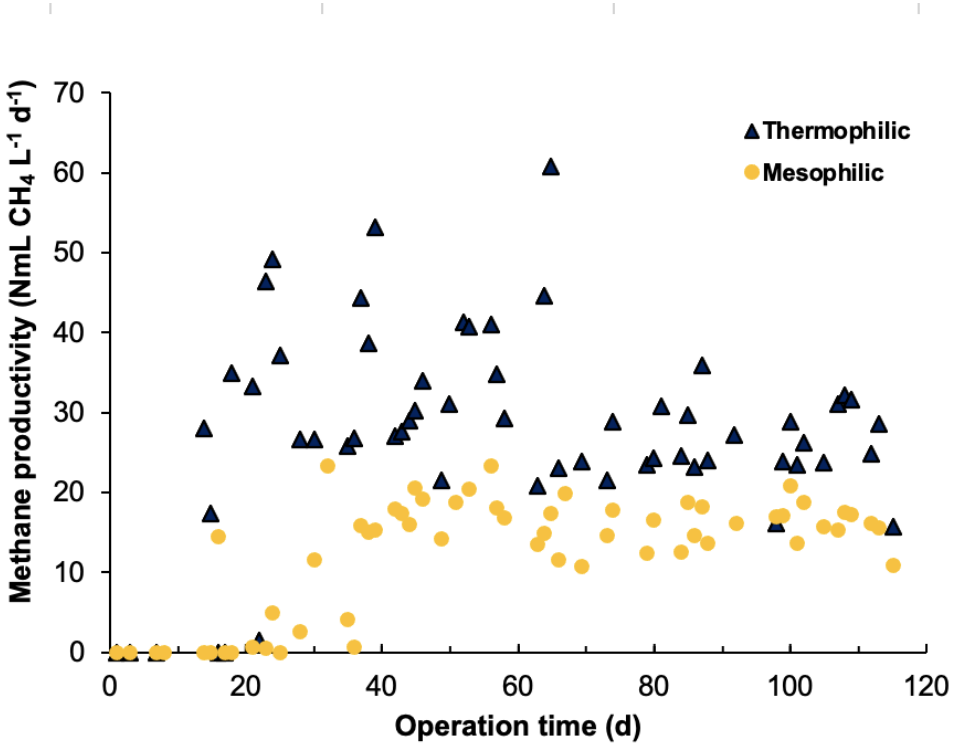

Figure S2: Venn diagram showing the number of shared and unique ARG (at the subtype level) among MABA, mesophilic and thermophilic bacteria. For each site, duplicate ARG were removed.

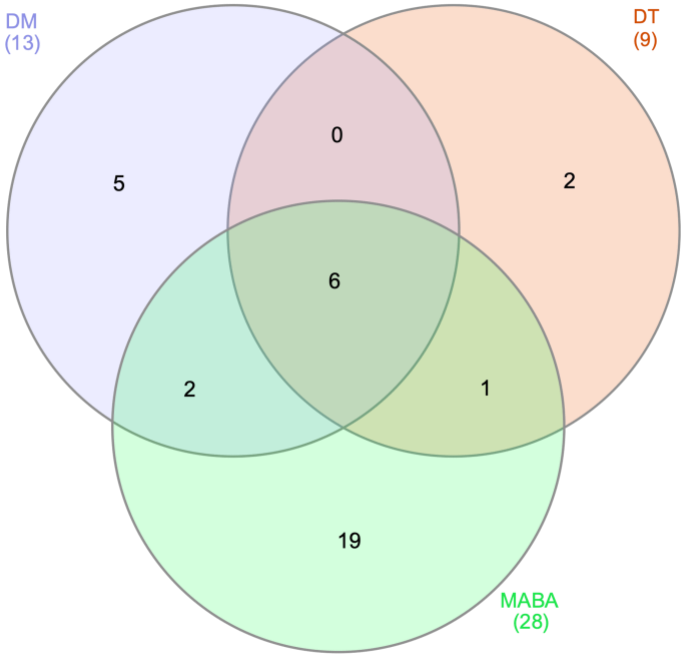

Figure S3: Principal Component Analysis (PCA) plot generated from the mobilome showing variation within and between the groups. Groups are differentiated by different colors.

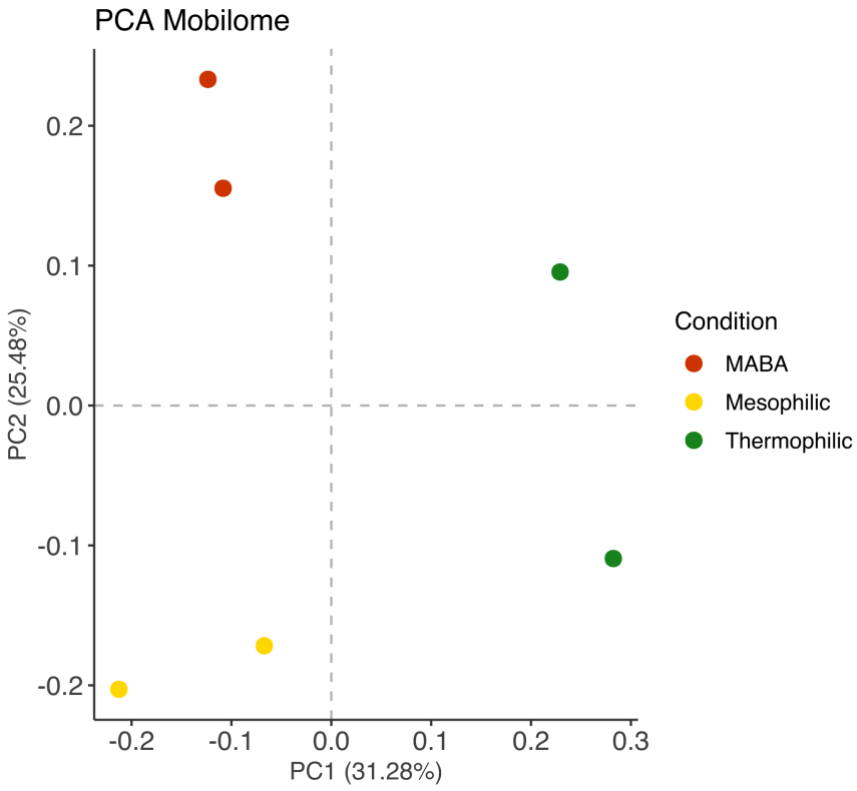

Figure S4: Relative abundance of metabolic pathways at KEGG level 3; ns (no significant difference, $p > 0.05$)

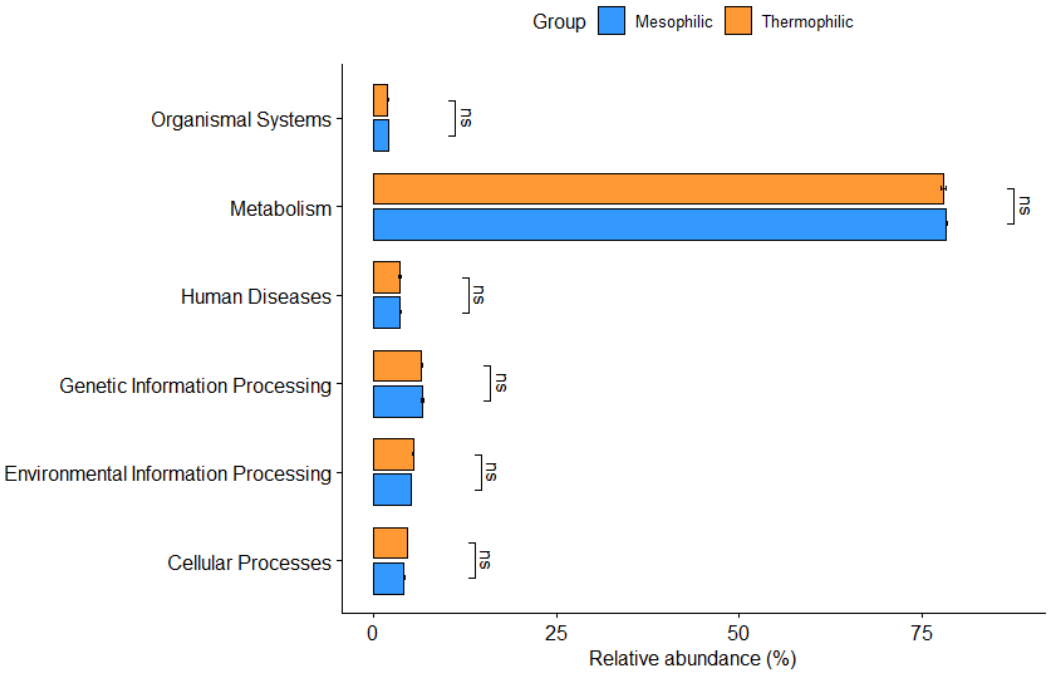

Table S1: Resistomes (ppm) in WWTPs systems coupled with anaerobic digestion for biosolid treatment.

WWTP system	Influent	Effluent	Biosolid	Digestate-Mesophilic	Digestate-Termophilic	Reference
Activated sludge	595.3	82.6	29.9	47.4	ND	Yang et al., 2014
Microalgae-bacteria	924.7	190.1	58.9[a]	36.9[a]	19.5[a]	Ovis-Sánchez et al., 2021; [a]This study
Activated sludge	NR	NR	24.1	24.4	22.9	Zhang et al., 2015

MD: Mesophilic digestate; TD: Thermophilic digestate.

Section 4

Appendix A. Metagenomics workflow

Raw shotgun sequences were trimmed to remove adapters and low-quality nucleotide stretches using Trim Galore (v0.6.5) (Krueger, 2015). MEGAHIT (v1.2.9) (Li et al., 2015) was used to assemble clean reads into contigs. The resulting contigs were aligned against the Comprehensive Antibiotic Resistance Database (CARD) using BLAST, with an e-value cutoff of 10^{-5} (McArthur et al., 2013). A read was considered as an ARG sequence if the result showed identity ≥ 80% and a bitscore ≥ 50.

Mobile genetic elements (MGEs) were identified using mobileOG-db (Data Version: Beatrix 1.6 v1) (Brown et al., 2022), and were divided into five major mobileOG categories which represent the key groups of MGE-associated molecular mechanisms: Replication/Recombination/Repair (RRR), Integration/Excision (IE), Stability/Transfer/Defense (STD), Inter-Organism Transfer (T), and Phage-related biological processes (P). A read was considered a mobilome sequence if the result showed an identity ≥ 90% and a bit score ≥ 50. The levels of ARG or MGEs sequences in the present study were described using the unit of "ppm" (one read in one million reads) as recommended by Yang et al. (2013), which was defined as the portion of ARG or MEGs sequences in "total metagenome sequences".

For taxonomic classification of contigs, Kaiju (v1.9), with the NCBI nr + euk database (2022-03-10) as a reference, was used, applying the default greedy run mode (Menzel et al., 2016). We also removed poorly identified taxa because they artificially increased the similarity between the samples. Specifically, the following taxa were excluded: “Unclassified”, “cannot be assigned to a (non-viral) genus”. OTUs with fewer than two reads in the total sample were removed.

Figure S1: Sample processing to obtain EPS fractions. Created using BioRender.com. F-EPS: Free-EPS; B-EPS: Bound-EPS.

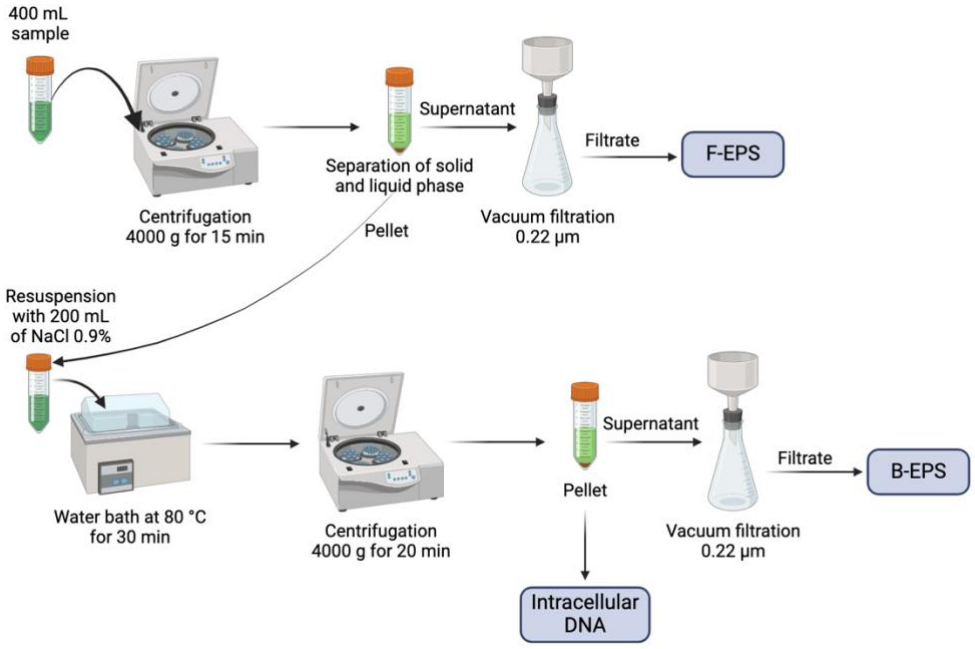

Figure S2: Diagram of extracellular DNA recovery. Created with BioRender.com.

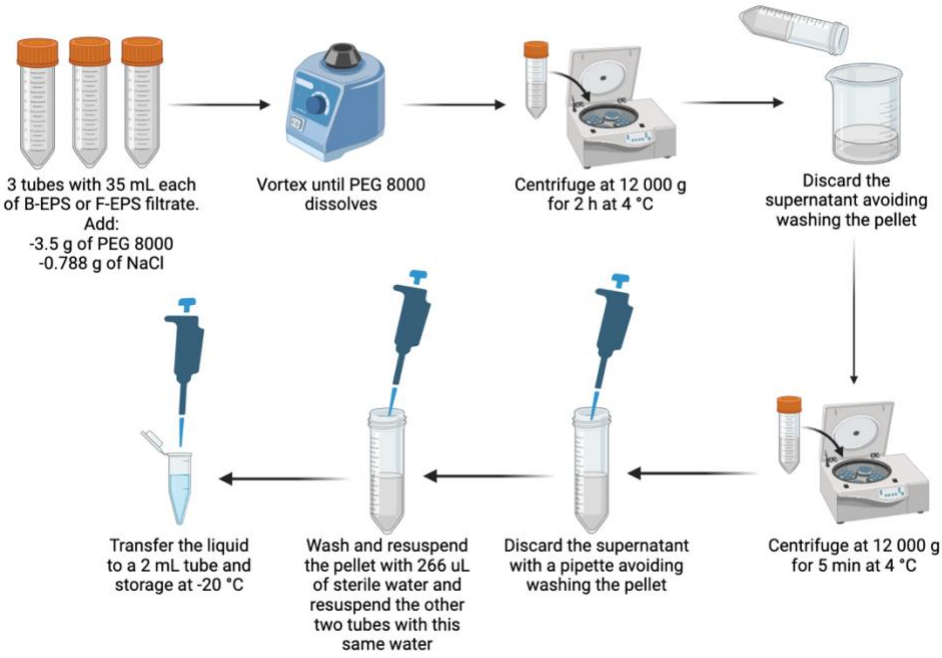

Table S1: DNA samples for Illumina sequencing.

Sample origin	Sample ID
Batch III (Reactor 1 and Reactor 2)	F-EPS-Initial
	B-EPS-Initial
	Intracellular-Initial
	F-EPS-Final
	B-EPS-Final
	Intracellular-Final
Domestic wastewater	WW-F-EPS
	WW-Intracellular
Selective plates from domestic wastewater	WW-CIP
	WW-SUL
	WW-AMP
	WW-TET
	WW-ERY
Selective plates from Batch III (Reactor 1 and Reactor 2)	TET-Initial
	CIP-Initial
	ERY-Initial
	SUL-Initial
	AMP-Initial
	TET-Final
	CIP-Final
	ERY-Final
	SUL-Final
	AMP-Final

Table S2: EPS concentrations in batch III.

EPS fraction (mg g SSV^{-1})	Batch III	
	Initial	Final
Free-Carbohydrates	72.4 ± 6.7	74.8 ± 1.4
Free-Proteins	34.4 ± 1.4	242.3 ± 6.7
Bound-Carbohydrates	60.2 ± 8.7	67.9 ± 2.1
Bound-Proteins	62.8 ± 3.9	208.2 ± 16.6

Table S3: ARB and THB Log removal in different wastewater systems.

System	Ampicillin	Ciprofloxacin	Erythromycin	Sulfamethoxazole	Tetracycline	THB	Reference
Monoalgal	4	1	1	2.5	1	1	(Cheng et al., 2020)
Activated sludge	ND	ND	ND	~2.5	~2.5	~2	(Gao et al., 2012)
Activated sludge	ND	ND	ND	~2	~2	1.3	(Proia et al., 2018)
Microalgae-bacteria	3.4	1.4	2.5	2.4	2.6	0.8	This study

ND: Not determinated.

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