WASTEWATER COMPOSITION IMPACTS THE MICROBIAL COMMUNITY ASSEMBLY, INFLUENT IMMIGRATION AND ANTIMICROBIAL RESISTANCE IN ACTIVATED SLUDGE SYSTEMS.

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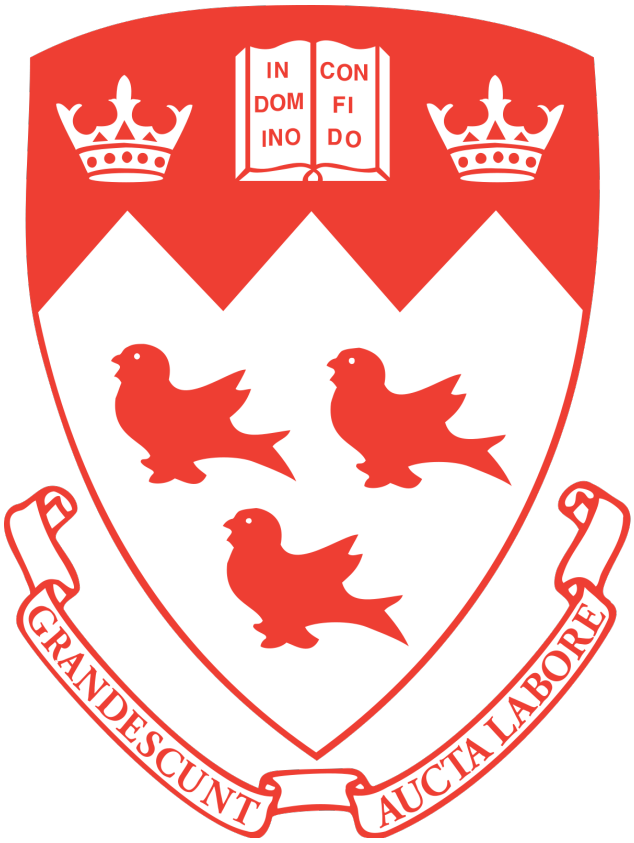

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ABSTRACT

Wastewater Treatment Plants (WWTPs) are believed to be a propagator of Antimicrobial Resistance Genes (ARGs) in the environment. However, the complex dynamics impacting ARG persistence in activated sludge are still not well understood. The present study investigated variations in microbial community compositions and the complement of ARGs they carried using bench-scale activated sludge systems receiving synthetic wastewater of two different compositions. The first synthetic wastewater composition was more readily biodegradable and rich in sugars whilst the other was slowly biodegradable and rich in proteins and lipids. These systems were supplied with influent solids from the sewers to simulate immigration from upstream sewer systems. Amplicon sequencing of the 16S rRNA gene revealed that the microbial communities in reactors receiving the slowly degradable synthetic wastewater was more diverse and exhibited higher rate of immigration than the community in reactors receiving the readily degradable wastewater. The nitrate production was also found to be influenced by wastewater composition with the slowly biodegradable composition promoting the growth of *Nitrosospira*, a low rate ammonia oxidizing bacterial genus. A qPCR approach to quantify ARGs found that, out of the 81 genes evaluated, 17 genes had a significantly higher concentration in the slowly biodegradable composition, whilst the readily biodegradable composition showed a significantly higher concentration in 9 genes. Immigration significantly increased the concentrations of 8 genes and decreased the concentration of 4 genes. Analysis of ARG sequence variants revealed evidence of direct immigration in only one gene (betOMPK). The remaining targeted genes were likely impacted by an ecological shift in the community composition. The data from the sequence variant analysis for the remaining genes were, unfortunately, inconclusive. Taken together, this study suggests that a more diverse community growing on slowly degradable substrates is more receptive to continuous immigration than a less diverse community growing on readily degradable substrates. Furthermore, the chemical wastewater composition and immigration influence the prevalence of ARGs carried by activated sludge communities. The diversity of the microbial community in activated sludge positively correlates with the diversity of ARGs.

RÉSUMÉ

Les stations de récupération de ressources de l'eau sont considérées comme des propagateurs de gènes de résistance aux antimicrobiens dans l'environnement. Cependant, la dynamique complexe qui influence la persistance des gènes de résistance dans les boues activées n'est pas encore bien comprise. La présente étude a examiné les variations dans la composition des communautés microbiennes et le contenu de gènes de résistance qu'elles portent en utilisant des systèmes de boues activées à petite échelle recevant des eaux usées synthétiques de deux compositions différentes. L'une des compositions était plus facilement biodégradable et riche en sucres, tandis que l'autre était lentement biodégradable et riche en protéines et en lipides. Ces systèmes ont été alimentés en matières solides provenant des égouts afin de simuler l'immigration en provenance des réseaux d'égouts en amont. Le séquençage d'amplicon de l'ARNr 16S a révélé que les communautés microbiennes des réacteurs recevant les eaux usées synthétiques lentement dégradables étaient plus diversifiées et présentaient un taux d'immigration plus élevé que celles des réacteurs recevant les eaux usées facilement dégradables. La production de nitrate a été influencée par la composition avec la composition lentement biodégradable favorisant la croissance de *Nitrosospira*, un genre bactérien à faible taux d'oxydation de l'ammoniac. Une approche qPCR visant à quantifier les gènes de résistance a permis de constater que, sur les 81 gènes évalués, 17 présentaient une concentration significativement plus élevée dans la composition lentement biodégradable, opposés aux 9 gènes de la composition facilement biodégradable. L'immigration a significativement augmenté les concentrations de 8 gènes et diminué la concentration de 4 gènes. L'analyse des variants de séquence des gènes de résistance a révélé des preuves d'immigration directe dans un seul gène (betOMPK). Les autres gènes ciblés ont probablement été affectés par un changement écologique dans la composition de la communauté microbienne. Les données de l'analyse des variantes pour les autres gènes n'étaient malheureusement pas concluantes. Dans l'ensemble, cette étude suggère qu'une communauté plus diversifiée se développant sur des substrats lentement dégradables est plus réceptive à une immigration continue qu'une communauté moins diversifiée se développant sur des substrats facilement dégradables. En outre, la composition chimique des eaux usées et l'immigration influencent la prévalence des gènes des résistance portés par les communautés de boues activées. Il semblerait que la diversité de la communauté microbienne dans les boues activées soit en corrélation positive avec la diversité des gènes de résistance.

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CONTRIBUTION OF AUTHORS

Emmanuel Díaz Mendoza created the experimental design, operated the reactors, prepared samples for sequencing, analysed the data and prepared the manuscript.

Claire Gibson helped with the experimental design, and revised the manuscript.

Susanne A. Kraemer developed the bioinformatics pipeline for analysis of amplicon sequencing data.

Simon Barnabé revised the manuscript.

Dominic Frigon obtained funding, supervised the research and revised the manuscript.

TABLE OF CONTENTS

Chapter 1. Introduction

Antimicrobial therapy is the main course of action for the treatment of infectious diseases and saves millions of lives every year across the world (Holmstrup and Klausen, 2018). However, antimicrobial resistance (AMR) has reduced our ability to treat infections and even rendered diseases to be untreatable. Thus, the worldwide rise of antimicrobial resistance has become a major health problem, causing 1.27 million deaths in 2019 alone (Murray et al., 2022). Among the causes that could contribute to the development of AMR is the indiscriminate use of antimicrobials in humans and livestock, as well as poor hygiene conditions and practices (Rehman et al., 2020). The World Health Organization (2015) has recognized this threat and created a global action plan to counter AMR at the World Health Assembly in May 2015, and several actions addressing the issue have been conducted since then.

A search of the phrase "Antimicrobial Resistance" on the scientific database ScienceDirect in January 2021 showed that this topic was not widely addressed until the early 2000s (Figure 1.1). For example, articles from the 1940's consider the intrinsic resistance of different bacterial species to penicillin. In fact, although a great deal of antimicrobials had been discovered and some introduced as clinical treatments by 1950 (Pazda et al., 2019), AMR was not considered to be a significant issue as it was believed that microorganisms could not evolve rapidly enough to develop defense mechanisms to respond to the chemical attack from antimicrobials (Hoek et al., 2011). The situation remained the same until the end of the 1970´s, and AMR gained recognition in the literature between 1980 and 2000. At the turn of the millennium, AMR was recognized as a developing problem (World Health Organization, 2001), and from the 2000s onwards the number of articles addressing it exponentially increased (Figure 1.1). However, most published articles still focus on medical issues, including dentistry, immunology, and microbiology. Conversely, the environmental dimension of antimicrobial resistance (which includes wastewater) accounted for only 3 % of the 16 thousand articles classified under "Antimicrobial Resistance" for the year 2020. To combat the spread of AMR, it is critical that we prioritize research efforts to address the role of the environment in its dissemination.

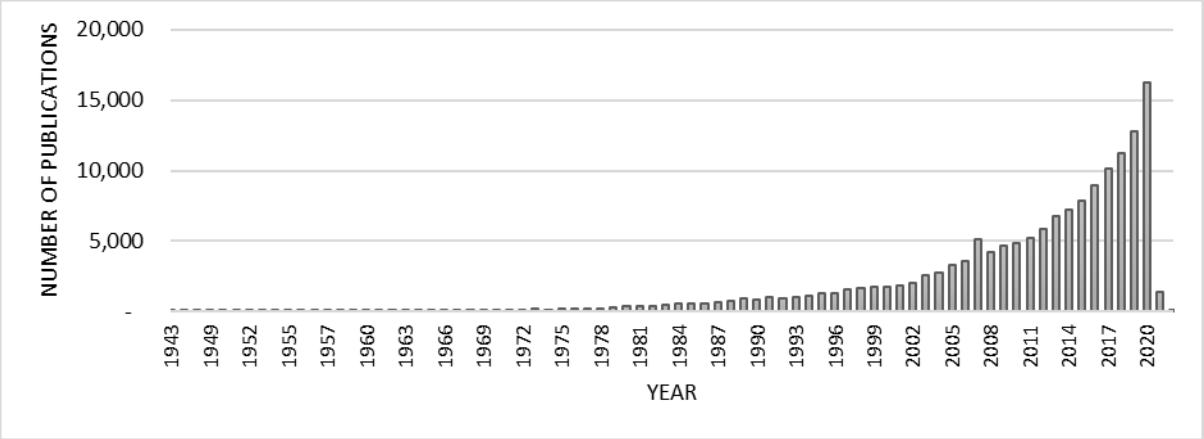

Figure 1.1. Number of publications related to "Antimicrobial Resistance" produced from data gathered from the Database ScienceDirect. Search made in January 2021.

To tackle the growing problem of AMR in WWTPs, a deep understanding of factors impacting the persistence and spread of ARGs is required. Nonetheless, the dynamics of ARGs in wastewater are still not well understood and there are many confounding variables that can affect their abundance in activated sludge. One of such dynamics is the relationship between the resistome's and the microbial community's composition. More research of activated sludge under highly controlled and reproducible conditions is needed in this regard. Additionally, different factors that affect the microbial community should also be considered. Indeed, stochastic factors such as sewer immigration and deterministic factors such as wastewater composition likely impact AMR as they impact the activated sludge's microbial community.

1.1 Problem Statement and Objectives

To study the environmental fate of Antimicrobial Resistance Genes (ARGs) in activated sludge, laboratory-scale wastewater treatment reactors were utilized with established wastewater compositions. In line with the literature review presented below, the main objective of the present study is to investigate the ARG dynamics in wastewater and the role of organic matter composition.

The three specific objectives of the study are as follow:

1. To study the impact of wastewater composition on immigration dynamics between the influent and activated sludge.
2. To determine the impact of synthetic wastewater biodegradability and composition on the concentration of ARGs in the activated sludge resistome.

3. To investigate the interaction between wastewater composition and sewer composition and their impact on the activated sludge microbiome and resistome composition.

1.2 Thesis organization

To address the problem statement, laboratory-scale wastewater treatment bioreactors (a total of 18) receiving two different synthetic wastewater compositions were operated for 105 days. During one of the phases of operation, real wastewater influent solids were added to a subset of the reactors to study the effect of immigration. The composition of microbial communities were determined by amplicon sequencing of the 16S rRNA genes, 81 ARGs and related genetic elements were quantified by qPCR array, and the specific dynamics of certain ARGs were investigated by amplicon sequencing.

This thesis is organized as follows. After the current introduction chapter, Chapter 2 presents an extensive literature review on ARGs, wastewater composition and the microbial community in wastewater treatment plants. Chapter 3 is a manuscript in preparation intended for submission to the journal "Water Research". Chapter 4 presents general conclusions, recommendations, and future work.

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Chapter 2. Literature Review

The following literature review is organized into two parts, with each identifying an important knowledge gap. The first part is divided into six relevant sections. The first section discusses the basic concepts of Antimicrobial Resistance (AMR) and Antimicrobial Resistance genes (ARGs). The following section considers the approaches most frequently used to study AMR. Subsequently, in the third section the role of wastewater treatment plants (WWTPs) in the spread of AMR is explored. The fourth section discusses the approaches that have been taken to manage AMR in WWTPs. The fifth section presents studies that have attempted to relate the microbial community to the presence and prevalence of ARGs. Finally, the sixth section examines factors affecting AMR and the microbial community in activated sludge and the first of two knowledge gaps is presented which relates to AMR dynamics in activated sludge.

The second half of Chapter 2 is divided into two sections. In the first section, the composition of wastewater and its intrinsic variability is discussed. The second section reviews the approaches taken to design a synthetic composition that is representative of real wastewater. Finally, the second knowledge gap is presented, which relates to the development of a new synthetic wastewater composition.

2.1 Basic Principles of AMR

The Word Health Organization (2021) defines Antimicrobial Resistance (AMR) as the change over time of bacteria, viruses, fungi and parasites to resist antimicrobial medicines, increasing the likelihood of disease transmission, severe infections, and higher fatalities. On this basis, Antimicrobial Resistance Genes (ARGs) can be defined as regions of DNA that encode a particular mechanism to counteract the effect of antimicrobials. However, for ARGs to be a health concern, they must conform with two considerations: they must occur in pathogens, or they must be mobile (have the capacity to be transferred to a pathogen) (Gibson et al., 2023c).

In general, as with other genetic traits, AMR is passed on from progenitor to offspring by reproduction or vertical gene transfer (VGT) as it is commonly known. However, DNA exchange via mobile genetic elements (MGEs) also occurs in a process called horizontal gene transfer (HGT). HGT has been well documented in the literature (Fuchsman et al., 2017; Hutinel et al., 2021; von Wintersdorff et al., 2016) and although it is generally accepted to occur in *Bacteria* and *Archaea*, it is also common among some eukaryotic organisms (Soucy et al., 2015). HGT is critical

in the dissemination of AMR, enabling the transfer of ARGs between non-pathogenic and pathogenic bacteria (Emamalipour et al., 2020; Kado, 2009) and the rapid adaptation of susceptible bacteria in the presence of antimicrobials (Williams, 2022). In diverse ecosystems, the potential for HGT is particularly important as the pool of genes readily available to microorganisms — considered to be the *supergenome* by Norman et al. (2009)— is extensive. WWTPs represent a particularly high potential for AMR development and dissemination because of the size of the gene pool (Mao et al., 2008; Moura et al., 2010), close contact between bacteria and the potential for the acquisition of new genes via HGT (Alessia et al., 2020; Alexander et al., 2022). For antimicrobial resistance genes to be exchanged through HGT, they must be associated with a Mobile Genetic Element (MGE) such as Insertion Sequences (IS), Transposons (Tn), plasmids, or Integrons (In). Insertion sequences and transposons are capable of moving to new locations in the same or different DNA molecules of a cell (Partridge et al., 2018) while plasmids are extrachromosomal DNA that contain useful additional genes that can be passed on to other cells (Esser, 1986). Integrons, on the other hand, move DNA fragments between different sites within a genome (Partridge et al., 2018).

There are four main mechanisms by which bacteria resist the action of antimicrobials. Firstly, bacteria with decreased membrane permeability due to modification or low expression of porins exhibit resistance to hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones (Pagès et al., 2008). Secondly, efflux pumps cause resistance to numerous antimicrobial classes (Soto, 2013) by actively expelling the antimicrobial molecule out of the cytoplasm (McMurry et al., 1980). Thirdly, bacteria may enzymatically modify or inactivate (degrade) antimicrobials. Reactions such as acetylation, phosphorylation, and adenylation decrease the affinity of the antimicrobial for its target (Wilson, 2014). Inactivation has been extensively studied in β-lactam antimicrobials. Through the enzyme β-lactamase, the amide bond is broken down, nullifying the antimicrobial molecule's effect (Abraham and Chain, 1988). Finally, the fourth resistance mechanism observed is the protection or modification of the antimicrobial target. Protection of the antimicrobial target often occurs by competition of proteins with the antimicrobial for the binding site (Li et al., 2013; Rodríguez-Martínez et al., 2011) or the overproduction of the target enzyme (Groot et al., 1988). Site modifications include mutations of the target site (Floss and Yu, 2005; Hooper, 2002), the enzymatic alteration of the target site

(Leclercq, 2002) or the development of alternative sites with similar biochemical functions but not inhibited by the antimicrobial (Peacock and Paterson, 2015).

2.2 Approaches to studying AMR in the environment

To study AMR, two types of techniques are commonly used: culture-based methods which rely on the isolation and growth of a particular organism or community, and molecular-based, which study microorganisms' genetic material. Culture-based techniques follow well established, standardized cultivation methods that ensure accuracy and prevent bias due to differences in the techniques (Szczepanowski et al., 2009; Wiegand et al., 2008). Culture-based techniques also allow the resistance phenotype to be assessed, which is critical to assess the minimum inhibitory concentration and understand the antimicrobial resistance spectrum of different taxa (Garcha et al., 2016; Li et al., 2009). In some cases, modifications to standard methods can improve the detection of specific organisms by providing conditions more similar to the sampled setting, for example in water and wastewater samples (Watkinson et al., 2007). However, one of the main limitations of these techniques is the fact that very few organisms are culturable. In fact, it is estimated that only 2 % of environmental bacteria can be cultured (Wilson et al., 1997).

Molecular-based methods, on the other hand, pose a great advantage for microorganisms that cannot be cultured or grow very slowly (Trevors, 2011). Two commonly used molecular-based methods for the detection of AMR in environmental samples are PCR (Lawung et al., 2009; Sianglum et al., 2009; Trevors, 2011) and shotgun metagenomic sequencing (Manoharan et al., 2021; Perez-Marron et al., 2022; Zhou et al., 2022). Other commercially available technologies are also used. Single-cell microfluidics with time-lapse imaging is a technology recently introduced to the market that can detect the transfer of genes both vertically and horizontally (Li et al., 2019b). Shotgun metagenomics approaches allow the detection of a large number of genes at once and gives information about sequence variants, however the detection limit is high and often a limiting factor. In comparison, polymerase chain reaction (PCR) based techniques have a low detection limit and can be high throughput, but specific gene targets must be selected for analysis. In addition, PCR based approaches provide no information on ARG sequence variants, however, multiplexed amplicon sequencing could be used alongside this technique to account for this shortcoming.

2.3 The role of WWTPs in AMR dissemination

The role of the environment in the spread of AMR has long been neglected. Numerous studies show ARGs to occur in surface waters receiving effluent wastewater (Bueno et al., 2020; Freeman et al., 2018; Koczura et al., 2012). Many studies aimed to compare ARGs or ARB from the effluent of WWTPs and their respective receiving water body, for example in Lake Xochimilco in Mexico (Rosas et al., 2015), Jinchuan and Yangtze Rivers in China (Chen et al., 2020), or Wascana Creek in Canada (Freeman et al., 2018). When the abundance of ARGs was compared between downstream and upstream of a river, some studies show a higher abundance downstream indicating the WWTP's effluent has an impact on the receiving water body (Bueno et al., 2020; Freeman et al., 2018; Koczura et al., 2012). For example, using molecular approaches, Bueno et al. (2020) analyzed the role of the wastewater treatment plant near a Chilean River on the spreading of ARGs. Out of the 24 ARGs tested, 17 showed a significant increase in the relative abundance downstream from the WWTP discharge. The spread of ARGs is also worsened by combined sewers overflow events, where raw wastewater is discharged in the receiving waters when the plant's capacity is exceeded (Eramo et al., 2017). Conversely, others suggest WWTP discharge to have no significant influence on receiving waters (Chen et al., 2020; Karkman et al., 2016). For example, Karkman et al. (2016) suggest that the influence of effluent wastewater discharge is not substantial to cause big environmental problems related to AMR. Although it is universally accepted that effluent wastewater contains high abundances of ARGs, the relative impact of their discharge on receiving waters varies.

Analyzing the waste biosolids produced during the wastewater treatment process, Karkman et al. (2016) found, contrary to expectation, that the ARG abundance in the biosolids was lower than in the raw influent and final effluent, which suggests that the risk of AMR dissemination into the environment by WWTPs would be more significant through the discharge of treated or untreated effluent than by land fertilization by biosolids. Karkman et al. (2016)'s results seem, however, to be in conflict with other studies where it was found that most of the ARGs' abundance and diversity remain in the biosolids after wastewater treatment with differences of up to seven orders of magnitude compared to the effluent (Mays et al., 2021; Munir et al., 2011; Zhang et al., 2009a; Zhang et al., 2009b). This suggests that the spread of AMR through fertilized soils would be more critical than that of the receiving waters. This is supported by studies which showed no correlation between the presence of a WWTP and AMR in the surrounding waters (Chen et al., 2020; Karkman

et al., 2016) and also those that did find significant influence but no strong correlation could be claimed (Bueno et al., 2020; Freeman et al., 2018; Koczura et al., 2012). Taken together, these results demonstrate that studying AMR in sludge rather than effluent wastewater may be more valuable, and critical to understanding the spread of AMR.

2.4 Approaches to managing AMR in WWTPs

Wastewater treatment processes do not achieve complete removal of ARB and ARGs before the discharge of effluent wastewater and waste biosolids into the environment (Freeman et al., 2018). Kucukunsal and Icgen (2020) compared different wastewater treatment processes and their seasonal patterns on the removal of seven common ARGs. The technologies analyzed were Conventional Activated Sludge (CAS), Biological Nutrient Removal (BNR), Sequential Batch Reactor (SBR), Membrane Bioreactor (MBR), and coagulation-flocculation/UV-disinfection (CF-UV). The absolute abundance of ARGs were reduced between three and five orders of magnitude with MBR providing the highest reduction. As for the seasonal influence, summer temperatures showed higher ARG removal efficiencies in all cases. This result is consistent with those of Yuan et al. (2014), who observed that the removal of ARB and ARGs increases with increasing Chemical Oxygen Demand (COD) load and decreases with decreasing temperature. Li et al. (2019a) compared the ARG removal efficiencies among four MBR WWTPs. Findings showed ARGs to persist in the effluent of all the assessed systems. In another study, Karkman et al. (2016) carried out a quantitative analysis of transposase and ARGs in a WWTP in Helsinki, Finland. Of the genes detected in the influent wastewater, 57.2 % were detected in the effluent wastewater after treatment. These results demonstrate the urgent need for process optimization to reduce the spread of AMR.

In an effort to minimize the release of ARGs, numerous studies have aimed to optimize the disinfection step of the wastewater treatment process. The primary goal of the disinfection process is to inactivate any remaining pathogen that may still be present in the water after the biological treatment. Oxidation processes are commercially used, and recently advanced oxidation processes (AOPs) have increasingly gained traction (Ike et al., 2019). Zhou et al. (2020) tested UV-activated persulfate to remove ARB and ARGs in the secondary effluent and used qPCR to evaluate the removal efficiency. Three frequent oxidants were tested: sodium hypochlorite (NaClO), 1,3-dibromo-5,5-dimethylhydantoin (DBDMH), and $KMnO_4$. The sulfonamide resistance gene *sul*1

was chosen as a marker of the presence of ARGs in the qPCR tests. While chlorine and DBDMH showed a *sul1* removal efficiency of 99 % in some cases, $KMnO_4$ proved to be inefficient.

Others focused on the use of primary treatments for the removal of ARGs. For instance, Li et al. (2017) studied the removal efficiency of ARGs using chemical coagulation. With 99 % removal of ARGs, the method has potential in a WWTP. However, the removal of ARGs by coagulation only removes them from the effluent but not from the sludge. In this context, studies that look at and analyze WWTPs as a set of different processes may be more revealing. With the latter in mind, Yuan et al. (2019b) assessed the fate and removal efficiency of ARGs for mercury, silver, tetracyclines, and sulfonamides in two systems with tertiary treatments. The systems analyzed were a biological-treatment/coagulation/sand-filtration/chlorine-disinfection system and a biological-treatment/sand-filtration/constructed-wetlands system. The ARG abundances in the effluent were 2 to 4 orders of magnitude lower than the raw influent depending on the gene. However, high abundances of ARGs remained in the sludge. These results highlight the need to study AMR and ARGs in different compartments of WWTPs, as the dynamics of ARGs cannot be unveiled by analyzing the influent and effluent alone.

The studies presented in this section highlight the need to study AMR in all compartments of WWTPs. In addition, the results in terms of removal efficiency varied greatly based upon environmental conditions, WWTP operation and ARG class. This could be related to the fact that the ARG dynamics and factors impacting their persistence are still not well understood. This emphasizes the need for studies focusing on factors impacting the prevalence of ARGs in WWTPs.

2.5 The relationship between Microbiome and Resistome

The microbial community composition has been shown to be a key determinant of ARG content in the environment in several studies. Correlations were observed between the ARGs present and the phyla *Firmicutes* and *Actinobacteria* in a landfill (Wu et al., 2017), and the phyla *Proteobacteria*, *Actinomycetes* and *Cyanobacteria* in sea samples (water and sediment) (He et al., 2022). In WWTPs, sulfonamide, tetracycline, mercury, and silver ARGs have been shown to be carried primarily by the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Parcubacteria*, and *Euryarchaeota (Yuan et al., 2019b).* This correlation between taxa and ARGs is also supported by *Zhang et al. (2019)*'s findings, which show that the fate of ARGs is strongly correlated with the fate of their host. This is of particular importance in biological WWTPs since microorganisms are the basis for pollutant

removal. Control over the microbial community composition may be critical in managing the spread of AMR.

2.6 Factors impacting the microbial community and AMR in activated sludge

Microorganisms play a crucial role in diverse ecosystems by driving biochemical processes, and wastewater is no exception. Although the microbial community that develops in these systems is unique depending on different environmental factors, similar ecosystems will roughly share similar microbial structures (Grady et al., 2011). In this sense, studies that look at both similarities and singularities among microbial communities in activated sludge are essential to comprehend the microbial dynamics and AMR in WWTPs.

The factors affecting microbial community assembly in activated sludge can be deterministic or stochastic. Factors deterministically affecting activated sludge systems will produce similar communities in different experimental units with the same factors. These factors include nitrogen content (Liu et al., 2010; Wittebolle et al., 2009), COD (Han et al., 2010; Liu et al., 2010), temperature (Alawi et al., 2009), SRT (Kim et al., 2011; Yuan et al., 2019a), hydraulic retention time (HRT) (Han et al., 2010), pH and dissolved oxygen (Zheng et al., 2011) which are discussed in greater detail in the subsequent sections.

2.6.1 Operational conditions

WWTP operational conditions such as temperature, solids retention time (SRT) and pH have a significant impact on the microbial community composition within the activated sludge. To gain a better understanding of the microbial community composition in activated sludge systems globally, Wu et al. (2019) conducted an extensive study surveying 269 WWTPs in 86 cities, 23 countries, and around the five continents. The microbial community was greatly influenced by absolute latitude, mean annual temperature, SRT, and influent COD and BOD concentrations. Strong correlations were observed which allowed the authors to predict temperature and SRT with an explained variance of 69 % and 25 %, respectively, using random forest analysis with species abundance as input. Similarly, Alawi et al. (2009) showed temperatures to affect specific populations such as the nitrifying bacteria. SRT is a critical operational parameter as it controls the growth rate of bacteria (Grady et al., 2011) and consequently community assembly in WWTPs. For example, nitrifying bacteria need a relatively high SRT as they are slow growers and consequently, at low SRTs, they will wash out from activated sludge (Kim et al., 2011). WWTP

microbial communities are highly complex with around 10^9 species (Wu et al., 2019) impacted by multiple parameters at any one time. To better understand the assembly of this complex community, highly controlled experiments are required.

Operational parameters such as SRT also influence AMR in WWTPs (Neyestani et al., 2017; Zhang et al., 2019). Considering the relationship between the microbial community composition and AMR previously discussed, this is not surprising. Neyestani et al. (2017) identified an increased presence of ARB in cultures inoculated with samples from activated sludge operated with a long SRT (20 days). These trends have also been observed under anaerobic conditions in samples obtained from anaerobic digesters (Zhang et al., 2019). WWTP operational factors appear to have a significant impact on ARGs and AMR, however many studies fail to report on these factors, especially when full-scale WWTPs are concerned. This is a major limitation as a better understanding of these conditions could help contextualize the outcome of each study.

2.6.2 Wastewater composition

Alongside operational parameters, Wu et al. (2019) also showed influent COD and BOD concentrations to have a significant impact on the microbial community composition in WWTPs. Microbial diversity in sludge communities has been shown to be linked to COD level in the influent with a high COD yielding a higher diversity (Han et al., 2010). Similarly, Yang et al. (2020) analyzed the bacterial community of three Chinese WWTPs and found that different pollutant loads lead to differences in the microbial community of an activated sludge systems. Higher pollutant loads appeared to yield more diverse communities, measured with Shannon´s diversity index. *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria* were the most abundant phyla in all three WWTPs in decreasing order of abundance. In general, *Proteobacteria* is present in numerous environments from soil to water bodies or the atmosphere and plays a significant role in organic and nutrient removal (Shin et al., 2015). *Bacteroidetes,* on the other hand*,* are present in human gut microbiota, soil and aquatic environments with specific organic matter degradation capabilities (Bolam and Koropatkin, 2012; Thomas et al., 2011). Finally, *Acidobacteria* has also been related to the removal of nitrogen and different sugars (Kielak et al., 2016). Another important finding from Yang et al. (2020)'s study is that the highest abundances of genes are from functions such as the metabolism of amino acids, carbohydrates, lipids, cofactors, vitamins, xenobiotic compounds, and nucleotides. Numberger et al. (2019) found similar results with the most abundant phyla being

Proteobacteria, *Firmicutes*, and *Bacteroidetes* in a WWTP with activated sludge technology in Germany. Similarly, Ma et al. (2018) found that pollutant loads, and the ecosystem determine the microbial community. The study was on a pilot-scale constructed wetland and the three most abundant phyla in this case were *Proteobacteria*, *Firmicutes*, and *Cyanobacteria*. The ratio COD/N is also one key factor in the community and process efficiency. A high COD/N ratio appears to favor heterotrophs and inhibit nitrifiers (Liu et al., 2010).

In the studies previously discussed, organic matter was measured as the lump parameter COD, and the organic matter composition was not considered. This is a significant drawback as the organic matter composition can alter the microbial community of aquatic environments and particularly of wastewater (Atashgahi et al., 2015; Layer et al., 2019; Wang et al., 2021; Yu et al., 2020) due to its high variability (Grady et al., 2011) and high diversity of pollutants (Huang et al., 2010). This is also problematic due to the intrinsic variability in wastewater composition in full-scale WWTP which is likely neglected when considering COD level alone.

Influent COD also significantly impacts AMR in WWTPs (Yuan et al., 2016; Yuan et al., 2014). Yuan et al. (2014)'s findings show a direct relationship between the COD and ARGs in the influent and an inverse relation between COD and ARGs in the effluent. A follow-up study by the same research group showed equivalent results in terms of COD, ammonia, and turbidity (Yuan et al., 2016). Nevertheless, the analysis of the influent and effluent is not representative of ARG dynamics in the mixed liquor. Different wastewater sources may also impact AMR within WWTPs. For example, using a culture-based approach, Savin et al. (2020) assessed the presence of highly infectious bacteria, ARB, antimicrobials, and ARG traces in wastewater from two slaughterhouses, and downstream municipal WWTPs. They found that bacteria showed a higher rate of resistance to ureidopenicillins, third generation cephalosporins, and fluoroquinolones in wastewater from the municipal WWTP than water from the slaughterhouses. Similarly, Koczura et al. (2012) analyzed samples from a WWTP that receives hospital wastewater and the respective effluent-receiving river and compared them with cultures from patients of the hospital. Their results showed that 99 % of all the samples contained strains of antimicrobial-resistant *Escherichia coli*. These results further highlight the importance of wastewater composition and source on AMR in WWTPs.

2.6.3 Influent microbial community

The main stochastic influence in WWTPs is attributed to microbial immigration from the sewers, however the relative importance of this process has been long debated. Studies have shown that although microbial community from the sewers is highly variable over short periods (Guo et al., 2019), the microbial community of a well-established WWTP is relative stable, resisting selective pressure and mass-immigration from the sewers (Yu et al., 2021). This suggested that the influent microbial community had a negligible impact on the WWTP community over time. Conversely, others show immigration to have an impact on the assembly of developing communities and on small fluctuations in abundance of some well-established species (Dottorini et al., 2021). In fact, Dottorini et al. (2021)'s results showed that even though 72.5 $\pm$ 6.6 % cumulative abundance of all species from the sewers disappeared in the WWTP and that the most abundant species (61.2 $\pm$ 9.3 % abundance) in the WWTPs only represents 2.1 $\pm$ 1.1 % in the sewers. These highly abundant species in the WWTP can be predicted using species abundances in the sewers influent. This way, the sewers were proven as a constant supplier of different microbial species that would either grow, die, or survive in the WWTP. Furthermore, under highly controlled conditions, Gibson et al. (2023a) showed immigrating populations to contribute up to 25 % of sequencing reads in the activated sludge. Taken together, these results suggest that influent immigration plays a significant role in the microbial community assembly of the activated sludge.

Stochastic processes such as immigration can be influenced by deterministic factors (Yuan et al., 2019a). Peces et al. (2022) showed that there was a correlation between the seasonal abundance of certain sewer microbial species and their survival in activated sludge: while most species that were abundant in sewers in the fall would survive, most species that were abundant in the sewers in the winter would not. They also found that temperature was one of the most determining factors driving community changes. This was attributed to the influence of temperature over growth, decay and degradation rates, biochemical transformations, or liquid-gas solubility among other factors. Finally, Peces et al. (2022) attributed negligible impact to COD, ammonia or phosphate concentrations in the influent to community fluctuations. Griffin and Wells (2017), on the other hand, agree with the central role of temperature in community fluctuations but relegates it to a second plane in favor of the main driving force that they claim is the ecological niche. These

studies highlight the complexity of studying microbial community assembly processes in full scale WWTPs, where multiple factors likely impact immigration dynamics at any one time.

AMR has also been shown to be impacted by influent immigration in activated sludge. Firstly, sewers contain a high abundance of ARB and ARGs, which likely impact the resistome in the activated sludge (Gibson et al., 2023c; Munk et al., 2022). Secondly, under highly controlled conditions, Gibson et al. (2023b) showed immigration to cause a significant increase in the abundance and diversity of ARGs in the activated sludge. However, this study did not consider the impact of other operational parameters such as COD or organic matter content on the immigration dynamics. Given the complex relationship between stochastic and deterministic factors, future studies should aim to investigate factors impacting immigration dynamics and AMR in the activated sludge

2.7 First Knowledge gap: wastewater composition, the microbial community and AMR

To tackle the growing problem of AMR in WWTPs, a deep understanding of factors impacting the persistence and spread of ARGs is required. Nonetheless, the dynamics of ARGs in wastewater are still not well understood and there are many confounding variables that can affect their abundance in WWTPs. One of the main shortcomings of the studies presented in the previous sections is that most do not contextualize on the WWTPs' operational parameters, which are crucial in the activated sludge process (Iacopozzi et al., 2007). Critical parameters such as the chemical oxygen demand, nutrient content, and the solids and hydraulic retention times could help better interpret these results and allow comparisons between studies. Furthermore, most studies are performed in full-scale WWTPs, where the parameters mentioned above are not completely controlled over time thus causing variability in results. Consequently, the optimization of existing or new technologies will be unproductive without a solid understanding of the factors impacting their prevalence.

In the context of this literature review, factors that affect the microbial community are central to ARG dynamics. Organic matter composition (not only COD), and microbial immigration from the sewers seem to be some of the main factors that affect the dynamics of ARGs in activated sludge systems. Microbial immigration from the sewers appears to alter the microbial community composition in WWTPs. Wastewater composition plays a critical role in the microbial community assembly process, and likely impacts the fate of immigrating bacteria. The COD fractions of

wastewater also shape the microbial community that develops in a WWTP. However, to date, no studies have considered the impact of these parameters together under highly controlled, reproducible conditions. Lumped parameters such as COD and total nitrogen are only effective to study sludge systems in terms of removal efficiency. An in-depth analysis of the microbial community requires their contextualization at the compound level.

In full scale WWTPs, immigration is likely impacted by multiple deterministic factors at any one time such as wastewater composition (nutrients and organic matter), temperature and SRT. To study the impact of specific variables on immigration and AMR, a laboratory-based approach rather than full-scale would be recommended since different confounding variables can mask the underlying dynamics. Consequently, in this study bench-scale reactors will be used. Reactors will be fed with two different synthetic compositions to achieve different microbial communities with the supply of influent solids from the sewers to simulate microbial immigration. The organic fractions of the synthetic compositions will be classified into slowly and readily biodegradable substrates as these categories are also important for mathematical modelling. The approach of comparing two synthetic wastewater compositions where one is readily biodegradable while the other is slowly biodegradable in a controlled environment adopted in this study aims to study microbial community assembly and AMR in the activated sludge in the context of wastewater composition and bacterial immigration.

2.8 Wastewater Composition

Wastewater composition is one of the main drivers of microbial community assembly. During reactor operation, to allow wastewater composition to be controlled over time, a synthetic wastewater will be used. In the following sections, the general composition and variability of municipal wastewater is discussed, with a strong focus on organic matter content. This information will be used to inform the design of a new synthetic wastewater composition which will be used during reactor operation.

2.8.1 Major wastewater constituents

Human activities determine the type and concentrations of pollutants found in wastewater. For example, milk farms' wastewater can contain eighteen times the COD level of domestic wastewater and they are also vastly different in terms of organic compounds (Gutiérrez et al., 2009; Henze et al., 2008). In general, municipal wastewater contains water from both origins: industrial

and domestic. Moreover, even if industrial wastewater undergoes treatment before being discharged into the municipal sewage network, these discharges still carry organic and inorganic pollutants. Wastewater quality also varies geographically and temporally. That is, composition changes from place to place, and fluctuates during the day and season of the year. These changes are due to the population's habits, but also other distinct factors such as economic development, industrial activity, legal framework, type of sewage system, and season of the year, among others (Henze et al., 2008).

The historical focus of WWTPs is on the removal of organic compounds, nutrients (in mineral and organic forms), and microorganisms because they deplete oxygen in water, promote the growth of photosynthetic organisms leading to eutrophication, and disseminate pathogens (Grady et al., 2011). Whereas pathogens are essentially removed by physico-chemical disinfection, organics and nutrients are removed by biological processes. Consequently, for the design of WWTPs, it is customary to measure these constituents as lumped quantities such as the biochemical oxygen demand (BOD) or the total Kjeldahl nitrogen (TKN). However, the ecological effects of the wastewater constituents can only be understood by characterizing the organic composition (Bunzel et al., 2013; Guo and Frigon, 2023; Schaider et al., 2017).

The wastewater's organic composition is also very heterogenous. In fact, there can be up to 3013 organic compounds in wastewater, which complicates the study of wastewater (Maizel and Remucal, 2017). They can be classified into several categories (Table 1), with the major categories in domestic wastewater being fibers, sugars, proteins, nucleic acids, volatile fatty acids, humic acids, tannic acids, linear alkylbenzene sulfonate, and lipids (Huang et al., 2010). This variability can be seen in studies reporting different proportions in organic carbon related to different families of compounds. Wen et al. (2023) reports up to 60 % of the organic carbon being attributed to fibers and up to 30 % attributed to proteins while Huang et al. (2010) reports 30 % and 16 % for the same compound families, respectively. These observations show the complexity of the organic constituents in the wastewater matrix, making it a daunting task to design a synthetic wastewater of relevant composition. For ecological study purposes, the answer to this daunting task could be to compare the results from synthetic wastewater of different compositions.

Table 2.1. Organic constituents of municipal wastewater. Adapted from (Huang et al., 2010).

Constituents	Concentration (μg/L)	TOC concentration (μg-C/L)
Non and semi volatile organic compounds		
Proteins	27800	13373
Sugars	38870	15548
Lipids	1350	960
Volatile fatty acids	19090	8436
Linear alkylbenzene sulfonate	2220	1238
Humic acids	11880	6475
Nucleic acids	19172	6691
Tannic acids	2500	1340
Fibers	64800	25920
Volatile compounds		
Alkyl and aromatic hydrocarbons	37	32
Alkenes	51	45
Alcohols	145	118
Organic acids	224	167.79
Ketones	10	8
Phenols	19	13
Nitrogenous compounds	22	12.94
Ethers	7	5
Amines	8	5
Esters	43	30

Another way to characterize the organic composition of wastewater is to focus on functional size distribution because particulate and colloidal matter encompass a large fraction of organics (Ravndal et al., 2018). Indeed, both molecular identity and functional size (dissolved [<0.22 μm], colloidal [<1.2 μm], or particulate [>1.2 μm]), are important because they influence the biodegradation rate. For example, Ravndal et al. (2018) found that particulate matter's biodegradation rates were inversely proportional to particle sizes. While those of polymeric compounds, on the other hand, depended on their molecular compositions. The impacts on degradation rates are important to classify organic compounds into readily, slowly, and non-degradable COD used in activated sludge modeling (Reijken et al., 2018) and then relate this classification to community assembly (Guo and Frigon, 2023). In this respect, a specific constituent that has been gaining attention over the last twenty years is the cellulose content from toilet paper (likely related to *fibers* in Table 2.1). Studies report that cellulose from toilet paper represents 7-17% of suspended solids (Honda et al., 2000), or 25-40% of COD (Ahmed et al., 2019) in raw wastewater. However, cellulose is slowly hydrolyzed (Reijken et al., 2018), making

the energy expenditure to gain access to the substrate relatively high. In fact, the extent of cellulose degradation depends on the process's SRT, and in consequence, cellulose is virtually not degraded at typical activated sludge operation conditions (SRTs between 7 and 20 days) (Li et al., 2019c).

The main categories of wastewater constituents other than organic matter are microorganisms, nutrients, metals and other inorganic compounds. Other pollutants of emerging concern are also present in wastewater, including microplastics (Hidayaturrahman and Lee, 2019), engineered nanomaterials (Westerhoff et al., 2011), pesticides (Youssef et al., 2019), aromatic hydrocarbons (Oh et al., 2016; Xia et al., 2019), and antimicrobials and other pharmaceutical products (Manzetti and Ghisi, 2014; Phoon et al., 2020; Szekeres et al., 2017; Zhang et al., 2018b). The presence of these contaminants of emerging concern can impact the dissemination of ARGs. For example, aromatic compounds have been found to enrich the abundance of ARGs in wastewater (Xia et al., 2019), and metal oxides nanoparticles have been shown to lower bacterial tolerance to antimicrobials (Yuan et al., 2020) and increase HGT due to transduction (Han et al., 2020).

2.8.2 Antimicrobials in wastewater

The presence of different classes of antimicrobials in wastewater is potentially important because they can promote selection and HGT of ARGs specially at concentrations below the minimum inhibitory concentration (MIC) (Hutinel et al., 2021; Jutkina et al., 2016). One of the main sources of antimicrobial residuals in wastewater are hospitals. Studies have found that concentrations range from 0.1 to 53 μg/L in hospital wastewater but decreased at the WWTP's influent up to ten times (Al-Maadheed et al., 2019; Aydin et al., 2019; Langbehn et al., 2021; Szekeres et al., 2017) most likely due to dilution because the fraction of the total flow that hospitals contribute to wastewater is from 1 % to 3.5 % (Aydin et al., 2019; Kumari et al., 2020). Regardless, detectable levels of antimicrobials remained in the influent wastewater, with sulfonamides, β-lactams, and glycopeptides being the main classes detected in these studies.

Taken together, these studies indicate that the presence of antimicrobials in wastewater is important and should be considered when trying to simulate wastewater by synthetic compositions, especially when AMR is to be studied. However, there may be central interactions between substrates, antimicrobials and reactor conditions that are not well understood so far. In this regard, studies have shown antimicrobials to affect the microbial community in wastewater, decreasing methane production in anaerobic digestion (Czatzkowska et al., 2022) and affect nitrification in

activated sludge (Zhang et al., 2020). Antimicrobials have also been shown to be removed via adsorption and biodegradation (Zhang et al., 2018a). All these factors could potentially affect the biodegradation of the organic fraction of wastewater. Consequently, an effective laboratory system with controlled wastewater compositions is needed to address these interactions properly. For this reason, as a first approach to the design of a new wastewater composition, antimicrobials will be excluded. After the microbial community and AMR dynamics have been fully elucidated, follow-up studies could introduce antimicrobials to the synthetic wastewater to study their impact.

2.9 Designing a Synthetic Wastewater

2.9.1 General interest

As previously discussed, wastewater compositions vary geographically and temporally. These variations in composition are sometimes problematic when studying biological phenomena in depth over time. When not adequately controlled and reproduced, the composition of the wastewater can become a confounding variable precluding firm conclusions. The use of synthetic wastewaters composed of well identified compounds in fixed concentrations is a solution to this lack of experimental control. Additionally, synthetic wastewater has the advantage over real wastewater of being convenient and somewhat safer. However, the previous discussion demonstrated that a major challenge is determining the compounds that would best represent the relevant properties of real wastewater.

With several experimental objectives in mind, many synthetic wastewater compositions have been developed. The most complex synthetic wastewater compositions aim to emulate the wastewater from different sectors of human activities such as pharmaceutical and personal care product manufacturing (Osachoff et al., 2014), acid mine drainage (van den Berg et al., 2016), and sugar waste (Guiot and Van Den Berg, 1985), among others. As for municipal wastewater, the most commonly used compositions are *Syntho* (Boeije et al., 1999) and *Synthes* (Aiyuk and Verstraete, 2004). *Syntho* was conceived to simulate a laboratory conventional activated sludge (CAS), and *Synthes* was developed to emulate an up-flow anaerobic sludge blanket (UASB). Syntho is now included in the ISO 11733:2004 as an alternative standard to the OECD composition (International Organization for Standardization and European Committee for Standardization, 2004). Nevertheless, the criticisms of reduced complexity remain applicable to *Syntho*.

Consequently, many studies have tried to develop their own composition or have used specific materials for particular applications.

2.9.2 Evaluation of different synthetic wastewater compositions

The variations in raw wastewater composition tend to also impact the COD level as different compounds have different oxygen demands depending upon whether they are easily or slowly biodegradable. In general, proteins and lipids are slowly biodegradable and, with the exception of fibers, carbohydrates are easily biodegradable. Table 2.2 displays the COD fractions of raw wastewater and primary effluent. Within municipal wastewater, the protein and lipid fractions appear to contribute the greatest factions of COD. Based on the municipal wastewater's COD fraction from Raunkjær et al. (1994), the proposed new composition aims to be a more slowly biodegradable composition with particulate organic matter and high slowly biodegradable dissolved organic matter.

In contrast to the new composition proposed in this study, *Syntho* and *Synthes* have more readily biodegradable compounds. Both use sodium acetate for volatile fatty acids (VFAs) content. Glycerol, diet fibers, and potato starch are used for carbohydrate content. Finally, milk powder is provided for protein and lipids content but also contains sugars in the form of lactose. In addition to milk powder, *Synthes* also uses soy oil for lipid content. *Syntho* uses other compounds to better emulate domestic wastewater. Linear Alkylbenzene Sulfonate (LAS) and Alcohol Ethoxylates (AE) are used to represent surfactants due to detergents used in households. Diatomaceous earth is used to represent sand that comes from runoff. *Syntho* is also inoculated through lyophilized mixed liquor solids.

To enhance microorganisms' abundance dry meat extract is used in *Syntho* and dried yeast extract is used in *Synthes,* these ingredients supply essential amino acids, vitamins and other growth factors. Common compounds used for nutrients (N and P) are either organic, such as peptone or urea, or inorganic such as ammonium, nitrate, and phosphate (Boeije et al., 1999). The former four compounds supply nitrogen, and the latter supplies phosphorus. However, peptone is also a marginal carbon and phosphorus source. Frequent trace metals include chromium, copper, manganese, nickel, lead, and zinc (Aiyuk and Verstraete, 2004).

Table 2.2. Chemical oxygen demand fractions of wastewater. Adapted from Sophonsiri and Morgenroth (2004).

Protein (%)	Carbohydrate (%)	Lipid (%)	Unidentified or Others (%)	Total COD mg/L	Type	Source
Raw wastewater						
31	16	45	8	203	Municipal	(Heukelekian and Balmat, 1959)[a]
30	10	N.D.	60	813	Municipal	(Narkis, 1980)
15	7	N.D.	78	394	Domestic	(Narkis, 1980)
8	12	10	70	530	Domestic	(Henze, 1982)
28	18	31	22	N.D.	Municipal	(Raunkjær et al., 1994)
18	16	7	59	967	Homogenized municipal	(Dignac et al., 2000)[b]
Primary effluent						
12	6	82	0[c]	309		(Sophonsiri and Morgenroth, 2004)
12	6	19	63	259		(Tanaka et al., 1991)

Notes:
N.D. Not determined.
[a] Estimated using conversion factors of average composition of carbohydrates, proteins, and lipids from Henze (2002).
[b] Estimated from standard COD content of carbohydrates, proteins, and lipids.
[c] Adjusted to zero to balance the total COD content and the sum of COD from carbohydrates, proteins, and lipids.

An analysis of carbonaceous COD content in *Syntho* (Table 2.3) not only demonstrated most to be comprised of soluble readily biodegradable substrates, but carbohydrates content was also the major fraction of organic matter. As a result, *Syntho* does not accurately represent wastewater in terms of COD fractions. The COD content presented in Table 2.3 is estimated, with a total COD of 413.6 mg/L.

Table 2.3. Analysis of estimated carbonaceous COD content in Syntho.

	Concentration	Carbohydrates	Proteins	Lipids	Total COD	Carbohydrates	Proteins or amino acids	Lipids	Total COD
Units	(mg/L)	(mgCOD/mg Compound)				(mgCOD/L)			
Sodium acetate	120	-	-	-	0.8	-	-	-	93.6
Dry meat extract	15	0.2	0.5	-	0.7	3	7.5	-	10.5
Glycerol	40	1.2	-	-	1.2	48.7	-	-	48.7
Starch	50	1.2	-	-	1.2	59.2	-	-	59.2
Low-Fat Milk Powder	120	0.6	0.6	0	1.2	72.8	66.1	2.4	141.4
Sodium Dodecyl Sulfate	10	-	-	2.2	2.2	-	-	21.8	21.8
Genapol® X-080	10	-	-	2.2	2.2	-	-	22.2	22.2
Peptone	15	0.2	0.8	-	1.1	-	-	-	16.2
TOTAL		3.4	1.9	0	10.5	183.7	73.6	2.4	413.6
		FRACTION				44%	18%	1%	

Notes.

* Although specifically used as soluble detergent: Sodium Dodecyl Sulfate and Genapol® X-080, the long chain similar to fatty acids lead to a bacterial metabolism similar to the ones of lipids.

* Carbohydrate, protein, and lipid content in dried meat extract (Pearson, 2003), low-fat milk powder (US Dairy Export Council, 2013; USAID, 2006), and peptone (Thermo Fisher Scientific, 2019) was estimated from typical composition of these constituents. Equivalent COD content for carbohydrates, proteins, lipids, sodium acetate, glycerol, starch, sodium dodecyl sulfate, and Genapol® X-080 was estimated using Rittmann and McCarty (2020)'s model. Average chemical compositions for carbohydrates, proteins, and lipids were used for the latter estimation. The formulas used are $C_6H_{10}O_5$, $C_4H_{6.2}O_{1.2}NP_{0.01}$, and $C_8H_{16}O$ (Rittmann and McCarty, 2020), respectively.

These results are in accordance with O'Flaherty and Gray (2013), who conducted a comparative experimental analysis of eleven synthetic wastewater compositions and two actual wastewaters from Ireland. O'Flaherty and Gray (2013)'s results show that, with some exceptions, BOD is in the range of medium load, while COD is slightly lower than medium load for all the synthetic wastewater compositions. *Synthes* is one of the compositions that exhibited a high COD load, most likely due to the increased concentration of its components and the use of soy oil. Given that no composition considered in this study was far from the actual wastewater range, in terms of COD load, the study suggests to base the choice of composition on the COD/BOD and C/N/P ratios or the specific oxygen uptake rate.

The results also indicate that carbon sources from these synthetic wastewaters are mainly soluble, which also introduces a bias in the microbial community as some populations would grow more quickly than others. This condition results in some populations being more abundant than they would be in actual wastewater. Indeed, studies have found that the form of organic matter has an impact on the microbial community of aquatic ecosystems (Saarenheimo et al., 2017; Yu et al., 2020) and their dynamics (Wu et al., 2018).

2.9.3 Alternative compositions to simulate municipal wastewater

Selected studies have used other components such as food or products derived from animals to study the hydrolysis or degradation of specific COD fractions. However, in these studies food ingredients or a mixture of ingredients alone are used to provide the wastewater with carbon, nutrients, and trace metal sources and no bought chemicals are used to supplement synthetic wastewater. Food constituents include eggs (Dimock and Morgenroth, 2006; Wanner and Novák, 1990), dog food (Gomec et al., 2002; Langenhoff, 2000; Vandergheynst et al., 1997), rice (Langenhoff, 2000), cat food (Prieto et al., 2019), milk powder (Ghyoot and Verstraete, 2000; Sprouse and Rittmann, 1990) and whey (Kato et al., 1997). All of these ingredients are complex in their compound composition, thus it is difficult to track the COD fractions. In addition, with the exception of milk powder and whey, they are slowly biodegradable as they tend to be in particulate form even when crushed, blended or sonicated. Finally, other constituents derived from animals include dextran (Confer and Logan, 1997b; Haldane and Logan, 1994), dextrin (Confer and Logan, 1997b), and bovine serum albumin (BSA) (Confer and Logan, 1997a). These last components are relatively pure and will mostly dissolve in solution, and consequently they may be somewhat more readily biodegradable.

2.10 Second knowledge gap: a more representative municipal wastewater

The studies presented in this second half of Chapter 2 exemplify the complexities associated with wastewater composition, particularly in terms of the organic matter portion. Municipal wastewater's composition depends on multiple factors, which are intricately linked meaning that one sampling site of the same network can have different wastewater compositions at different times. In research, this variability is problematic and can be seen as a potential confounding factor. Since we cannot control the composition of actual wastewater, there is a need for synthetic wastewater that can produce firm conclusions and allow reproducibility allowing comparability among different studies.

The synthetic wastewater compositions discussed thus far, highlight the need for a new composition that is representative of actual wastewater. As the analysis of *Syntho* shows, synthetic wastewater compositions tend to lack key components of actual wastewater. For example, the absence of particulate or dissolved organic matter may skew results and produce a microbial community which is not always representative of that which real raw wastewater would engender.

This is also the case for those compositions that represent only a readily biodegradable fraction such as the carbohydrates portion. This lack of heterogeneity in the COD fractions and in the organic matter form (either particulate or dissolved) highlights the need for new synthetic wastewater compositions that resemble more closely the composition of actual wastewater.

To address the limitations of synthetic wastewaters currently available, the present study proposes a new composition which better represents the different COD fractions (readily and slowly biodegradable) and forms (dissolved and particulate) of actual wastewater. The presence of particulate organic matter was considered to be one important factor, which is included in this new composition. The new composition was designed with COD fractions that represent carbohydrates 29 %, proteins 18 %, lipids 31 % and other carbonaceous compounds 22 %, following the fractions reported by (Raunkjær et al., 1994) in municipal wastewater. Details on each of the components, COD level and fractions are included in the appendix in Table S1 and Table S2.

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Chapter 3. Wastewater Composition Impacts the Microbial Community Assembly, Influent Immigration and Antimicrobial Resistance in Activated Sludge Systems.

3.1 Introduction

Antimicrobial Resistance (AMR) has been recognized as a global health problem by the World Health Organization (WHO) since 2015. Since then, the WHO created the "Global Antimicrobial Resistance and Use Surveillance System" and provided venues to address the issue. Indeed, two million people are infected by Antimicrobial Resistant Bacteria (ARB) each year (Akova, 2016) and 1.27 million deaths were estimated to be linked to AMR in 2019 alone (Murray et al., 2022). However, despite an exponential increase in the number of publications on this topic over recent years, the majority of studies focus on AMR within clinical settings. Other relevant routes of exposure such as the environment have been neglected for the most part. A quick search of the words "Antimicrobial Resistance" (January 2021) in the scientific database 'ScienceDirect' shows that only around 3 % of the total publications on AMR are related to environmental sciences. Consequently, our knowledge of the role of the environment in the spread of AMR is lacking.

Wastewater Treatment Plants (WWTPs) are known hotspots of AMR (Hultman et al., 2018) and have been shown to contain important loads of Antimicrobial Resistance Genes (ARGs) (Auerbach et al., 2007; Mispagel and Gray, 2005; Schwartz, 2003; Tennstedt et al., 2003). It is believed that hospital wastewater contributes to the ARG content in municipal wastewater (Koczura et al., 2012; Zhang et al., 2020). However, in reality ARGs in wastewater likely originate from numerous settings, and are associated with a highly diverse microbial community (Yuan et al., 2019b). In this context our understanding of ARG dynamics in WWTPs and specifically in activated sludge systems is still lacking in some regards. The dynamics in activated sludge are important as the major fraction of ARGs are retained in the solid fraction (Mays et al., 2021; Zhang et al., 2009). Biosolids could, consequently, introduce large number of ARGs and negatively affect the environment when used as fertilizer.

Within the WWTP, factors such as the Solids Retention Time (SRT) (Gibson et al., 2023a; Neyestani et al., 2017; Zhang et al., 2019) and the organic load (Yuan et al., 2016; Yuan et al., 2014), measured in the form of the Chemical Oxygen Demand (COD), can impact AMR in the activated sludge. Thus, it is critical to consider the impact of wastewater composition and operational parameters in WWTPs when studying AMR. Furthermore, the microbial community

composition has been shown to correlate with AMR in many environments (He et al., 2022; Wu et al., 2017; Yuan et al., 2019b). Consequently, a better understanding of the microbial community assembly process and impact of wastewater parameters in activated sludge could be critical in the management of AMR.

Two crucial factors known to impact the microbial community in activated sludge are influent bacterial immigration (Dottorini et al., 2021) and wastewater composition (Peces et al., 2022; Tamminen et al., 2022). It is also likely that these two factors interact, with the wastewater composition potentially impacting the fate of immigrating bacteria. Despite our knowledge of their importance, the high variability in wastewater composition and the variety of pollutants present (Grady et al., 2011) hinder comparability among similar studies. Consequently, our understanding of the relationship between wastewater composition, immigration and AMR remains limited. To enable the complex dynamics of this relationship to be evaluated, a high level of control and reproducibility is desirable. These two requirements could be fulfilled by utilizing a fixed wastewater composition.

Numerous studies use synthetic wastewaters such as Syntho (Boeije et al., 1999) and Synthes (Aiyuk and Verstraete, 2004) to simulate wastewater and maintain a stable composition for the duration of experiments. However, these compositions may not be representative of actual municipal wastewater. Thus, in the present study, we developed a new synthetic wastewater composition designed to be more representative of actual wastewater in terms of the organic matter composition, COD fractions and diversity of organic compounds. Laboratory-scale wastewater treatment bioreactors were operated with two different synthetic wastewater compositions—Syntho and the new composition named "Simulated Municipal Wastewater (SMWWat)". To study the impact of immigration on the activated sludge microbial community under different substrate landscapes, influent solids from a Quebec WWTP were supplied to a subset of the reactors. Amplicon sequencing of the 16S rRNA gene was used to analyze the microbial community composition, and a qPCR approach was used to investigate the ARG concentration and sequence diversity . The results revealed the organic composition of the wastewater to impact nitrification, sewer immigration and ARG prevalence in activated sludge systems. These findings are critical to our understanding of factors impacting the prevalence of AMR in WWTPs and its spread in the wider environment.

3.2 Materials and Methods

3.2.1 Experimental Design and Reactor Operation

A total of 18 reactors were operated between December 2021 and March 2022 for a total of 105 days (15 SRTs, where one SRT = 7 days) with an average Hydraulic Retention Time (HRT) of 1.86 days. The operation was divided into three phases. In *Phase 1*, the reactors were inoculated with 160 mL of mixed liquor, containing 2050 mg/L of total suspended solids (TSS) and 1730 mg/L of volatile suspended solids (VSS). The experimental design is shown in Figure 3.1 for better understanding. Reactors were fed one of two synthetic wastewater compositions (detailed in section 3.2.2). That is, nine reactors per composition. After 4 SRTs, at the beginning of *Phase 2* the mixed liquors of the reactors that were fed the same wastewater composition were mixed and separated again to create homogenized microbial communities across all biological replicates and experimental conditions as described by Kaewpipat and Grady (2002). The aim of *Phase 2* was to investigate the effect of immigration on the reactors while having two controls for comparison. The nine reactors per composition were divided into three subsets (three replicates per condition). Each subset was either supplied with influent solids to simulate immigration, sterile influent solids, or no solids (synthetic wastewater only). The sterile solids control was included to distinguish whether the impact of immigration was due to the addition of influent solids alone (i.e. an extra source of substrates) or due to actively growing immigrating genera. After operating reactors for 7 SRTs during *Phase 2*, the reactors were then fed for 4 additional SRTs with only synthetic wastewater during *Phase 3*. The objective of this phase was to see if the changes introduced by immigration would remain over time or be reversed .

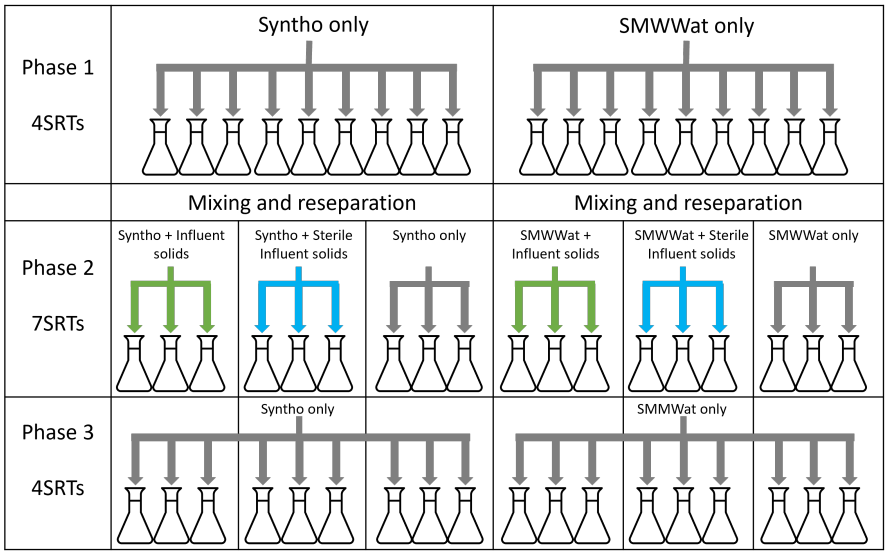

Figure 3.1. Schematic of reactors operation. In Phase 1, half of the reactors were fed with Syntho *and the other with SMWWat (synthetic wastewaters). At the end of Phase 1, the reactors with the same composition were mixed. In Phase 2, reactors were fed with one of the two synthetic wastewater compositions supplemented with either influent solids, sterile influent solids, or no solids. Finally, in Phase 3, reactors were fed with only synthetic wastewater.*

Reactors were incubated in Erlenmeyer flasks at 21°C with shaking at 100 rpm on an orbital shaker (Multitron AJ118). Solids were removed daily (1/7 of the total volume) to provide an overall solids retention time of 7 days. Reactors were fed daily with one of the two synthetic wastewater compositions (70 mL), and the supernatant was wasted every other day (100mL). Supernatant was wasted by settling the solid fraction of the reactors' content in a 100mL graduated cylinder for 45 min. The mixed liquor and influent solids were harvested from the Cowansville WWTP in Quebec, Canada. On site, the influent solids were concentrated by settling in 20 L buckets for 20 min. The liquid fraction was then discarded (4/5 of the volume) and the fraction concentrated with solids was stored for future use. All samples were transported to McGill University on ice and stored at 4 °C until processing. For the inoculum, a grab sample was obtained from the aeration tank of the WWTP and processed within 24 hours of collection. The influent solids were processed immediately by washing in synthetic wastewater, centrifuging in 50-mL aliquots at 21,100 × g for 10 min, and discarding the supernatant. This wash step was repeated three times, and solids were resuspended in synthetic wastewater. The washed influent solids were stored at 4 °C for no more than two weeks and diluted to a concentration of 120 mg/L before use. A batch of 300 mL from the influent solids was autoclaved in glassware at 121 °C and 15 psi for 30 min for the sterile solids supply.

3.2.2 Wastewater Composition

All reactors were fed with one of two different synthetic wastewater compositions. The first composition was Syntho, which was prepared following Boeije et al. (1999)'s formula. The new synthetic wastewater composition was named Simulated Municipal Wastewater (SMWWat). The concentration of each component and an analysis of the COD content for both Syntho and SMWWat are shown in Table 3.1. The new wastewater composition was developed following the same principles that led to the creation of Syntho, aiming to include compounds related to those that would occur in actual wastewater. A central principle was to include different source of substrates in the two compositions that would make them similar but distinct in the way they are degraded.

Syntho and SMWWat were compared to one other, and also to previously reported wastewater compositions (Table S1 and Table S2) with respect to carbohydrate, protein, lipid, and mineral and organic nitrogen and phosphorus contents. The literature review of five articles reporting COD fractions of carbohydrates, proteins and lipids suggested a target composition with COD mainly from lipids and proteins (Dignac et al., 2000; Henze, 1982; Heukelekian and Balmat, 1959; Narkis, 1980; Raunkjær et al., 1994). In our evaluation, Syntho was rich in carbohydrates and composed only of soluble compounds. Therefore, the composition of SMWWat aimed to be closer to the municipal influent wastewater composition with higher protein and lipids. This new composition was also designed to contain more slowly degradable compounds when compared to Syntho. The fractions chosen were 18 %, 29 %, 31 %, and 22 % for carbohydrates, proteins, lipids and other carbonaceous compounds, respectively. A summarized comparison of the nitrogen, phosphorus and COD content of Syntho and SMWWat is shown in Table 3.1.

Table 3.1. Comparison of total nitrogen, total phosphorus and organic matter measured as COD between Syntho and SMWWat.

Component	Syntho		SMWWat		Units
	Concentration	COD Fraction*	Concentration	COD Fraction	
Nitrogen	51.0	NA	51.6	NA	mg-N/L
Phosphorus	8.4	NA	8.3	NA	mg-P/L
Carbohydrates	214.9	48 %	81.1	18 %	mg-COD/L
Proteins	97.5	22 %	131.6	29 %	mg-COD/L
Lipids	44.8	10 %	139.3	31 %	mg-COD/L
Others	91.3	20 %	97.1	22 %	mg-COD/L

Total cCOD	448.5	100 %	449.0	100 %	mg-COD/L

*COD fractions were estimated from the measured total COD and typical portion and their associated COD. Details are shown in Table S1 and Table S2.
* COD measures were obtained using the Standard Methods 5220D (American Public Health Association et al., 2017).

Following the logic and desired properties described above, the composition of SMWWat was selected as follow. Propionate was chosen to represent volatile fatty acid as it has a longer chain than acetate but also because it has an odd number of carbons (three instead of two of acetate). This ensures the degradation pathway is different than that of acetate. Instead of glycerol, which is the backbone of glycerides (lipids) (Kenwood and Merrill, 2016), prenol was chosen, which is the backbone of prenol lipids (Donato et al., 2017), and is present in many fruits (Baeza-Jiménez et al., 2017; Wanke et al., 1998) and commercial fragrances (Mimoun, 1996). Prenol was also chosen to ensure there were a range of substrates which differed between the two compositions. Inulin was selected to represent the carbohydrates fraction. Inulin is a dietary fiber, which is, nonetheless, one of the most biodegradable fibers (Tanaka et al., 1975; Uchiyama, 1975; Uchiyama et al., 1973). Tween 80® was chosen not only to represent the surfactant fraction that is released from detergents, pharmaceuticals and cosmetics (Gautier and Bellamy, 2000; Gough et al., 1982) but also because of its chemical structure, which is related to lipids (Taoka et al., 2011). Dimethyl phthalate was chosen to represent the other organic fraction contributed by industrial effluents. Dimethyl phthalate is also present in domestic wastewater due to its use as plasticizer, in insecticides, cosmetics, soap, and household cleaning supplies (Schettler, 2006). Finally, egg yolks and egg whites were chosen due to their high lipid and protein content, respectively (Yamamoto, 1997). Egg whites have been used in previous studies to represent the biodegradable particulate fraction of wastewater (Dimock and Morgenroth, 2006; Wanner and Novák, 1990).

The feed for both compositions was prepared from stock solutions of each of the wastewater's components. The stock solutions were prepared at 200 x for carbonaceous, nitrogenous and trace metals constituents, and at 50 x for the phosphorous constituents. These were autoclaved and stored in 500 mL bottles at 4 °C until use. Autoclaving sterilization was performed at 121 °C and 15 psi for 30 min. The trace metals solution was sterilized by filtration (0.2 μm). No stock solutions were prepared for uric acid and diatomaceous earth due to their low solubility. Consequently, they were added after the feed was prepared by diluting the stock solutions. The egg yolk and egg white

were cooked, blended using a home cooking blender, dried at 25 °C for 24h, and sieved (300 μm). They were stored at ambient temperature and added to SMWWat when the feed was prepared.

3.2.3 Analytical methods

Once per SRT (every 7 days), supernatant samples representing the effluent wastewater, and mixed liquor from the bioreactors were collected. The effluent wastewater was tested for COD using Standard Methods 5220D (American Public Health Association et al., 2017), and for nitrogen species (ammonium, nitrate, and nitrite) using the microplate techniques by Shand et al. (2008). Mixed liquor samples were analyzed for total suspended solids (TSS) and volatile suspended solids (VSS) using Standard Methods 2540B and 2540E (American Public Health Association et al., 2017), respectively.

3.2.4 Microbial Community Analysis

Influent and mixed liquor suspended solids were centrifuged, the supernatant removed, and samples were stored at −80 ℃ for future DNA extraction. DNA was extracted from centrifuged solids using DNeasy PowerSoil Pro Kit (Qiagen, Germantown, MD, USA). PCR of the 16S rRNA gene V4 region was done using the modified Caporaso primers: 515F and 806R (Apprill et al., 2015; Parada et al., 2016). PCR cycling conditions were as follows: 94 ℃ for 3 min followed by 35 cycles of 94 ℃ for 45 sec, 50 ℃ for 60 sec, 72 ℃ for 90 sec. 72 ℃ for 10 min and a final hold at 4 ℃. Amplicons were sequenced on the Illumina MiSeq PE250 platform at McGill University and Génome Québec Innovation Centre (Montréal, QC, Canada).

Sequencing data was processed using QIIME2 (Bolyen et al., 2019) and R software (packages "vegan" and "ape") (Oksanen et al., 2015). The QIIME2 pipeline was as follows: quality filtered with DADA2 (Callahan et al., 2016) (trimmed at 25f and 20r, and truncated at 220f and 210r) and taxonomy was assigned using MiDAS 4.8.1 (Dueholm et al., 2022). After quality-filtering, ASV tables were exported and rarefied to 40,000 reads and analyzed using R "vegan" package and Bray-Curtis dissimilarity.

After taxonomy was assigned, the microbial community was classified into different populations. Using the microbial community assembled in *Phase 1*, genera that were present in 80 % of the reactors of each composition and had a relative abundance of 0.1 % were classified as *Core Residents* while those that did not meet these criteria would be considered *Non-Core*

Residents. This is based upon the criteria established by Wu et al. (2019) and Gibson et al. (2023a). Within the Core Residents category, two further categories were distinguished. The first category was the *Universal Core Residents*, which were present in both compositions as core resident. The second category was the *Substrate-Specific Core Residents*, which would only meet the previously explained criteria for one of the compositions and not the other.

Genera that were not present at the beginning of *Phase 2* (before the addition of influent solids) but appeared at the end of *Phase 2* (after the addition of influent solids) were considered *Immigrants*. As with the *Core Residents*, the *Immigrant* genera were further divided into two categories. The *Universal immigrants* were those that met the immigrant criteria for both compositions while *Substrate-Specific Immigrants* were the ones that only met the criteria for one of the compositions. These communities were also analyzed using the RRN Operon database (Stoddard et al., 2015) to determine the 16S gene copy number of the reactors and the communities.

3.2.5 ARGs Quantification Analysis

The concentration of ARGs in the reactor and influent samples were analyzed using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). Two samples per wastewater composition taken from the end of *Phase 1*, one sample per composition from the beginning of *Phase 2*, and all samples obtained at the end of *Phase 2* and *Phase 3* were analyzed. Samples at the end of *Phase 1* were before reactor mixing and samples at the beggining of *Phase 2* were collected after reactor mixing. PCR cycling conditions were as follows: 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 30 sec and 60 ℃ for 30 sec. 72 ℃ for 10 min and a final hold at 4 ℃. The ARG concentration was normalized to copies per 16S rRNA gene. In total 71 genes were analyzed, which included 9 mobile genetic elements and integrons, 56 antimicrobial resistance genes and 6 metal and other resistance determinants. Primers are indicated elsewhere (Muziasari et al., 2017).

Additionally, 15 genes were analyzed using Droplet Digital PCR (ddPCR) using the QX200 Droplet Digital PCR system (Bio-Rad Laboratories Inc., Pleasanton, CA, USA) according to the manufacturer's instructions. DNA dilution and the optimal annealing temperature was determined by running an initial PCR with a temperature gradient from 56℃ to 63℃, with 3 different dilutions (from 10^0 to 10^3). The optimal dilution and annealing temperatures chosen were those which showed the best separation between positive and negative droplets with the least 'rain' (i.e.

intermediate droplets). PCR cycling conditions were as follows: 95 ℃ for 5 min followed by 50 cycles of 95 ℃ for 30 sec, 59-63 ℃ for 60 sec and 72 ℃ for 30 sec. The 50 cycles were followed by 5 min at 4℃, 5 min at 90 ℃ and a final hold at 12 ℃. The annealing temperature for each gene is indicated in Table S3 in the appendix.

The same 15 genes were analyzed using multiplexed amplicon sequencing using the QIAGEN® Multiplex PCR Kit to analyze the gene variants in the reactors and influent samples. PCR cycling conditions were as follows: 95 ℃ for 15 min followed by 40 cycles of 94 ℃ for 30 sec, 60 ℃ for 90 sec and 72 ℃ for 90 sec. The 40 cycles were followed by a final extension of 72℃ for 10 min and a final hold at 4 ℃. Subsequently, samples were barcoded in a second PCR reaction. Barcode PCR conditions were as follows: 94 ℃ for 3 min followed by 15 cycles of 94 ℃ for 30 sec, 59 ℃ for 20 sec and 68 ℃ for 45 sec. The 15 cycles were followed by 5 min at 68℃ and a final hold at 3 ℃. Samples were sequenced on the Illumina MiSeq PE250 platform at Génome Québec Innovation Centre (Montréal, QC, Canada). Primers are indicated in Table S4 in the appendix.

The sequencing data obtained from Génome Québec was separated into separate files for each gene and sample using a custom python script (available upon request). Complete rationale and process is detailed elsewhere (Gibson et al., 2023b). Briefly, the files were trimmed to remove low quality base pairs using Trimmomatic 0.39 (Bolger et al., 2014). Then the forward and reverse reads were merged, sorted by length and dereplicated by clustering (with an id of 1) using vsearch v.2.22.1 (Rognes and et al., 2016). Real sequence variants from sequencing errors were filtered using a custom R script. Variants were filtered following a Poisson distribution centered around the mean of all counts, with many sequence variants only having one or two reads. OTU tables were constructed after filtering using the clusterfast method in vsearch (with an id of one). All sequence variants were compared to a database of ARGs based on a dereplicated version of the CARD database with a minimum id of 70 % using tblastx (blast v.2.12.0). Sequence variants that did not have hits to their respective target gene at this id level were removed.

3.2.6 Statistical Analysis

To test whether the difference in ARG concentration was significant among all samples, a three-way ANOVA test was performed using the PROC GLM command in SAS software (SAS Institute Inc., 2013). The concentration was tested for between-subject effects for wastewater composition effect, the influent solids condition effect and the interaction of these two. The concentration was also tested for within-subjects effects for the phase effect and interactions with the other factors. The analysis was done for each ARG. Only data from *Phase 2* and *Phase 3* were used as the no-influent-solids reactors were used as control. Homoscedasticity was verified using the White test in SAS Software (ARCHTEST statement from the PROC AUTOREG command) (SAS Institute Inc., 2013). If the homoscedasticity condition was not met, a nlog transformation was applied to the concentration. If no concentration was detected, the log transformation of the detection limit was used in the ANOVA analysis for that sample. The significance of the difference between communities was analyzed using the ANOSIM test with Bray-Curtis dissimilarity in R "vegan" (Oksanen et al., 2015).

3.3 Results

3.3.1 Operational parameters and steady state

During *Phase 1* of reactor operation, all reactors received synthetic wastewater only to ensure that stable operation was reached before immigration was introduced during *Phase 2*. After 4 SRTs steady state seemed to have been reached in terms of COD removal, TSS concentration and the nitrification process (Figure 3.2 A, B and C), however the microbial community continued to evolve over time. Considering the Bray-Curtis dissimilarity, the microbial communities continued to significantly change between successive SRT intervals (ANOSIM; $p<0.05$) until after 5 SRTs (35 days) when dissimilarities started to stabilize for the reactors (Figure 3.2 D). After this point the difference in the Bray-Curtis dissimilarity between successive sampling points decreased. Overall, the distance between each sampling point did not fully stabilize until after 11 SRTs (77 days) of operation, at which time the distances became highly similar. This trend was also shown using principle coordinate analysis (Figure 3.3 A), as the microbial communities are shown to cluster from the 7^{th} and 9^{th} SRT onwards. It should be noted, however, that among the reactors fed with SMWWat, after stabilization there was more variability in the microbial community of triplicate reactors when compared to those fed with Syntho (Figure 3.2 D and Figure 3.3 A).

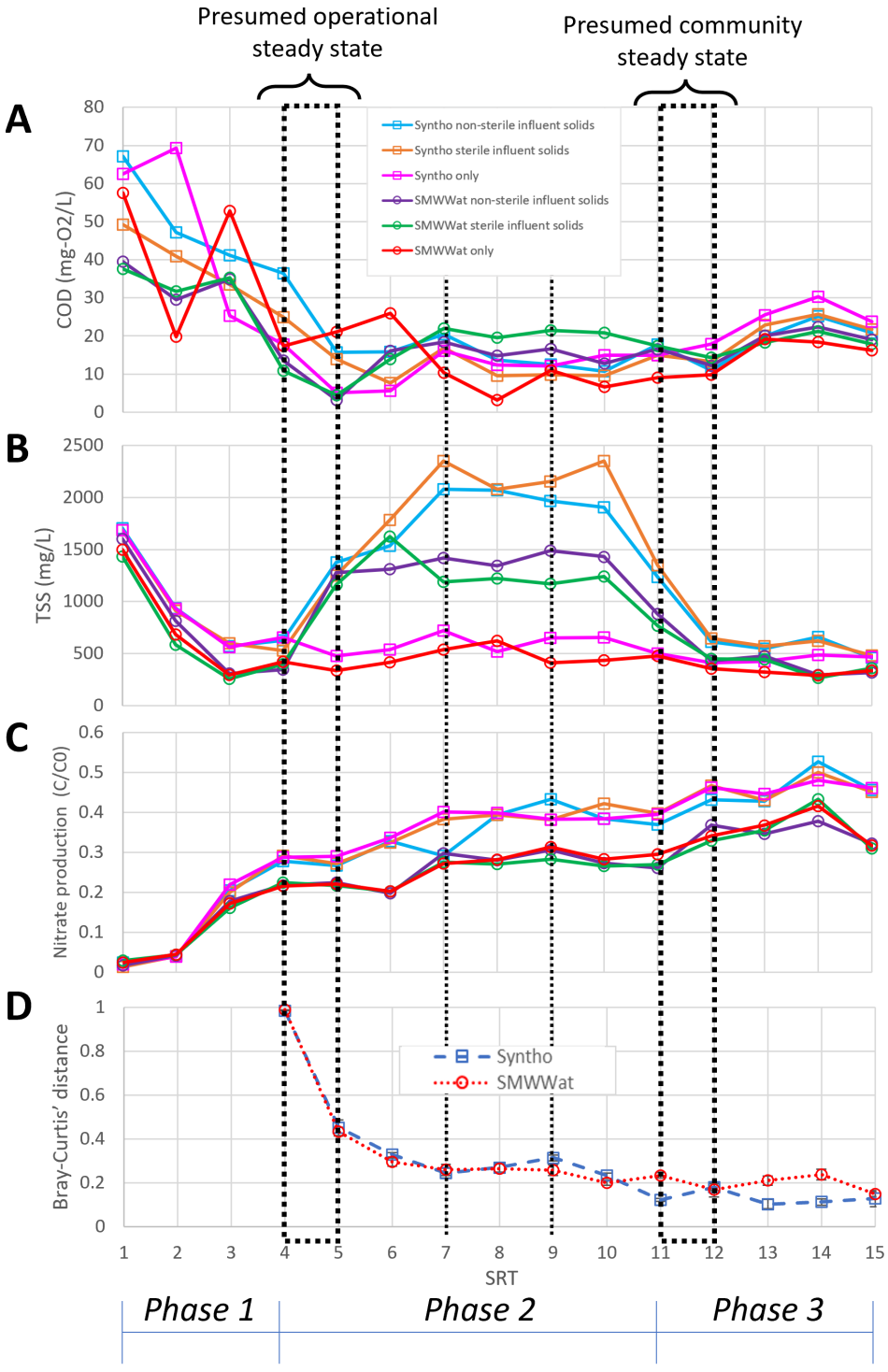

Figure 3.2. Operational and microbial community's steady state. A) Soluble COD of the wastewater treatment effluent. B) Reactor's mixed liquor 's total suspended solids. Fraction of volatile suspended solids is 85%. C) Reactor's nitrate production measured in the effluent. C indicates nitrate produced (mg-N/L) and C0 the total nitrogen in the feed (mg-N/L). D) Bray-Curtis' distances of the reactors fed with synthetic wastewater only between successive sampling points. Maximum difference at 1 is the distance between the inoculum and sample at SRT 4. Standard error is indicated by the bars.

When analyzing the reactors that were only supplied with synthetic wastewater throughout, alpha diversity metrics show reactors fed with SMWWat to have been more diverse than those fed with Syntho alone (Figure S3). Despite the Shannon's diversity, Chao1's richness and Pielou's evenness decreasing in all reactors when compared to the inoculum, the diversity index of reactors fed with SMWWat were higher. For example, Shannon's diversity index decreased from 3.9 to 3.2 at 11 and 15 SRTs, respectively for Syntho. While for SMWWat, the index went from 4.9 to 4.1 at 11 and 15 SRTs, respectively. The indexes also somewhat stabilized after the 12th SRT point. Taken together, these results suggest that the slowly biodegradable SMWWat composition better supports diverse communities than the readily degradable Syntho composition.

3.3.2 Population dynamics with different wastewater compositions

Using Bray-Curtis dissimilarity, the microbial community composition of the reactors proved to be significantly different using (ANOSIM; $p<0.05$) based upon the synthetic wastewater composition received. Although the major change in the microbial communities over time was due to community development discussed above represented along axis 1 (explaining 37.2 % of variation), a clear difference in the community based upon the wastewater composition could still be observed along axis 2 (representing 24.8 % variation; Figure 3.3 A), showing the importance of community assembly. It is worth noting that using Bray-Curtis dissimilarity, the most variation explained was for the ecological succession. On the other hand, using Jaccard dissimilarity, the most variation explained was due to the wastewater composition. This suggests that the ecological succession of the community over time was associated with the dynamics of the most abundant genera rather than the introduction of new taxa.

To better define the differences in the microbial community between the reactors, specific populations were defined as described in Section 3.2.4. Briefly, *Core Resident* genera were defined as those occurring within 80 % of the reactors (defined per synthetic wastewater composition received; i.e. 80% of the 9 reactors per feed) in a relative abundance of at least 0.1%. The *Core Resident* population could then be divided into two subgroups. The *Universal Core Residents* were members of the core resident population under all reactor conditions (i.e. with both synthetic wastewater compositions), whilst the *Substrate-Specific Core Residents* formed part of the core resident population in only one set of the reactors. It should be noted that this classification was based upon the community at the end of *Phase 1* (after 4 SRTs) which invariably changed over

time. This evolution of the microbial community is likely to have caused small changes in the relative abundance of the *Core Resident* genera over time. However, 95 % of the *Universal* and *Substrate-specific Core Residents* remained categorized as such after SRT 5, which is the last sampling point where the community was significantly different to that at SRT 4. After SRT 7, 90% of the *Universal Core Residents* were still classified as such.

Despite the significant difference explained by wastewater composition (ANOSIM; $p<0.05$), the reactors shared somewhat similar communities (in average 80% of the reads at the end of *Phase 1*). The *Universal Core Resident* population, present with both wastewater compositions, accounted for around 80% of the sequencing reads in the reactor microbial communities (Figure 3.3 B).This represents 13% and 11% of the total genera observed for reactors fed with SMWWat and Syntho, respectively. The *Substrate-specific Core Residents* accounted for 15 $\pm$ 3 % and 12 $\pm$ 2.7 % of the microbial community in the SMWWat and Syntho reactors, respectively. This represented 9% of the total genera observed in all reactors at the end of *Phase 1*. Genera which did not meet the criterion for the *Core Resident* population were considered to be *Non-Core Residents.* Reactors fed with Syntho had a greater proportion of reads contributed by this population, demonstrating either a more variable community (i.e. genera occurred in fewer than 80% of the reactors) or a greater number of lower abundance genera (< 0.1 % of total relative abundance) were present. Nonetheless, a higher diversity was still seen in the mixed liquor of the reactors receiving SMWWat (Figure S3).

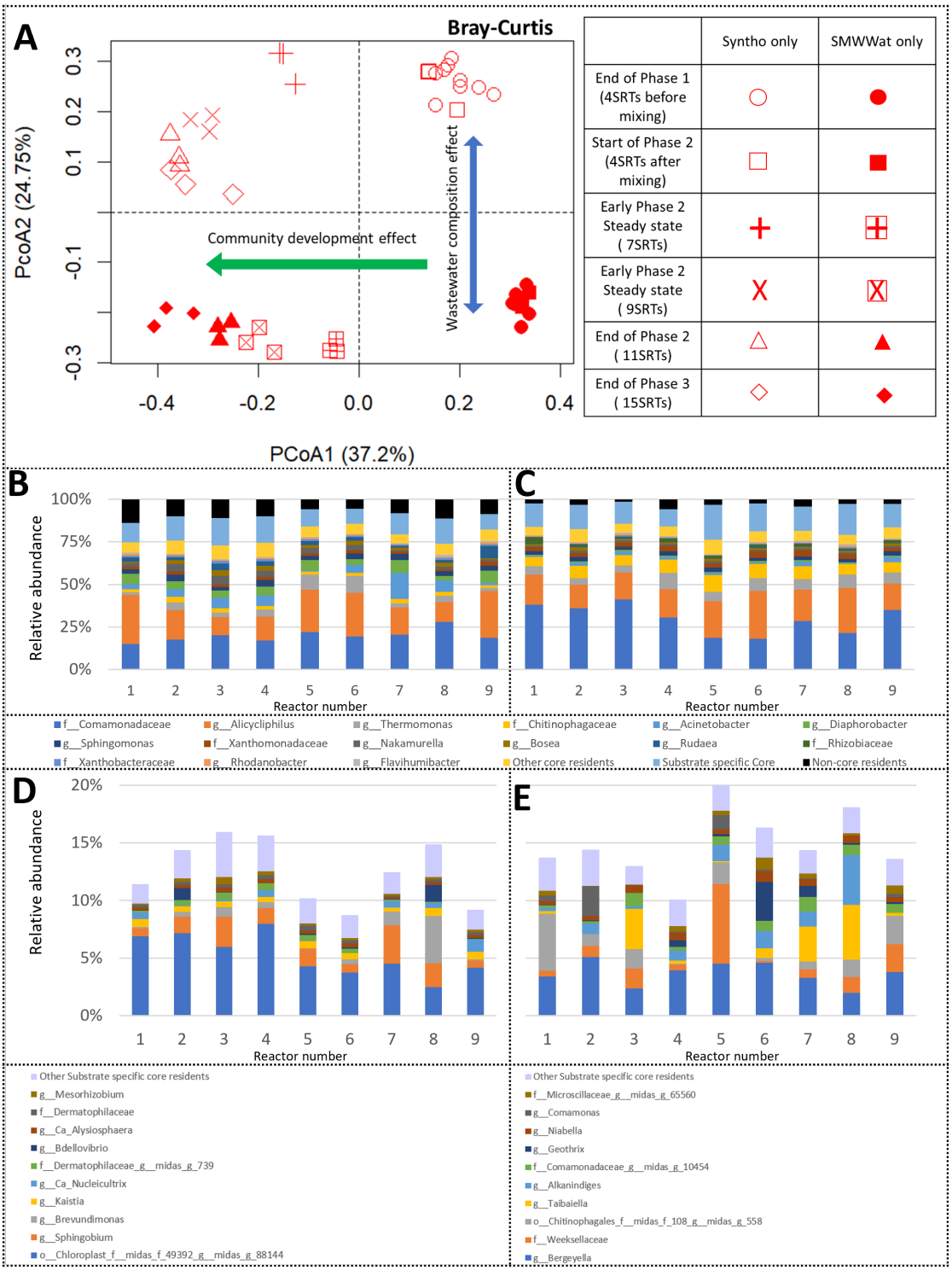

Figure 3.3. Core microbial community. Where possible, taxonomy was assigned up to genes level. A) Principal coordinate analysis using Bray-Curtis dissimilarity. B) Relative abundance of the Universal Core Residents for Syntho C) Relative abundance of the Universal Core Residents for SMWWat D) Relative abundance of the Syntho-Specific Core Residents. E) Relative abundance of the SMWWat-specific Core Residents.

The average 16S rRNA operon copy number (rrn) was used to track the ecological succession of the reactors' microbial community and better describe the *Residents* and *Immigrants*' response to substrate availability. At the end of *Phase 2*, the average 16S rRNA operon copy number (rrn) in the reactors was similar to that of the inoculum (Figure 3.4 A), which may be indicative of community stability (Nemergut et al., 2016; Shrestha et al., 2007). The highest copy number for Syntho reactors was seen in those with immigration with an average of 2.80 while for the reactors with no influent solids, the average was 2.75. For SMWWat, it was the reverse, the highest copy number was in the reactors without influent solids with an average of 2.92, and those receiving influent solids had an average of 2.81. There is also a clearly higher copy number for reactors fed with SMWWat only than those fed with Syntho only, which may be indicative of the high level of competitive fitness of the microbial community in SMWWat reactors (Klappenbach et al., 2000), potentially due to the slow biodegradability.

When the communities are analyzed using the definitions of *Core Residents*, *Non-Core Residents* and *Immigrants* (Figure 3.4 A), the *Immigrants* (*Universal and Substrate-specific*) and SMWWat *Subtrate-specific Core Residents*' rrn copy number has the biggest fraction of populations with more than seven rrn copies compared to the other populations. The major difference is also seen between the Syntho *Substrate-specific Core Residents* and the SMWWat *Substrate-specific Core Residents.* This difference in the *Subtrate-specific Residents´*s copy number illustrates the direct relationship between slowly biodegradable substrates and a high rrn copy number. This is exemplified to such a degree that the copy number of the Syntho *Substrate-specific Residents* does not go beyond four.

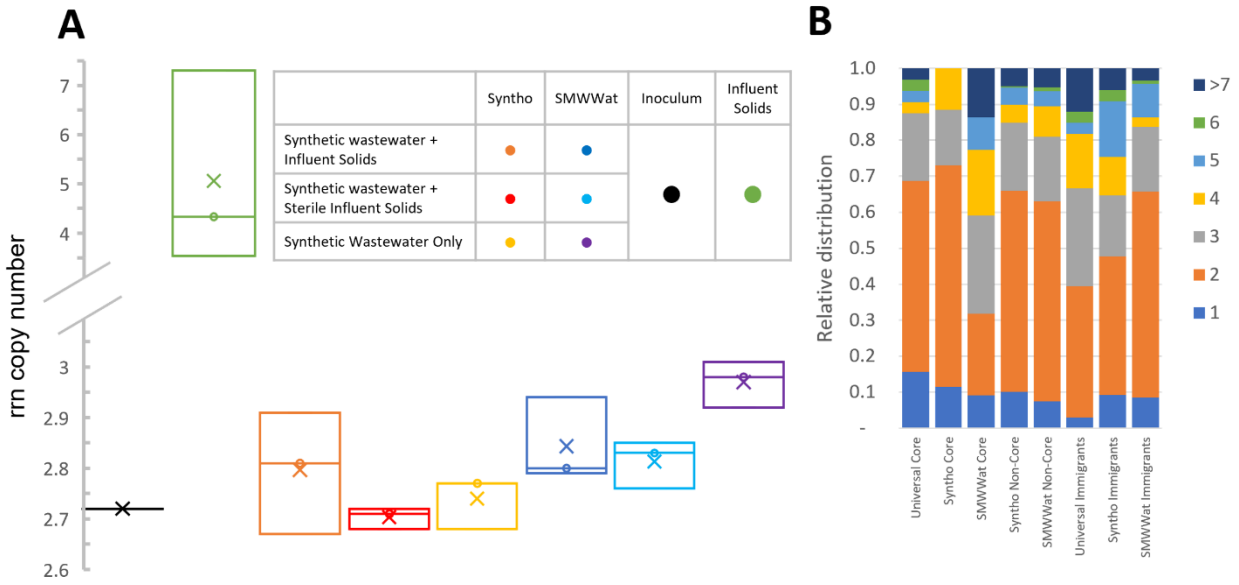

Figure 3.4. 16S rRNA operon copy number. A) Weighted average rrn copy number for reactor and influent samples at the end of Phase 2. B) Relative distribution of rrn copy number by defined population for reactors received influent solids during Phase 2.

3.3.3 Sewer immigration and the impact of wastewater composition

After *Phase 1* (4 SRTs), during *Phase 2* triplicate reactors were supplied with either influent solids, sterilized influent solids or no solids for both synthetic wastewater compositions as shown in Figure 3.1. During *Phase 3* (4 more SRTs), the influent solids supply was removed, and the reactors were fed only synthetic wastewater as in *Phase 1*.

Principle coordinate analysis was used to visualize the overall changes in the microbial community composition with immigration during *Phase 2* (Figure 3.5 A). Observing the Bray-Curtis plot, the most variation explained was due to the continued community development (ecological succession) over time and wastewater composition (Figure 3.5 A). Immigration appeared to have a minor impact on the overall microbial community composition, with the effect most likely due to changes in the relative abundance of the already existing genera. This is also reflected in Figure 3.6 which shows a decrease of the *Universal Core Residents* for both compositions and an increase in the SMWWat *Substrate-specific Core Residents* and in the *Non-core Residents* for Syntho. Finally in *Phase 3*, when influent solids were removed and all reactors received synthetic wastewater only once again, the impact of immigration appears to be reversed and the microbial community of all reactors became more similar.

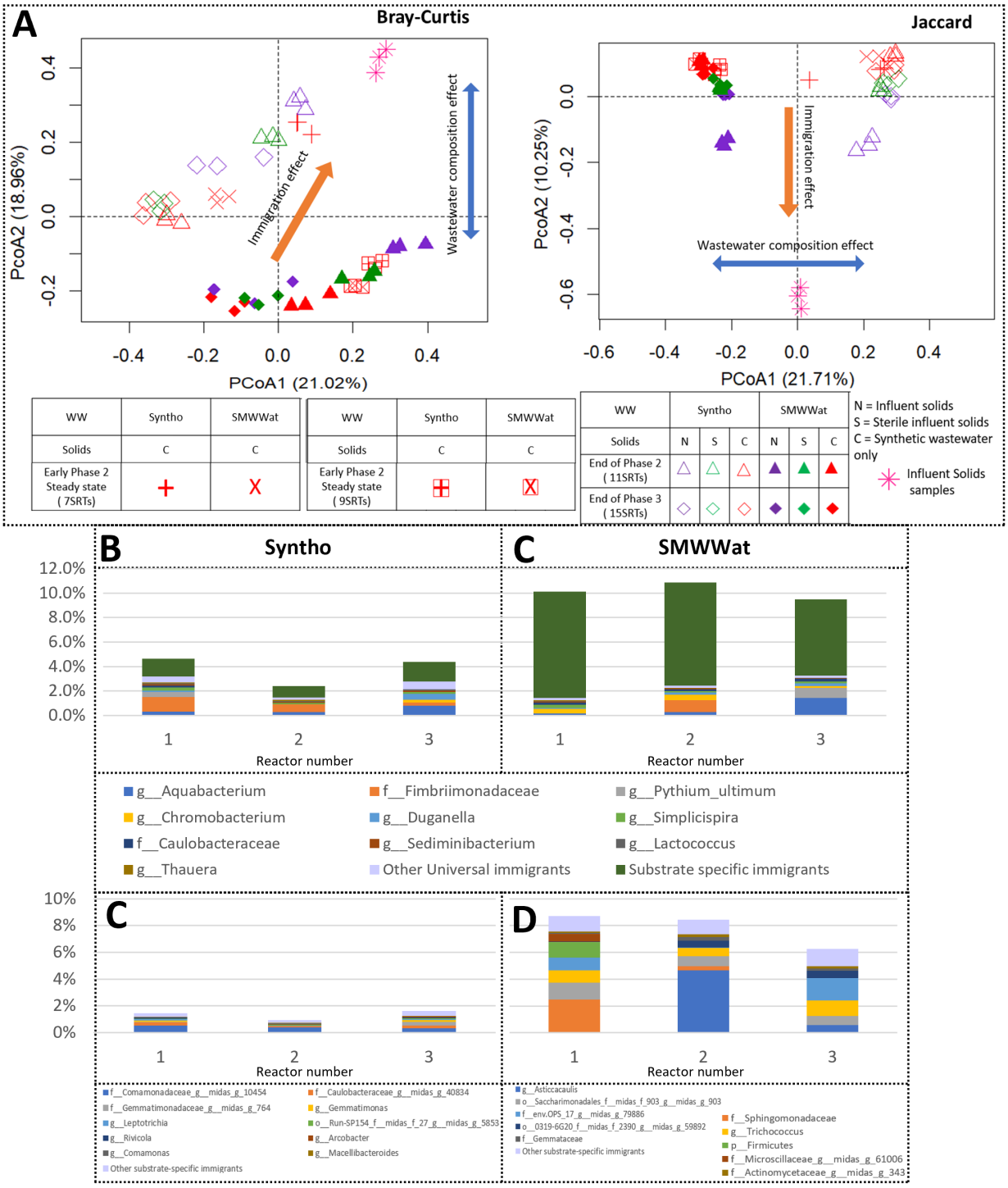

Figure 3.5. Impact of immigration on the microbial community assembly and the interaction with wastewater composition. Relative abundances are for reactors supplied with influent solids at the end of Phase 2. A) Principal coordinate analysis using Bray-Curtis and Jaccard dissimilarity. B) Relative abundance of the Universal Immigrants for both compositions. C) Relative abundance of the Syntho-specific Immigrants. D) Relative abundance of the SMWWat-specific Immigrants.

To investigate the impact of immigration in higher resolution, the community at the end of *Phase 2* was divided once more into specific populations. *Immigrant* genera were defined as those which were not present at the beginning of *Phase 2 but* appeared at the end of *Phase 2* in the reactors receiving influent solids. The *immigrant* population was further divided into two subcategories, as with the *Core Resident* population. The *Universal Immigrants* were genera identified as immigrants in reactors operated with both wastewater compositions, while the *Substrate-specific Immigrants* successfully immigrated with only one of the two wastewater compositions. The definitions for the *Core Resident* and *Non-Core Resident* populations remained the same as in *Phase 1*.

Figure 3.6 shows the population distribution for reactors that received influent solids. After *Phase 1*, the *Core Residents* represented more than 94% of the total sequencing reads. Of the total *Core Resident* population, on average 80% belonged to the *Universal Core Residents* for reactors fed with either composition. After immigration was introduced, the *Universal Core Residents* saw a decrease in their abundance for both compositions while the *Substrate Specific Core Residents* increased in the SMWWat reactors, and the *Non-Core Residents* in Syntho reactors. Based upon the population definitions, this shows that SMWWat saw an increase in genera already occurring in high abundance, while Syntho saw an increase in lowly-abundant genera. When immigration was removed during *Phase 3* (Figure 3.1), the fraction of *Universal Core Residents* returned to similar levels as those in *Phase 1*. However, the *Non-Core Residents* (representing the low abundance genera at the beginning of *Phase 1*) saw a meaningful increase in both synthetic wastewater feeds which was maintained in the absence of immigration. These results are consistent with those observed in Figure 3.6A.

The dynamics of the *Core Resident* population with immigration could be linked to specific genera. For example, among the *Substrate-specific Core Residents* , in the SMWWat reactors the abundance of the genus g__midas_g_65560 (family *Microscillaceae*) increased as a result of immigration (Figure 3.6). An analysis of the amplicon sequencing variant (ASV) composition of this genus showed high diversity with 17 different ASVs observed (Figure S5). The highest ASV diversity was seen in reactors that received influent solids regardless of wastewater composition. However, a single dominant ASV, which was present in all reactors, accounted for 91% of reads in the reactors with immigration. This implies that immigration introduced new ASVs which

occurred in relatively low abundance and did not impact the core ASV present in all reactors. This is also supported by the fact that after immigration was stopped, the dominant ASV increased its abundance to similar levels as those in *Phase 1* (before immigration).

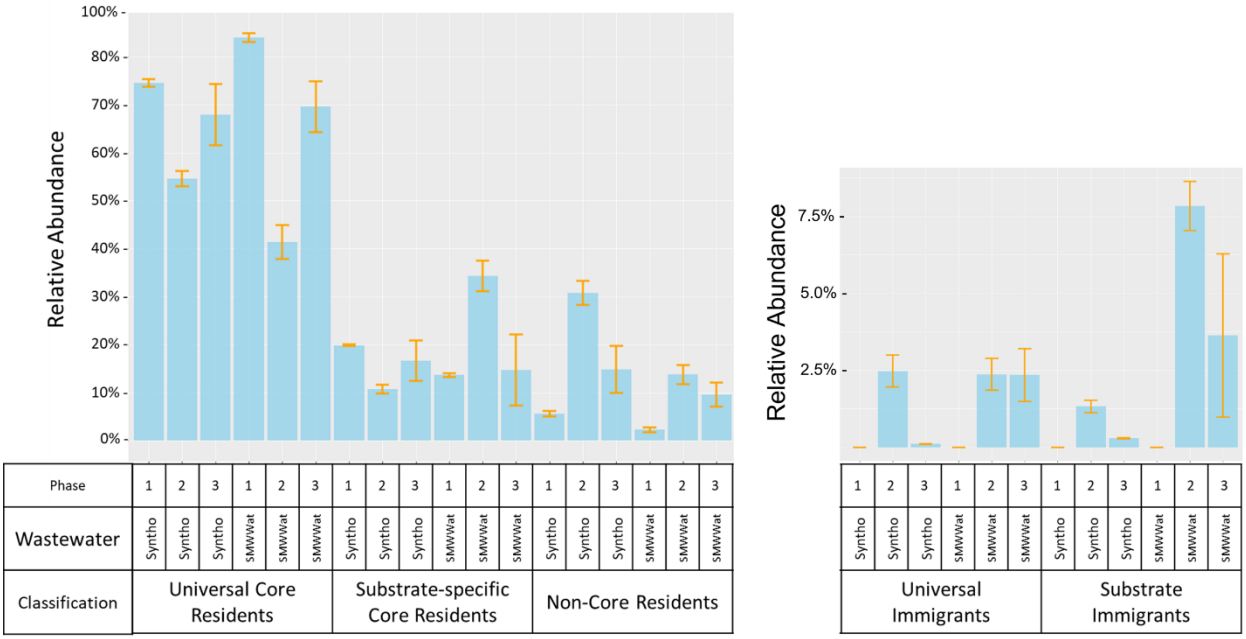

Figure 3.6. Population reads contributed for reactors that received influent solids in Phase 2. Error bars show standard error.

Immigrating genera were classified as either *Universal Immigrants* or *Substrate-specific Immigrants*. The *Universal Immigrants* accounted for a similar number of reads in reactors operated under both wastewater compositions (Figure 3.5 B and C). SMWWat had, however, a considerably higher proportion of *Substrate-specific Immigrants* in *Phase 2* (Figure 3.5 D) than that of Syntho (Figure 3.5 E). When immigration was stopped in *Phase 3*, the newly introduced genera persisted for longer in reactors fed with SMWWat while in reactors fed with Syntho the majority were lost (Figure 3.6). This is consistent with the results shown in Figure 3.6A, which demonstrate the impact of immigration to be somewhat reversed during Phase 3 as previously discussed.

Immigration increased the microbial community diversity within the reactors, as demonstrated using various alpha diversity metrics (Figure S4). SMWWat reactors also had a greater increase in the diversity index when compared to reactors operated with Syntho. This is consistent with the results in Figure 3.6 which show increased immigration in these reactors. After immigration was

removed during *Phase 3,* the diversity reduced in all reactors and returned to similar levels of those that received synthetic wastewater throughout. An increase in the Pielou and Shannon diversity indices was also noted in the reactors recevieing sterilized influent solids. This is likely due to residual DNA remaining after sterilization, or an increase in genera utilizing the influent solids as an additional food source.

3.3.4 Variations in nitrifiers' populations and nitrification performance

Within the reactors, nitrification was also impacted by the microbial community dynamics. Although COD removal was not disturbed (Figure 3.2 A), nitrification was shown to be sensitive to the community changes due to wastewater composition. The nitrate production (Figure 3.2 C) was specially affected by these differences. At the beginning of operation, no significant difference in nitrate production was observed. However, as reactor operation progressed, by the end of *Phase 1* greater nitrate production was shown in the reactors fed with Syntho. From 6 SRTs onwards, the difference between nitrate production with the two wastewater compositions stabilized and stayed the same for the remainder of reactor operation. Immigration had no observable effect on nitrate production. However, ammonia increased in concentration at SRT 5 (Figure S1) and stabilized at SRT 6. This may be attributed to the mixing of the reactors after SRT 4.

At the end of *Phase 1*, the abundance of two of the three main nitrifying genera varied between the reactors. Indeed, although *Nitrosospira* had a similar relative abundance for reactors fed with Syntho and SMWWat (0.04% and 0.05%, respectively) (Figure 3.7 A), *Nitrosomonas* and *Nitrospira* had a higher abundance for Syntho than for SMWWat at the end of *Phase 1* (Figure 3.7 B and C). In addition, *Nitrospira*, responsible for the nitrite oxidization step, was not detected in reactors fed with SMWWat, while in reactors fed with Syntho, it was detected at low relative abundances.

In *Phase 2, Nitrosospira* and *Nitrosomas* had similar relative abundances. However, in *Phase 3, Nitrosomonas* became more dominant for Syntho reactors. In *Phase 2* and *3*, the abundance of *Nitrospira* increased in the reactors fed with Syntho and influent solids. *Nitrospira* was not detected within the SMWWat reactors, even where influent solids were supplied. In the SMWWat reactors, *Nitrosospira* had the highest relative abundance, followed by *Nitrosomonas*. Finally, for both compositions, the greatest difference in *Nitrosospira* and *Nitrosomonas* abundance was observed in the reactors fed with synthetic wastewater only throughout the experimental run.

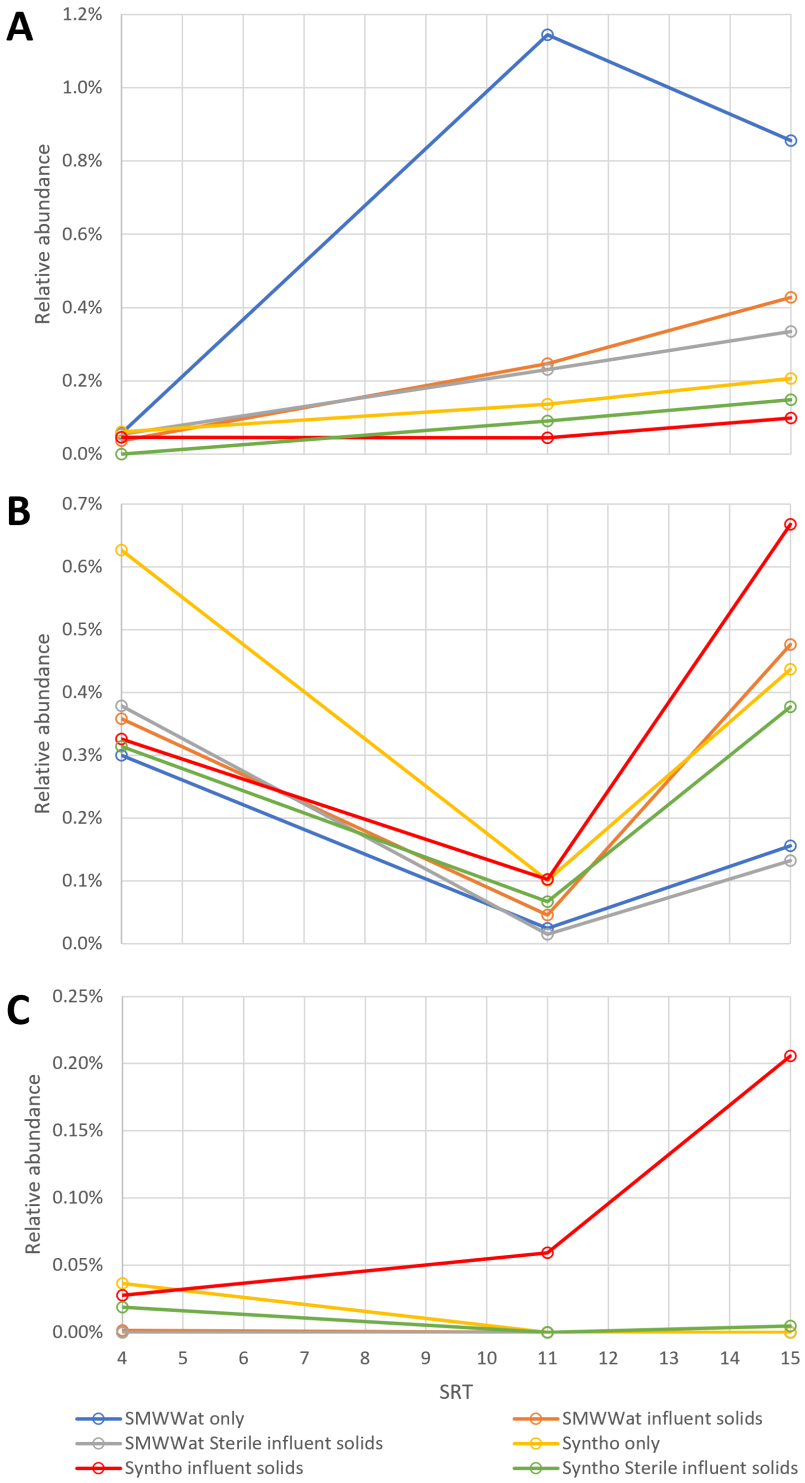

Figure 3.7. Main nitrifiers' relative abundance at the end of each Phase. Values indicated are the average of the three biological replicates. A) Relative abundance of the genus Nitrosospira. Average standard error was 0.05%. B) Relative abundance of the genus Nitrosomonas. Average standard error was 0.03%. C) Relative abundance of the genus Nitrospira. Average standard error was 0.01%.

3.3.5 The impact of wastewater composition and immigration on ARGs

The microbial community composition has increasingly emerged as a key determinant of AMR. Given the changes in the microbial community observed with different wastewater compositions and immigration, it was hypothesized that the abundance of ARGs would also likely vary. To test this hypothesis, qPCR array was used to quantify 71 different ARGs and MGEs within the reactors.

Using the qPCR array an average of 62.7 ± 4.1 (STD, n = 46) genes were detected in the mixed liquor of reactors receiving SMWWat, whilst only 59.6 ± 4.2 (STD, n = 46) genes were detected in the communities of reactors receiving Syntho (Figure 3.8 A). The highest concentrations (copies/16S rRNA gene) were found for Mobile Genetic Elements (MGEs), and Multidrug Resistant genes. For MGEs, the concentration exceeded 1, which shows the presence of several units of the same genetic elements per single bacterial cell. Using ddPCR for the detection of 15 genes (Figure 3.8 B), the influent solids were shown to have the highest concentration (copies/16S rRNA gene) of ARGs, followed by the reactors that received influent solids in *Phase 2* regardless of wastewater composition.

To test the impact of each independent variable on the abundance of ARGs and MGEs in the reactors, a three-way ANOVA was conducted. In this test, the effect between subjects was tested for the wastewater composition and influent solids condition plus the interactions between these two factors. The within subjects effect was tested for the effect of the *Phase* of operation and its interaction with the two previous factors. The ANOVA showed SMWWat to cause a significant increase in a wider variety of ARGS and MGEs than Syntho (Table 3.2). SMWWat was responsible for a significantly higher concentration in 17 genes, whilst in the Syntho reactors the composition significantly increased the concentration of only 9 genes regardless of solids conditions and the *Phase*. Particularly, the integron integrase gene intI3 which had a significantly higher concentration (> 1 copies/16S rRNA gene) in reactors fed with SMWWat whilst in reactors that received Syntho, the gene was observed sporadically. In *Phase 2,* intI3 displayed a significant increase in abundance in the SMWWat reactors that received influent solids (Table 3.2), and it was only detected in the Syntho reactors when they received influent solids (Figure 3.8 A). Finally, intI3 was undetected in the majority of reactors at the end of *Phase 3* for Syntho reactors.

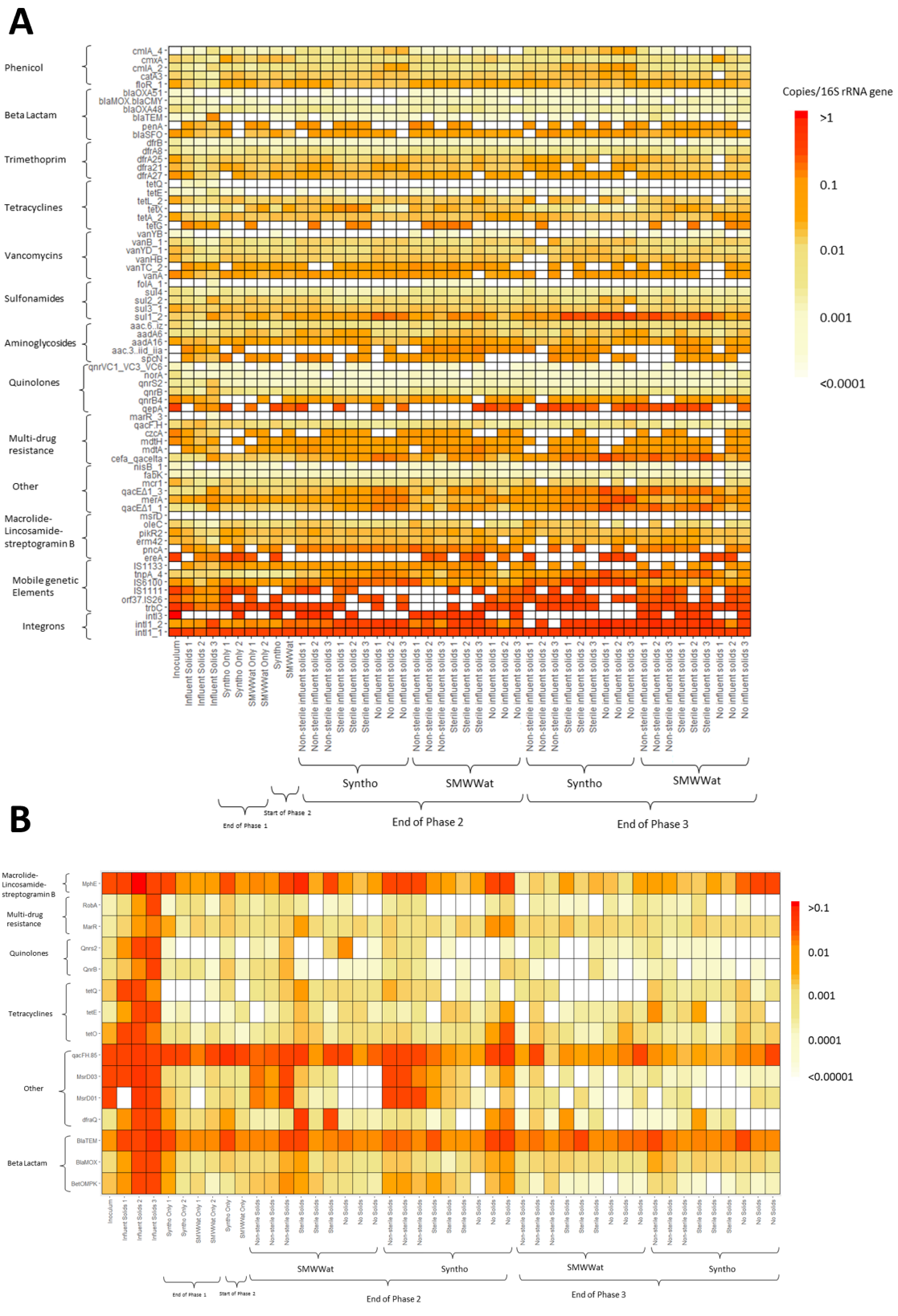

Figure 3.8. ARG content in the reactors. A) heatmap of the qPCR runs for 71 ARGs. B) heatmap of the ddPCR runs for 15 ARGs.

Table 3.2. ARGs that had a significant ($p<0.05$) impact on the concentration depending on wastewater composition or immigration as determined using a three-way ANOVA test

Category	Increased abundance due to wastewater composition		Immigration's effect	
	Syntho	SMWWat	Increasing	Decreasing
Aminoglycoside		aac(3)-iid_iia		
		aadA16		
Beta Lactam		blaMOX/blaCMY	BetOMPK	
			blaOXA48	
Integrons	intI1_1	intI3	intI3	intI1_2
MDR		marR_3		
MGE	IS6100			
MLSB	msrD	pikR2		
Other	merA	nisB_1	MsrD01	merA
			MsrD03	
Phenicol	cmlA_2	cmxA		cmlA_2
	cmlA_4	floR_1		
Quinolone		norA		
		qnrB		
		qnrS2		
Sulfonamide		folA_1	sul4	
		sul4		
Tetracycline	tetQ	tetA_2	tetQ	
Trimethoprim	dfra21	dfrB	dfrB	
Vancomycin	vanYD_1	vanYB		vanHB

MDR = Multidrug resistance
MLSB = Macrolide-Lincosamide-streptogramin B

To test whether immigration caused a significant increase in the abundance of ARGs in the reactors regardless of wastewater composition and *Phase*, the concentration was compared to that of the control groups. If immigration had an impact on the abundance of ARGs, a lower concentration would be expected in the reactors that received sterile influent solids and the lowest concentration should be in the reactors that received synthetic wastewater alone. Conversely, if immigration causes a decrease in the concentration of ARGs, the reactors that received synthetic wastewater only would be expected to have the highest abundance, followed by those that received sterile influent solids. Results from this analysis showed immigration to cause a significant

increase in the abundance of 8 genes, and a significant decrease in the abundance of 4 genes (Table 3.2). Out of the 8 genes increased by immigration only sul4 showed a significant difference between the wastewater compositions (SMWWat) and immigration, which indicates an interaction between these two factors. Of the genes decreasing in abundance with immigration three showed a significant difference with wastewater composition (Syntho) and immigration (cmlA_2, intI1_2 and vanHB).

In an effort to track the amplicon sequence variants of ARGs entering the reactors, multiplexed amplicon sequencing was used to identify sequence variants among the 15 genes quantified using ddPCR. As a recent study demonstrates, ARG-ASVs are important to understand the ARG dynamics between the influent and mixed liquor (Gibson et al., 2023b). Analysis of the gene variants using multiplexed amplicon sequencing revealed between 1 to 12 ASVs per ARG (Figure 3.9). The majority of genes analysed contained only one ASV. Various patterns in ARG-ASV dynamics were observed between the influent and activated sludge. For example, counter selection was observed in the betOMPK and blaTEM genes, with influent ARG-ASV's not detected within the reactors receiving these solids. In other cases, for example with the tetE and qnrS genes, the dominant ARG-ASV was already shared between the influent and reactor samples, thus immigration caused no change in the diversity of these genes. Other genes such as qacFH and dfrAq increased in ASV diversity during *Phase 2* in all reactors regardless of whether they received solids or not, suggesting that the new ASVs detected were present below the detection limit in *Phase 1* and increased in abundance over time. Finally, direct immigration was observed in the betOMPK gene, where ASV5, originating from the influent solids, was found to be introduced into only the reactors that received influent solids in *Phase 2*. The overall concentration for the gene betOMPK also showed a significant increasing effect of immigration (Table 3.2).

The remaining genes not discussed unfortunately, produced inconclusive results as gene variants were not detected using the multiplex amplicon sequencing approach despite being previously detected using ddPCR. Further optimization of the amplicon sequencing protocol is needed to ensure optimal primers and PCR conditions are used. In addition, improvements in the pipeline for the analysis of the sequencing data is required as the filters applied in this study seem to be somewhat stringent.

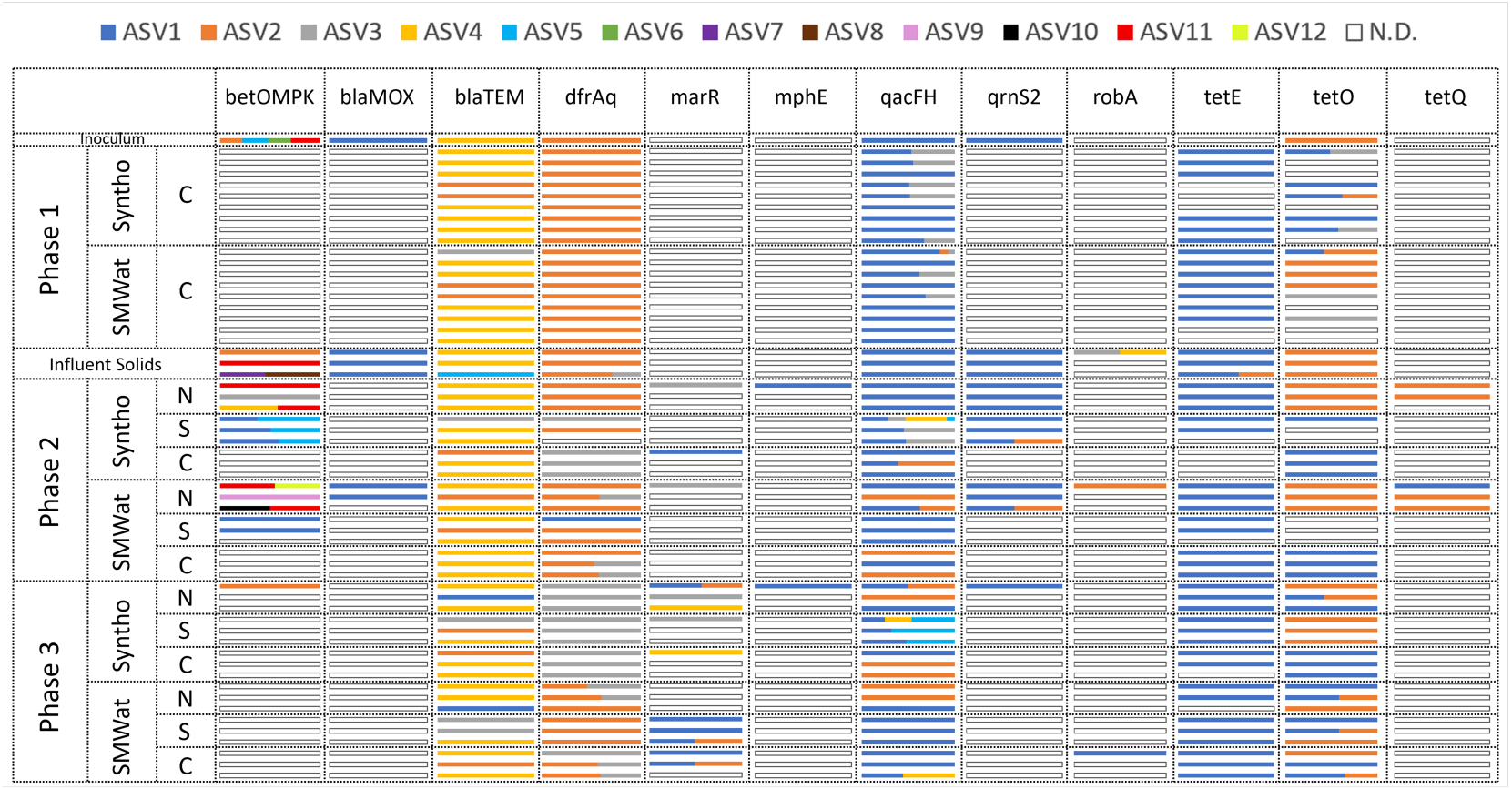

Figure 3.9. Relative abundance of different ARGs' amplicon sequence variants, legend shows variant number. N = Influent solids, S + Sterile influent solids, C = Wastewater only.

Diversity also appeared to be a driver of ARG abundance in the reactors, particularly when considering the Shannon diversity index which correlated with the genes detected (Figure 3.10 A) and the genes concentration (Figure 3.10 B). With the exception of genes associated with integron class 1, the number of genes directly correlated with diversity. This was also observed within the same antibiotic family (sulfonamides in the case of Figure 3.10 B). This trend appeared to be consistent for genes belonging to different classes of ARGs such as qacEΔ1 ($r = -0.705$, $p < 0.01$), qacF/H ($r = 0.471$, $p < 0.01$), qnrVC1_VC3_VC6 ($r = 0.492$, $p < 0.01$), sul3 ($r = 0.445$, $p < 0.01$), dfrB ($r = 0.494$, $p < 0.01$), and blaOXA48 ($r = 0.526$, $p < 0.01$) (Figure S6).

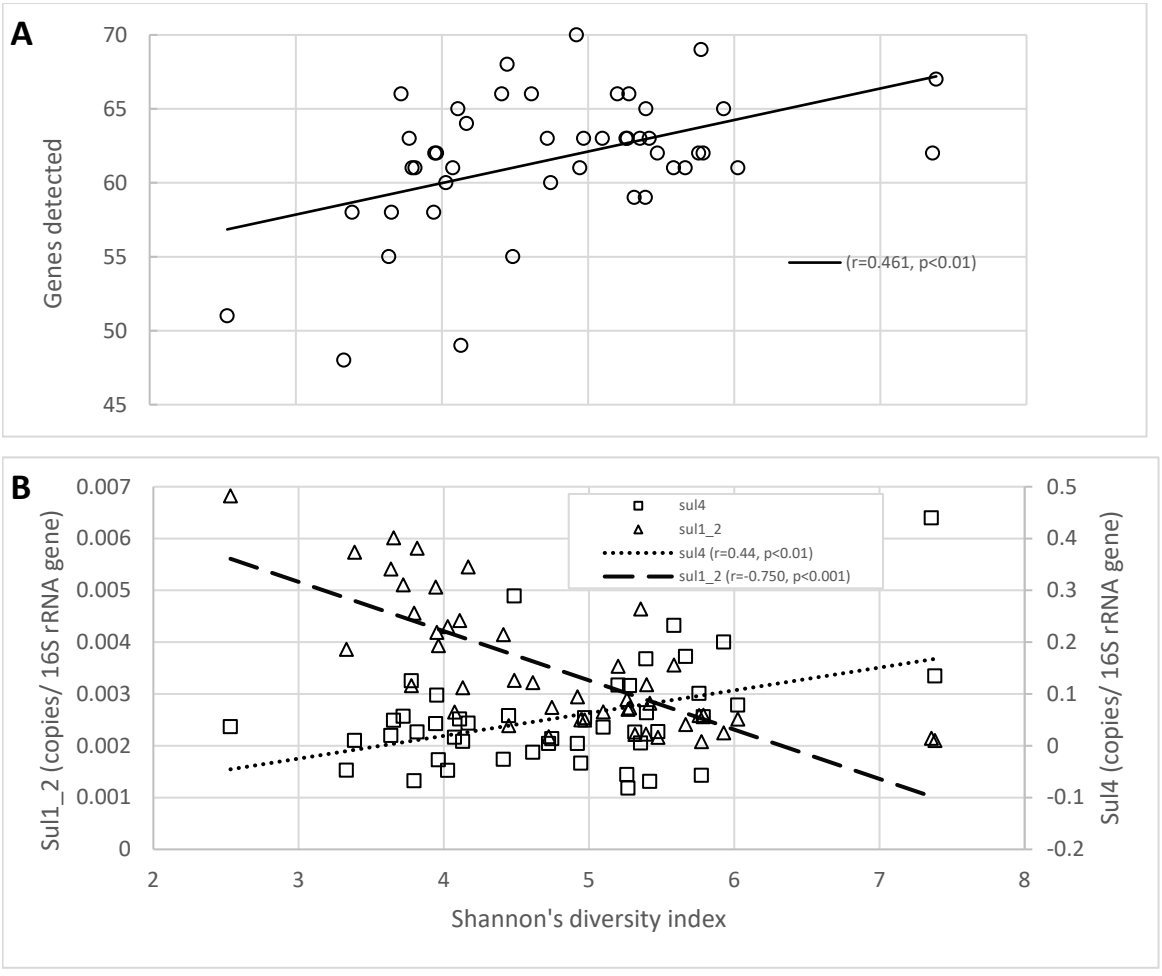

Figure 3.10. Correlation between ARGs and diversity. Legend indicates the Pearson correlation coefficient (r) and the significance probability (p). A) Correlation between the microbial community's diversity and the number of genes detected. B) Correlation between the microbial community's diversity and the relative concentration of ARGs belonging to sulfonamides category.

3.4 Discussion

3.4.1 Microbial community's assembly and stability

Whilst steady state of reactor operation appeared to be reached after 4 SRTs (28 days), the microbial community composition continued to significantly evolve until around 11 SRTs. These results showed that the microbial communities' stability of an activated sludge system cannot be judged on the basis of steady state of the operational parameters. Based upon reactor theory, it is typically assumed that steady state is achieved within 3 SRTs (Iacopozzi et al., 2007). However, results from this study demonstrate this assumption only applied to specific parameters. When considering the Bray-Curtis's distances (Figure 3.2 D) and the stabilization of the nitrifiers's population (Figure 3.7), a more stable microbial community was reached after 12 SRTs. Future studies should consider longer stabilization periods to ensure a stable microbial community is established.

Even when the community is relatively stable, and no disturbances are imposed to the system (like immigration or change in conditions), the microbial community seems to be in a constant state of change (Figure 3.2 D). This change appears to be due to small fluctuations in the relative abundance of the dominant genera and even ASVs. Since a wastewater highly diverse in substrates produces a highly diverse community, this would imply these aforementioned fluctuations would be higher in more heterogenous wastewaters. This last conclusion is supported by our results that showed SMWWat to yield a greater Bray-Curtis distance between two successive sampling points (Figure 3.2 D).

Wastewater composition was also shown to have a significant impact on the microbial community composition of the reactors. Although a large fraction of the community was shared (*Universal Core Residents*; Figure 3.3 B), there was also a statistically significant difference between the communities yielded depending on wastewater composition supplied (*Substrate-specific Core;* Figure 3.3 A). Results suggested that a more diverse wastewater composition will yield a more diverse microbial community. This is consistent with previous studies which demonstrate wastewater composition to have a significant impact on the activated sludge microbial community (Wu et al., 2019; Yang et al., 2020; Yu et al., 2020b). However, many of these studies consider wastewaters with vastly different compositions, for example from different sources. In the current study, the organic compounds used in both wastewater compositions were from the

same chemical family with similar molecular structures and the total COD was equal (Table 3.1, Table S1 and Table S2). Despite this, significantly different communities were yielded. These results highlight the need to characterize the composition of the organic matter to fully understand the ecological niches in wastewater and accurately assess the assembly of the microbial community in activated sludge.

Taken together, these results demonstrate the need to consider each wastewater constituent and not only the COD level when studying microbial community assembly in the activated sludge.

3.4.2 Heterotrophic immigration

Results from this study demonstrate that immigration has only a small impact on the overall microbial community of the activated sludge. These results are consistent with Yu et al. (2021)'s findings that a well-established WWTP's microbial community is stable, resisting selective pressure and mass-immigration. Furthemore, Dottorini et al. (2021)'s showed that immigration represents only small fluctuations in abundance of some well-established species. This was true even for reactors fed with SMWWat, which showed the highest proportion of immigration, as only small changes were seen in Bray-Curtis and Jaccard's principal coordinate analysis (Figure 3.5 A). However, even if small, these community changes due to immigration seem to be important because they are likely to influence other phenomena related to the microbial community.

The SMWWat organic matter composition appeared to create a better environment for immigrant taxa to thrive even during *Phase 3*, after the incoming supply of bacteria from the sewers was stopped (Figure 3.6). This composition also sustained higher diversity over time, even while the microbial community was still developing (Figure S3). This is most likely due to the variety of ecological niches available due to the more complex wastewater composition, but also to the availability of substrate for immigrating bacteria due to the low biodegradability rate. These results are consistent with those of Yuan et al. (2019a)'s who demonstrate interactions between stochastic and deterministic factors and highlight the need to consider these parameters when studying microbial community assembly in the activated sludge. In the case of this study, immigration and wastewater composition, respectively.

3.4.3 rRNA operon copy number as a functional analysis

The results showed a decrease in the 16S rRNA operon copy number after *Phase 2*, which may be indicative of the community changes stabilizing (Nemergut et al., 2016; Shrestha et al., 2007). Additionally, in *Phase 2*, the highest copy number was observed for the sewer influent solid samples, followed by reactors fed with SMWWat and reactors fed with Syntho in that order (regardless of solids condition). The difference in copy number could be particularly observed in the *Substrate-specific Core Residents*, where Syntho *Substrate-specific Core Residents* had copy numbers below 4, while SMWWat *Substrate-specific Core Residents* had the highest proportion of taxa having a copy number of 7 or more. This copy number has also been shown to be indicative of a high level of competitive fitness in adverse environments (Klappenbach et al., 2000), and particularly to a higher chemotactic motility, genome streamlining but low carbon utilization efficiency (yield) (Roller et al., 2016). The higher copy number in the SMWWat reactors would indicate a high competitive fitness of the microbial community due to the slow biodegradability and high heterogeneity of the feed. Based on this observation, the remarkably high copy number of the influent solid samples, would imply that the sewer microbial community is likely enriched by slowly-biodegradable-substrate consumers. This is contrary to what was previously thought based on this same metric (Guo and Frigon, 2023). This high copy number in the SMWWat reactors would also suggest an ecological similarity between the sewers and SMWWat.

3.4.4 The relationship between immigration, wastewater composition and nitrifiers

Nitrification was also impacted by the organic composition of the wastewaters. The population of nitrifying bacteria appeared to limit the nitrification process, and potentially, the ammonification rate. Among the SMWWat reactors, the difference in the relative abundances of ammonia oxidizing bacteria (Figure 3.7 A and B) may have impacted the production of nitrate (Figure 3.2 C). For example, reactors fed with SMWWat showed a higher relative abundance of the genus *Nitrosospira* while reactors fed with Syntho had a higher abundance of *Nitrosomonas*. In previous studies, *Nitrosospira* have been shown to have a lower ammonia oxidation rate than *Nitrosomonas* (Taylor and Bottomley, 2006). Consequently, the nitrification rate is likely to be impacted by these community differences caused by different wastewater compositions. However, it is important to note that these results do not consider ammonia oxidizing archaea, which are important to nitrification (Huang et al., 2021), either because they occurred in abundances below the detection

limit, or due to the bias of primers used towards specific groups of bacteria (Baker et al., 2003). Taken together, these results demonstrate how differences in the organic matter composition alone can impact the performance of critical processes such as nitrification in the wastewater treatment process.

The ammonification step may also have impacted nitrification within the reactors. Previous studies suggest ammonification not to be a limiting step in the nitrification process (Katipoglu-Yazan et al., 2012), however results from the current study suggest otherwise. In the study by Katipoglu-Yazan et al. (2012), the ammonia source (peptone, dried meat extract and urea) is likely to have been more readily bioavailable than in SMWWat, as the latter contained particulate organic matter. Consequently, these compositional differences may have added an impact on the ammonification process. This illustrates the sensitivity at the genus level of the nitrification process to differences in the organic matter composition.

In terms of immigration, *Nitrospira* was particularly and positively affected by the addition of influent solids (Figure 3.7 D). This is consistent with previous studies where immigration was predominantly significant for this genus (Yu et al., 2018; Yu et al., 2020a). However, immigration also appeared to be impacted by wastewater composition. Indeed, the reactors fed with Syntho and supplied with influent solids exhibited the highest proportion of immigrating *Nitrospira*. Additionally, the genus *Nitrosomonas* also seemed to have been positively impacted by immigration regardless of wastewater composition. Immigration appeared to have less of an impact on the ammonia oxidizing genera than nitrite oxidizing genera. This may potentially be due to their low relative abundance in the system, which in turn would support the hypothesis that immigration mainly impacts developed communities by supplying with an influx of already existing taxa (Dottorini et al., 2021; Yu et al., 2021).

3.4.5 The impact of wastewater composition and immigration on AMR in activated sludge

Using PCR based approaches, the current study showed that wastewater composition can greatly impact prevalence of ARGs. Wastewater with a more heterogenous composition with a high slowly biodegradable COD fraction (SMWWat) yielded higher ARG diversity and increased concentrations of certain genes (Figure 3.8 A and Table 3.2). Particularly communities fed with aromatic compounds have linked to exhibit co-occurrence of ARGs and aromatic degradation genes (Xia et al., 2019). This conclusion is also supported by a previous study where bacteria that

thrives in adverse environments would carry more ARGs (Allen et al., 2019), which is related to competitive fitness costs. Another explanation for a highly heterogenous wastewater yielding higher ARGs, is the fact that the microbial communities engendered are also highly diverse (Figure S3). This would seem to suggest that these resistant genes are related to a specific host with specific ecological niches because a high diversity would hinder the growth of an already dominant bacterial taxa as there would be more competition for a particular ecological niche. This would, in turn, allow for low abundant taxa to compete for the same ecological niche. This correlation between ecological niches and ARGs would be supported by Zhang et al. (2019), who showed that the fate of ARGs is strongly correlated with the fate of their host. In this sense, an analysis of the relative abundance of two of the most abundant phyla in the microbial community and related to the carrying of the gene tetQ (*Proteobacteria* and *Bacteroidetes*) (Yuan et al., 2019b), revealed an increase in the presence of these phyla in the reactors fed with Syntho (Table S5). Coupled with the results from the PCR array, where the concentration of the gene tetQ was significantly increased in Syntho-fed reactors, these results seem to support the conclusion of a relationship between a host with specific ecological niches related to certain ARGs.

In the current study, microbial diversity affected the persistence of ARGs. As previously discussed by Klümper et al. (2023), diversity may act as barrier to the introduction of ARGs in soils when no significant bacterial immigration happens. However, in aquatic environments, their results were inconclusive on the relative impact of diversity on AMR. Our results suggest that contrary to what occurs in soil, in aquatic environments, rich and diverse in organic matter such as wastewater, microbial diversity acts as a driving force to the increase of the presence of ARGs (Figure 3.10). In this sense, a more complex wastewater matrix would yield a higher abundance and presence of these resistance genes. This is also in line with the results from the ANOVA analysis where the reactors that were fed with SMWWat had a significantly higher concentration in 17 genes compared to the 9 genes that had a significantly higher concentrations in the reactors that were fed with Syntho.

Immigration increased the abundance of 8 genes in the activated sludge and decreased the abundance of 4 genes out of the 81 genes tested (Table 3.2). The increase in gene diversity with immigration could be related to bacterial diversity, as an increase in diversity indexes was observed with immigration (Table S4). Additionally, given that an interaction was found between

wastewater feed and immigration (an increasing effect for SMWWat and a decreasing effect for Syntho), sewer immigration would seem to increase ARG content in the presence of a heterogenous and slowly biodegradable organic matter wastewater that can nurture a high microbial diversity. Using multiplexed amplicon sequencing, ARG ASV immigration was observed for only one gene, betOMPK. This, once again, suggests that the increase in the abundance of ARGs with immigration was associated with the changes in microbial diversity rather than the introduction of genes from the sewers. This increase could be associated with the number of potential hosts available, and their level of fitness in the environment (Gibson et al., 2023b).

Based on these results, organic matter composition and immigration significantly impacted AMR within the activated sludge reactors. Organic matter content was a key determinant of microbial community diversity, which correlated with AMR within the reactors. Consequently, in order to better manage the release of ARGs into the environment by WWTPs, the control of organic matter is key to reducing the prevalence of these genes. Particularly, careful attention should be paid to the presence of aromatic and other slowly biodegradable compounds.

3.5 Conclusions

This study's results showed that the wastewater organic matter composition, particularly the slowly and rapidly biodegradable fractions, greatly impacted the microbial community and ARG dynamics in activated sludge:

- Significantly different microbial communities were assembled in reactors receiving slowly and rapidly biodegradable substrates.
- Slowly biodegradable substrates foster a higher rate of immigration and a lower nitrate production by favoring the growth of *Nitrosospira*.
- Slowly biodegradable substrates also enhanced the concentration of ARGs in activated sludge.
- The microbial community can be understood to be in constant drift with small fluctuations in the abundance of dominant taxa.
- A minimum of 12 SRTs is recommended to achieve community stability.
- Microbial diversity is an important driver of ARG content in the activated sludge.

- Immigration affects the assembly of microbial communities and is impacted by wastewater composition.
- Immigration appears to impact the relative abundance of *Nitrospira* more than other nitrite oxidizing bacteria.
- The increase in ARG diversity and abundance with immigration was likely associated with the changes in the microbial community composition. Only the betOMPK gene appears to be increased by direct immigration between the influent and activated sludge

3.6 References

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Chapter 4. Conclusions

This study investigated the influence of wastewater organic matter composition and immigration on the microbial community and resistance genes in activated sludge. Our findings show that the organic matter composition governs the microbial community assembly, the rate of immigration of new and existing taxa, the nitrification process, and the presence and abundance of ARGs. In this sense, a more chemically heterogenous and relatively slowly biodegradable wastewater composition will yield a higher microbial diversity, a higher rate of immigration of new taxa, a lower nitrate production and a higher number of ARGs detected.

Ecological succession in the microbial community composition was observed up to 12 SRTs. The microbial community should be understood as in constant drift with small fluctuations in the most abundant taxa. Future studies should consider the impact of these drifts in their experimental design. To ensure a stable microbial community is formed, operating reactors for a minimum of 12 SRTs would be recommended.

Results suggest that wastewater containing more slowly biodegradable substrates may be more likely to contain higher concentration of ARGs and mobile genetic elements. Whilst one ARG, betOMPK, appeared to increase in abundance due to direct immigration of the gene, all others which increased appeared to be impacted by the microbial community changes alone. Microbial diversity was observed to be a driving force for the detection of ARGs in activated sludge. As a consequence, a wastewater composition which procures a microbial community with high diversity will likely also exhibit a higher diversity in ARGs detected and higher concentrations of such genes. In order to better manage the discharge of ARGs into the environment, careful attention should be paid to the impact of deterministic factors on microbial diversity in activated sludge systems.

Finally, since the main aim of this study was to set the basis to analyze the dynamics of ARGs and the microbial community due to immigration and organic matter composition, no antimicrobials were spiked. In consequence, further studies with concentrations of antimicrobials under the minimum inhibitory concentration should be performed as a next step.

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Appendix

Additional figures

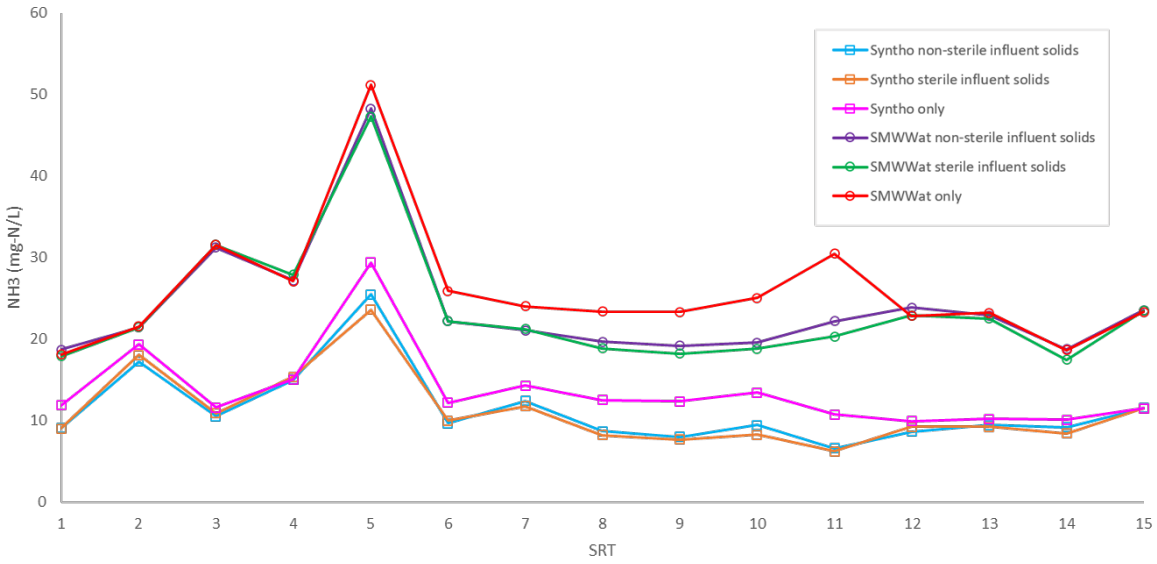

Figure S1. Ammonia concentration of the wastewater treatment effluent for the reactors fed with different wastewater compositions and different influent solids conditions.

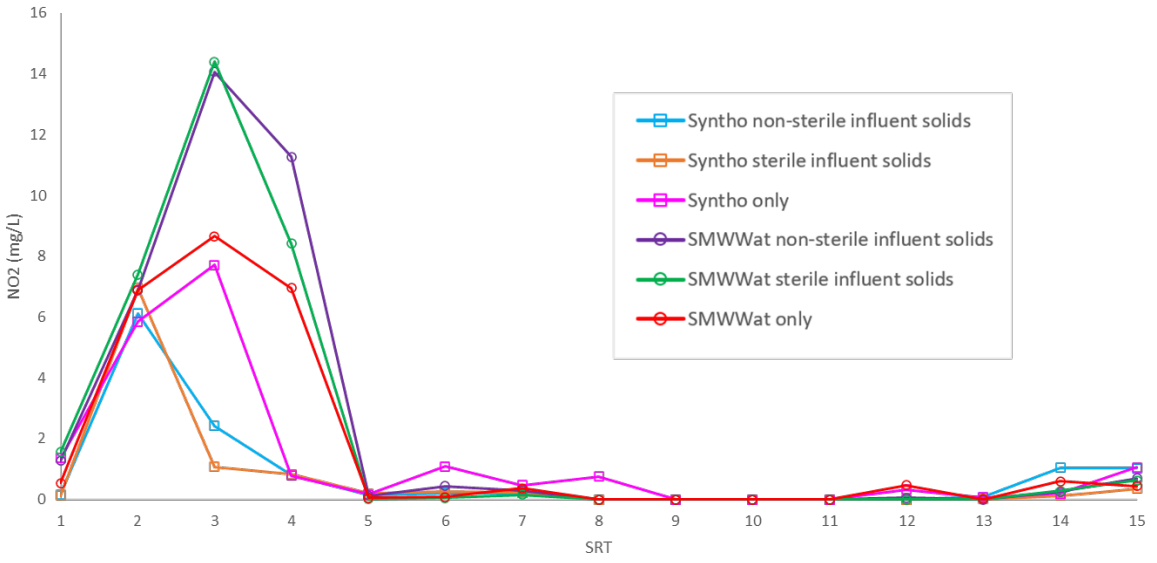

Figure S2. Nitrite concentration of the wastewater treatment effluent for the reactors fed with different wastewater compositions and different influent solids conditions.

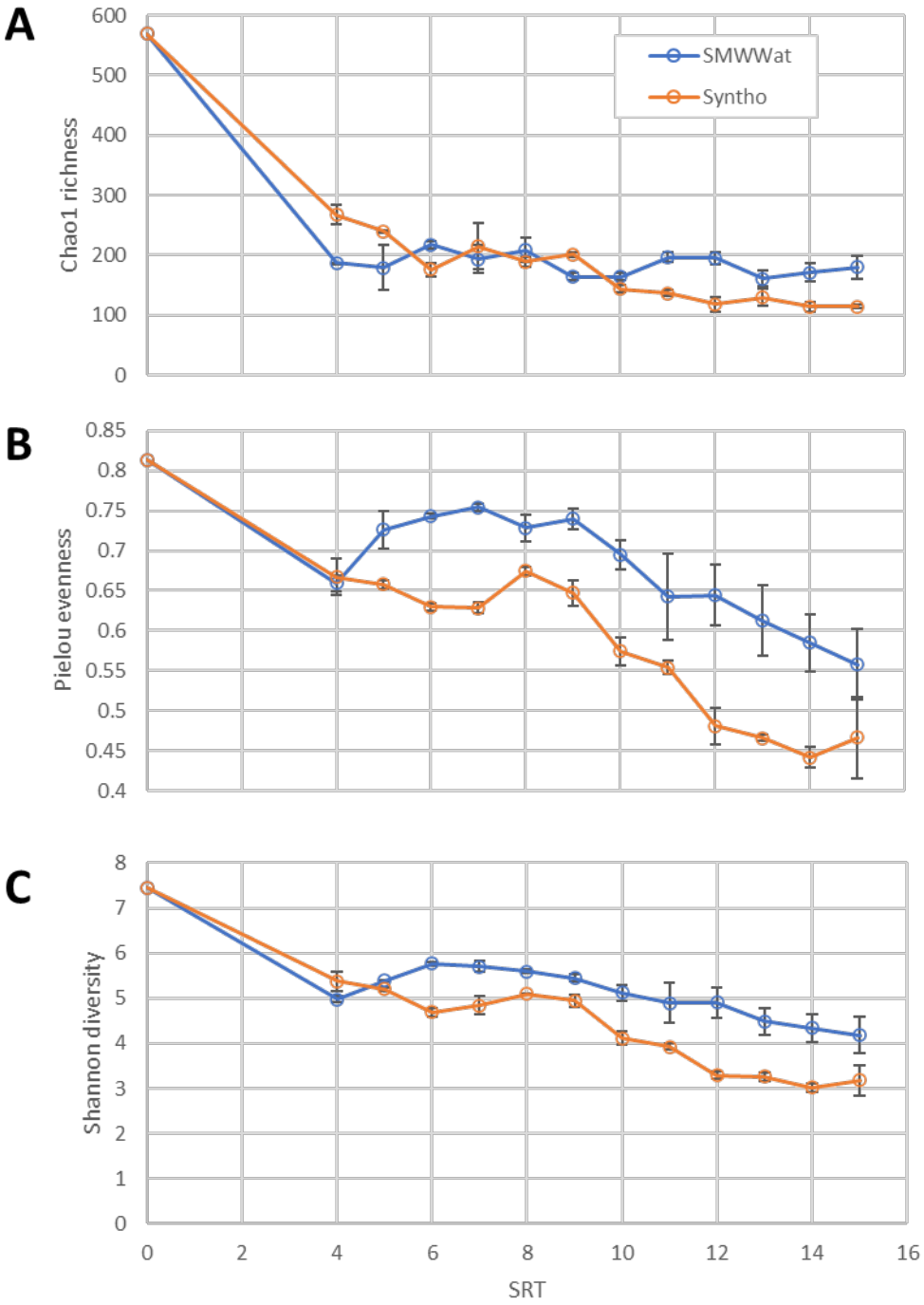

Figure S3. Alpha diversity metrics of the reactors fed with only synthetic wastewater.Bars indicate standard error. A) Chao1 richness index. B) Pielou evennes index. C) Shannon diversity index.

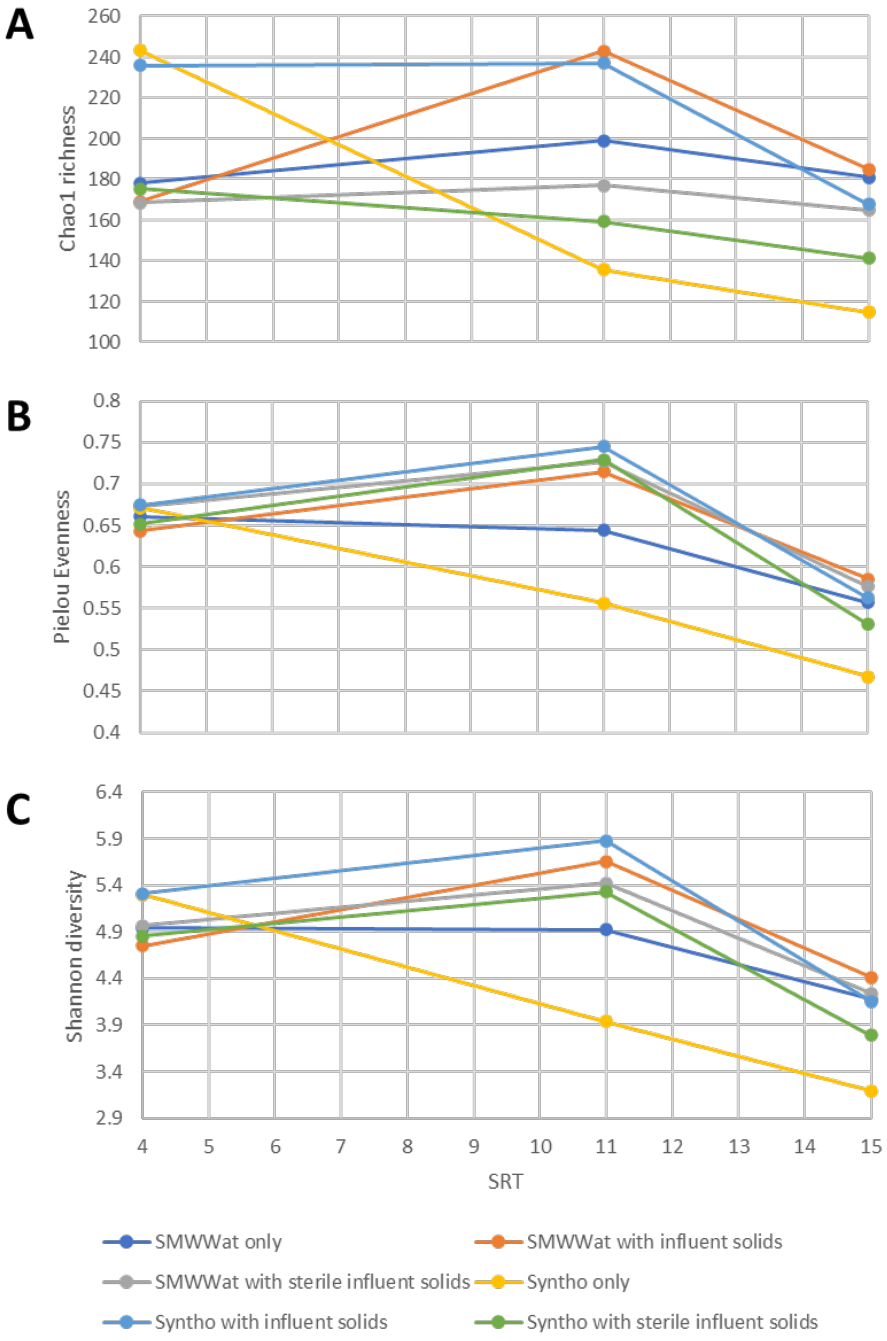

Figure S4. Alpha diversity metrics of the all reactors at the ened of Phase 1, 2 and 3. A) Chao1 richness index. B) Pielou evennes index. C) Shannon diversity index.

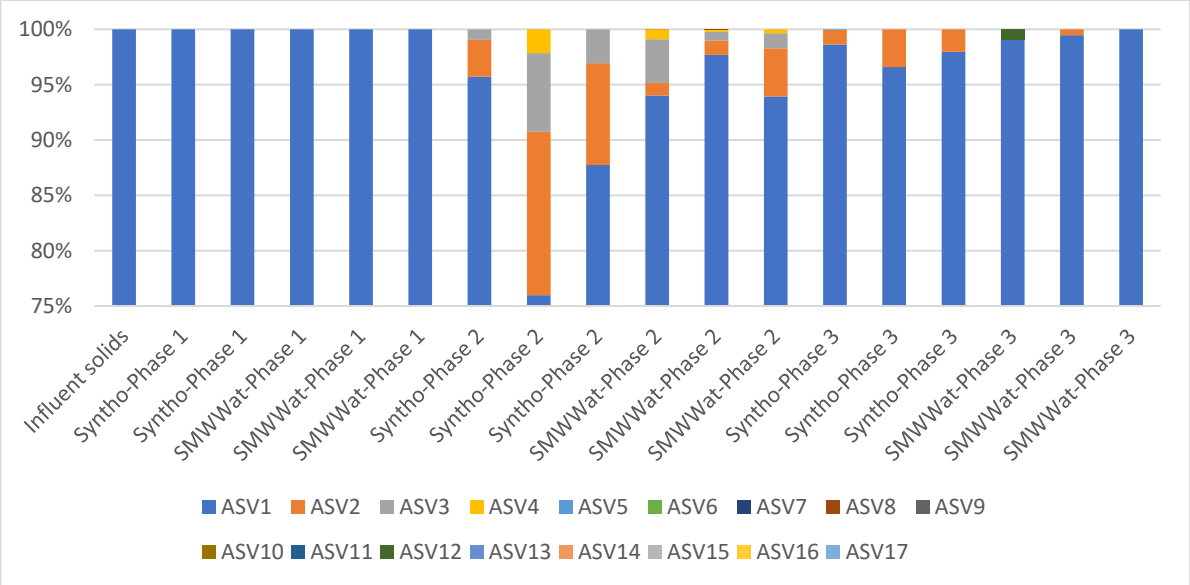

Figure S5. Amplicon sequence variant composition for the genus g__midas_g_65560 (MIDAS database, family Microscillaceae).

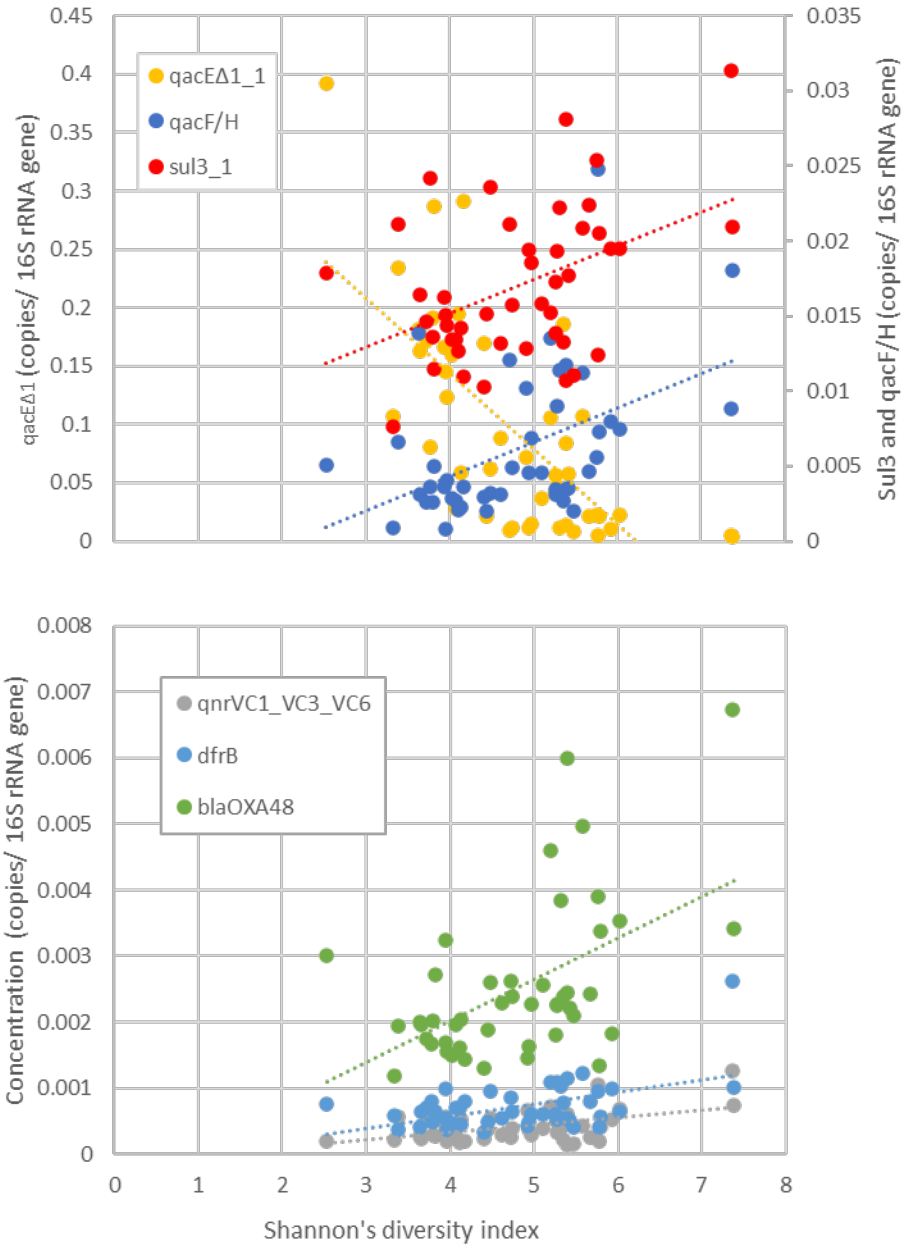

Figure S6. Correlation between the microbial community's diversity and the relative concentration of ARGs. Pearson correlation coefficient (r) and the significance probability (p) for each gene are as follows qacEΔ1 ($r = -0.705$, $p < 0.01$), qacF/H ($r = 0.471$, $p < 0.01$), qnrVC1_VC3_VC6 ($r = 0.492$, $p < 0.01$), sul3 ($r = 0.445$, $p < 0.01$), dfrB ($r = 0.494$, $p < 0.01$), and blaOXA48 ($r = 0.526$, $p < 0.01$).

Additional tables

Table S1. Composition of Syntho*: nitrogen, phosphorus, and COD content.*

Constituent	Supplier	Concentration (mg/L)	Total	Fractions					
			cCOD	Nitrogen	Phosphorus	Carbohydrates	Proteins	Lipids	Others
			(mg-COD/L)	(mg-N/L)	(mg-P/L)	(mg-COD/L)			
Acetate	Fisher Sci, 136118	120	91.3	-	-	-	-	-	91.3
Dry meat extract	BD, 211520-500G	15	13.7	1.9	-	4.0	9.7	-	-
Glycerol	Fisher Sci, BP229-1	40	46.6	-	-	46.6	-	-	-
Starch	Fisher Sci, S516-500	50	78.0	-	-	78.0	-	-	-
Low Fat Milk Powder	Nestle, 43402073	120	160.7	6.8	1.2	82.8	75.1	2.8	-
Sodium Dodecyl Sulfate	Aldrich Chemistry, 289957-500G	10	17.9	-	-	-	-	17.9	-
Genapol ®X-080	Sigma Aldrich, BCBN8857V	10	24.2	-	-	-	-	24.2	-
Peptone	Fisher Sci,BP142-500	15	16.2	1.4	-	3.6	12.6	-	-
Urea	Anachemia, 96238-380	75	-	35.0	-	-	-	-	-
Uric Acid	Sigma Aldrich, U2625-25G	9	-	3.0	-	-	-	-	-
Ammonium Chloride	Fisher Sci, 135887	11	-	2.9	-	-	-	-	-
Tripotassium Phosphate	Acros Organics, 387685000	20	-	-	2.7	-	-	-	-
Magnesium Hydrogen Phosphate	Chem Cruz, SC-250282	25	-	-	4.5	-	-	-	-
Diatomaceous earth	Fisher Sci, S75114	10	-	-	-	-	-	-	-
TOTAL			448.5	51.0	8.4	214.9	97.5	44.8	91.3
COD fraction						48%	22%	10%	20%

Notes.
*COD fractions were estimated from the measured total COD and typical portion and their associated COD.
*Carbohydrate, protein, and lipid content in dried meat extract (Pearson, 2003), low-fat milk powder (US Dairy Export Council, 2013; USAID, 2006), and peptone (Thermo Fisher Scientific, 2019) was estimated from typical composition of these constituents. Equivalent COD content for carbohydrates, proteins, lipids, sodium acetate, glycerol, starch, sodium dodecyl sulfate, and Genapol® X-080 was estimated using Rittmann and McCarty (2020)'s model. Average chemical compositions for carbohydrates, proteins, and lipids were used for the latter estimation. The formulas used are $C_6H_{10}O_5$, $C_4H_{6.2}O_{1.2}NP_{0.01}$, and $C_8H_{16}O$ (Rittmann and McCarty, 2020), respectively. The average measured COD for the whole composition was 450 mg/L.
* COD measures were obtained using the Standard Methods 5220D (American Public Health Association et al., 2017).

Table S2. Composition of SMWWat: nitrogen, phosphorus and COD content.

Constituent	Supplier	Concentration (mg/L)	Total	Fractions					
			cCOD	Nitrogen	Phosphorus	Carbohydrates	Proteins	Lipids	Others
			(mg-COD/L)	(mg-N/L)	(mg-P/L)	(mg-COD/L)			
Propionate	Sigma-Aldrich, 1880	27.0	32.7	-	-	-	-	-	32.7
Prenol	Fisher Sci, AAA1608914	14.0	50.3	-	-	-	-	-	50.3
Inulin	Fisher Sci, CAAAA18425-09	62.0	67.7	-	-	67.7	-	-	-
Dimethyl phthalate	Tci America, P0302500G	5.0	6.8	-	-	-	-	-	6.8
Tween 80	Sigma-Aldrich, P1754	4.5	7.3	-	-	-	-	-	7.3
Egg Yolk	BRUNBRAE	130.0	188.2	2.0	0.8	7.5	41.4	139.3	-
Egg White	BRUNBRAE	280.0	72.2	6.3	0.4	-	72.2	-	-
Dry meat extract	BD, 211520-500G	9.5	8.7	1.2	-	2.5	6.2	-	-
Peptone	Fisher Sci,BP142-500	14.0	15.1	1.3	-	3.3	11.8	-	-
Urea	Anachemia, 96238-380	75.0	-	35.0	-	-	-	-	-
Uric Acid	Sigma Aldrich, U2625-25G	9.0	-	3.0	-	-	-	-	-
Ammonium Chloride	Fisher Sci, 135887	11.0	-	2.9	-	-	-	-	-
Tripotassium Phosphate	Acros Organics, 387685000	20.0	-	-	2.7	-	-	-	-
Magnesium Hydrogen Phosphate	Chem Cruz, SC-250282	25.0	-	-	4.5	-	-	-	-
Diatomaceous earth	Fisher Sci, S75114	10		-	-	-	-	-	-
TOTAL			449.0	51.6	8.3	81.1	131.6	139.3	97.1
COD fraction						18%	29%	31%	22%

Notes.
*COD fractions were estimated from the measured total COD and typical portion and their associated COD.
*The estimated COD content of propionate, prenol, inulin, dimethyl phthalate and Tween 80 were estimated using Rittmann and McCarty (2020)'s model. The COD content of the lipid, carbohydrate and protein constituents was estimated using the average chemical compositions for carbohydrates, proteins, and lipids (Rittmann and McCarty, 2020) and the common fractions in eggs (Yamamoto, 1997). Concentrations were determined to meet Raunkjær et al. (1994)'s fractions. The average measured COD for the whole composition was 450 mg/L.
* COD measures were obtained using the Standard Methods 5220D (American Public Health Association et al., 2017).

Table S3. Annealing temperature for the Droplet Digital PCR run.

63 (°C)	62 (°C)	61 (°C)	59 (°C)
BlaTEM	BetOMPK	MarR	qacFH85
dfraQ	BlaMOX	MsrD01	
QnrB	MphE		
Qnrs2	MsrD03		
tetE	RobA		
tetO			
tetQ			

Table S4. Primer list of the 15 genes analyzed using ddPCR and multiplex amplicon sequencing.

Gene-Direction	Sequence
BetOMPK-F	ACCGGTTACGGCCAGTGGGA
BetOMPK-R	GCAAAGTTCAGGCCGTCAACCAGA
BlaMOX -F	ACCAGCTCGGCGGATCTG
BlaMOX -R	GAGCCGGTCTTGTTGAAGAGC
BlaTEM-F	GAACCGGAGCTGAATGAAGCC
BlaTEM-R	CGGGAGGGCTTACCATCTGG
dfraQ -F	ACATACCCTGGTCCGCGAAAG
dfraQ -R	CGCCACCAGACACTATAACGTGA
MarR-F	CACAGTTTAAGGTGCTCTGCTCTATCC
MarR-R	GCAAATACTCAAGTGTTGCCACTTCG
MphE-F	AAGTGAGCAATTGGAAACCCGCTA
MphE-R	AGGCCGCTGCTCTTTCTAAAGTC
MsrD01-F	GGCAAGCTAGGTGTTGAGCAATTAG
MsrD01-R	CCTTCACGATCTAAATGGCTCGTA
MsrD03-F	GGCAAGCTAGGTGTTGAGCA
MsrD03-R	TCCTTCACGGTCTAAATGGCTCGTA
qacFH-85-F	CTGTTTCAATCTTTGGCGAGGTCA
qacFH-85-R	TTTAGAACGGCGACACCACTG
QnrB-F	CGACCTGAGCGGCACTGAATTTA
QnrB-R	GCTCGCCAGTCGAAAGTCGAA
QnrS2-F	ATGCCAGCTTGCGATGGCAAA
QnrS2-R	GTGGCATAAATTAGCACCCTGTAGGC
RobA-F	TCAAATGCGCGTGCAGTTCTGG
RobA-R	GTAGCGCTCAATATCCTGACCTTTAC
tetE-F	TGATTGCTGGACCAGTCATTGG
tetE-R	CCATACGAAGCGCTCTTCTCC
tetO-F	GCAGGGACAGAACTATTAGAGCCATATC
tetO-R	GCTAACTTGTGGAACATATGCCGAAC
tetQ-F	TGGATTGAAGACCCGTCTTTGTCC
tetQ-R	AGCAGGTGTACTTACCGGGCTATA

Table S5. Relative abundance of two phyla related to the presence of the gene tetQ at the end of Phase 3 for reactors that received Synthetic wastewater only.

	Proteobacteria			*Bacteroidetes*		
SMWWat	71%	±	4%	19%	±	3%
Syntho	87%	±	3%	10%	±	3%

Where ± represents the standard error in the percentage reads detected between the three reactors.

Table S6. Significance of the ANOVA analysis for all the ARGs analyzed.

Gene	Between Subjects			Within Subjects			
	Wastewater	Solids	Wastewater*Solids	Phase	Phase*wastewater	Phase*Solids	Phase*Wastewater*Solids
BetOMPK	0.4831	0.0049	0.388	<0.0001	0.9051	0.1866	0.8204
BlaMOX	0.2667	0.3593	0.0243	0.6384	0.4269	0.0354	0.005
BlaTEM	0.5585	0.3825	0.0353	0.5116	0.3667	0.5163	0.3632
dfraQ	0.3481	0.0338	0.1255	0.0089	0.4453	0.0985	0.0308
MarR	0.7945	0.3122	0.1423	0.4267	0.1423	0.5324	0.1751
MphE	0.0739	0.658	0.0087	0.0001	0.067	0.0302	0.3182
MsrD01	0.4323	0.0007	0.1168	0.0029	0.0433	0.0014	0.4166
MsrD03	0.2788	0.0014	0.182	0.0002	0.041	0.0027	0.313
qacFH-85	0.7611	0.3259	0.1281	0.0014	0.9736	0.3578	0.4004
QnrB	0.853	0.0267	0.8093	0.2351	0.4281	0.2148	0.8396
Qnrs2	0.4091	0.1027	0.2175	0.2979	0.6731	0.385	0.7407
RobA	0.8413	0.016	0.6779	0.8954	0.9943	0.3733	0.2006
tetE	0.2634	0.4467	0.096	0.5731	0.8881	0.8305	0.5726
tetO	0.5973	0.0224	0.2947	0.0749	0.3231	0.6097	0.2281
tetQ	0.0171	<0.0001	0.2803	0.0068	<0.0001	0.2176	0.0014
aac(3)-i	0.0397	0.022	0.2706	0.7344	0.0163	0.3977	0.3854
aac(6)-i	0.9153	0.0694	0.1427	0.001	0.0161	0.4627	0.8464
aadA16	0.0015	0.0001	0.5146	0.2482	0.4204	0.4392	0.109
aadA6	0.0955	0.0599	0.5076	0.3392	0.1988	0.0708	0.0387
blaMOX/b	0.023	0.1297	0.109	0.301	0.5995	0.1534	0.2258
blaOXA48	0.7101	0.0215	0.2515	0.0044	0.1671	0.0089	0.0006
blaOXA51	0.4626	0.2705	0.8314	0.0009	0.0004	0.963	0.2604
blaSFO	0.1582	0.895	0.8363	0.3932	0.0097	0.038	0.0955
blaTEM	0.9944	0.5192	0.3422	0.6553	0.0723	0.5799	0.3273
catA3	0.053	0.71	0.2733	0.8974	0.2816	0.4073	0.359
cefa_qac	0.1647	0.0141	0.0007	<0.0001	0.0023	0.0003	0.001
cmlA_2	<0.0001	0.0045	0.0003	0.005	0.0095	0.5312	0.495
cmlA_4	<0.0001	0.1446	0.0004	0.0582	0.0095	0.6648	0.013
cmxA	0.0055	0.1209	0.083	0.0751	0.9312	0.1446	0.3033
czcA	0.0717	0.9449	0.2313	0.0815	0.5234	0.8977	0.9183
dfra21	0.0005	0.0341	0.0124	0.7147	0.3644	0.1138	0.4966
dfrA25	0.2621	0.0067	0.3526	0.1474	0.0973	0.0796	0.3309
dfrA27	0.4085	0.996	0.304	0.7507	0.1834	0.2676	0.9967
dfrA8	0.4002	0.4159	0.174	0.6521	0.1241	0.0489	0.0084
dfrB	0.0409	0.0045	0.9669	0.5218	0.1907	0.7711	0.6262
ereA	0.2069	0.3414	0.3959	0.2755	0.0097	0.0107	0.4836
erm42	0.9434	0.656	0.2413	0.7089	0.3234	0.4077	0.6949
fabK	0.1829	0.5785	0.1828	0.6136	0.311	0.7733	0.4703
floR_1	<0.0001	0.0055	0.0054	0.9823	0.0608	0.655	0.1745
folA_1	0.009	0.0105	0.3856	0.0059	0.0015	0.1279	0.0381
intI1_1	0.0125	0.4348	0.0048	<0.0001	0.0736	0.5907	0.0168

intI1_2	0.721	0.0395	0.0001	<0.0001	0.0719	0.7251	0.0015
intI3	0.0035	0.0222	0.2534	0.0031	0.0563	0.0113	0.2313
IS1111	0.2911	0.4689	0.2357	0.3422	0.0821	0.0858	0.2447
IS1133	0.0546	0.1893	0.6571	0.0318	0.7555	0.5232	0.2125
IS6100	<0.0001	0.4722	0.0809	0.038	0.0019	0.1847	0.1785
marR_3	0.0032	0.3047	0.429	0.1555	0.8377	0.0055	0.0035
mcr1	0.2896	0.1095	0.7595	0.058	0.4963	0.2	0.7345
mdtA	0.4416	0.2139	0.8945	0.199	0.237	0.578	0.1399
mdtH	0.2656	0.1473	0.8111	0.0471	0.3555	0.144	0.6081
merA	<0.0001	<0.0001	0.1693	0.0004	0.1365	0.6713	0.2144
msrD	0.0192	0.2066	0.2143	0.032	0.3636	0.0352	0.127
nisB_1	0.01	0.559	0.7543	0.4771	0.0461	0.1492	0.5315
norA	0.0143	0.0296	0.1442	0.0309	0.5797	0.6135	0.6903
oleC	0.2309	0.2093	0.1784	0.1098	0.7232	0.0755	0.1549
orf37-IS	0.2932	0.2102	0.0748	0.0064	0.969	0.0159	0.6646
penA	0.7465	0.0784	0.8787	0.4054	0.4041	0.1422	0.4419
pikR2	0.0305	0.0127	0.0015	0.1241	0.9808	0.9703	0.8813
pncA	0.1152	0.7829	0.7816	0.005	0.1136	0.8316	0.8447
qacE11	0.24	0.0164	0.0007	<0.0001	0.0058	0.0002	0.001
qacE13	0.1821	0.0162	0.0008	<0.0001	0.0031	0.0001	0.0009
qacF/H	0.3596	0.5894	0.0648	0.0039	0.0037	0.0073	0.2545
qepA	0.9529	0.431	0.9969	0.0803	0.9699	0.0624	0.3184
qnrB	0.0009	0.0964	0.6456	0.7139	0.0866	0.3797	0.7708
qnrB4	0.557	0.204	0.6131	0.3969	0.7758	0.0412	0.4314
qnrS2	0.02	0.3569	0.3635	0.2885	0.0283	0.1094	0.0316
qnrVC1_V	0.0733	0.0502	0.4038	0.3183	0.4667	0.2171	0.8831
spcN	0.3875	0.0857	0.417	0.2685	0.9345	0.0007	0.1107
sul1_2	0.06	0.0389	0.0018	<0.0001	0.059	0.001	<0.0001
sul2_2	0.0792	0.0048	<0.0001	0.2489	0.1395	0.025	0.006
sul3_1	0.6808	0.2423	0.4276	0.245	0.5951	0.5887	0.4616
sul4	0.0154	0.0004	0.0499	0.6213	0.0002	0.2602	0.2423
tetA_2	0.001	<0.0001	<0.0001	0.1507	0.7029	0.0186	0.0022
tetE	0.6412	0.0341	0.2112	0.4319	0.0315	0.0255	0.3515
tetG	0.2699	0.485	0.9273	0.3204	0.3477	0.031	0.2226
tetL_2	0.4006	0.7276	0.6903	0.1058	0.5247	0.8154	0.867
tetQ	0.1029	0.0813	0.0813	0.1029	0.1029	0.0813	0.0813
tetX	0.2145	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
tnpA_4	0.6228	0.0639	0.0021	<0.0001	0.0124	0.0019	0.0052
trbC	0.1873	0.761	0.0102	0.1559	0.0355	0.0232	0.0257
vanA	0.562	0.5155	0.558	0.1226	0.6595	0.9903	0.4424
vanB_1	0.6402	0.2686	0.6895	0.6174	0.6374	0.5444	0.4575
vanHB	0.0573	0.029	0.0203	0.191	0.1937	0.0548	0.0312
vanTC_2	0.101	0.6083	0.3425	0.0786	0.6045	0.7786	0.4916
vanYB	<0.0001	0.0649	0.7462	0.5086	0.0206	0.1733	0.7498

vanYD_1	0.0044	0.2701	0.0413	0.0115	0.0035	0.0026	0.0438

Note. * indicates interaction of the different factors.

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