University of Glasgow

Microbial communities and off-grid wastewater treatment

Linghui Shi MSc, BEng

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy (PhD) by Research

James Watt School of Engineering

College of Science and Engineering

University of Glasgow

January 2025

Abstract

Water and sanitation have improved significantly in the past century due to advanced wastewater treatment. Centralised wastewater treatment systems deal with large amount of wastewater in small footprint are always preferred in urban areas. In contrast, decentralised wastewater treatments are more common in rural areas. Among these, septic tanks are one of the most common off-grid solutions, however, their performance various and hardly meets any wastewater discharge standards. This thesis presents a study on the performance and microbial community of small-scale constructed wetland systems treating wastewater from septic tank. Additionally, a lab-based batch experiment to study the change of microbial community and antimicrobial resistance (AMR) during acute amoxicillin exposure.

Three small-scale constructed wetland systems were built and operated in Mexico. Two identical household systems (WS1 and WS2) collected wastewater from single households. They consisted of an equalization tank and a biodigester (AR), followed by a horizontal constructed wetland (CW1), a vertical constructed wetland (CW2) and a holding tank (HT) for the reuse of treated wastewater in gardening. WS3, while similar to WS1 and WS2, received toilet wastewater from a school building and included an additional aeration tank between AR and CW1. All three systems were monitored from the initial three months of the operation, with WS1 and WS2 undergoing an extended monitoring of ten months. Two household systems (WS1 and WS2) were receiving wastewater with high chemical oxygen demand (COD) concentration (average 1131.4mg/L in WS1 and 1894.7mg/L in WS2), NO_2^- concentration (average 51.1mg/L in WS1 and 76.7mg/L in WS2) and NO_3^- (average 106.4mg/L in WS1 and 214.8mg/L in WS2). Despite higher influent concentrations than full-scale constructed wetland, our constructed wetland system removed at least 73.4% COD, 79.9% NO_2^- and 78.8% NO_3^- in average. School system (WS3) received wastewater with much lower COD (153.0-587.0mg/L), NO_2^- (3.0-54.4mg/L) and NO_3^- (13.7-62.9mg/L) concentrations than the household systems, whilst achieved similar removal rates to household systems (58.8-89.9% of COD, 39.3-58.8% of NO_2^- and 29.7-80.6% of NO_3^-).Removal of COD, NO_2^-, NO_3^- in all three systems were comparable to long-term operational full-scale constructed wetland systems in Mexico from

the first month. It took 10 months to achieve a stable removal of COD, NO_2^-, NO_3^- in both household systems. Phosphate accumulated in the systems in long-term and NH_4^+ was not removed. Microbial communities were analysed through *16S rRNA* sequencing, where AR and HT (influent and effluent of constructed wetlands) were analysed over the monitoring time with additional analysis of within CW1 and CW2 of each system in the last seven months. Microbial community composition in WS3 shared less similarity with household systems, where despite influent was different in two household systems the microbial community compositions in WS1 and WS2 were similar to each other. *Bacteroidota* was the most dominant phylum in the first three months, while *Proteobacteria* became the most dominant phylum in the long-term study. Only eight ASVs from the first three months were shared with all 46 most abundant ASVs in long-term study, which suggests the change of microbial community happened overtime. Microbial communities took longer (14 months) to stabilise than pollutant removal, and despite different microbial communities were found in these two systems they achieved comparable pollutant removal rates.

Laboratory preliminary experiments aimed to develop a rapid approach to detect AMR in mixed microbial community from wastewater were undertaken. The growth of anaerobic microbial communities under aerobic condition was monitored by optical density (OD) to mimic transition of wastewater from a septic tank to constructed wetland. A gradient of amoxicillin concentrations (0-32.0mg/L) was tested, revealing that the growth rate of the microbial community varied across three distinct ranges with increased amoxicillin concentrations in which beneficial range (0-1.5mg/L), detrimental range (1.5-4.5mg/L) and no further change range (greater than 4.5mg/L). Meanwhile the length of lag phase increased solely with rising amoxicillin concentrations. A further experiment with selected amoxicillin concentrations examined the change of microbial communities through *16S rRNA* sequencing and the expression of antimicrobial resistance genes (ARGs) via high throughput quantitative polymerase chain reaction (HT-qPCR), respectively. A shift of the microbial community composition was observed after amoxicillin exposure, where relative abundance of *Paenibacillus azoreducens* increased while *Bacillus cereus* group decreased in all amoxicillin exposed microbial communities in both DNA and RNA. However, data were insufficient to conclude that amoxicillin

exposure was the only factor for this shift. ARG expression increased with amoxicillin concentration, however ARG abundance was not available due to poor data quality. The results suggest that the length of lag phase may serve as a potential measure for assessing AMR in mixed microbial community, although further work is needed to validate this method.

This study highlights that small-scale constructed wetland systems are capable of removing pollutants from wastewater after septic tank, which could be a potential solution for off-grid sanitation. The potential risk of spreading pathogens and AMR associated with small-scale constructed wetland remain unknown. Further research is needed to understand and assess those risks in the future.

Table of Contents

List of Tables

List of Figures

Acknowledgement

First and foremost, I am profoundly grateful to have Professor Cindy Smith and Dr Stephanie Connelly as my primary supervisors, I could not have completed this PhD without their encouragement, support and guidance. I would like to thank Dr Stephanie Connelly for all her invaluable guidance in experiment design and her endless support in my first three and half year of my study. I am especially appreciative of her efforts in organising all online informal chats during the challenging Covid-19 time. I wish her a better recovery and return to work in the near future. I am immensely thankful for Professor Cindy Smith's supervision over the past two years, without her help, I would not been able to sort out all my data and complete my thesis. I would also like to thank my supervisor Dr Umer Ijaz for his guidance in bioinformatics and Professor William Sloan for his insightful suggestions regarding the setup of my experiments.

I would like to thank the team at Universidad Autónoma Metropolitana-Cuajimalpa in Mexico for their efforts in negotiating with the families participating our study of constructed wetland systems, as well as for their assistance with the setup, operation, measurement of all the physio-chemical parameters and DNA extraction. Without their invaluable contributions, this study would not be possible.

I am grateful to the entire Water and Environment team at the University of Glasgow for providing a friendly, supportive and collaborative working environment. Thank you, Dr Marta Vignola for your assistance with the flow cytometer and to Dr Ayo Ogundero, Dr Dominic Quinn, Dr Fabien Cholet, Dr Melissa Moore and William Barr for their support and lovely chats in the lab. I also with to acknowledge all the technicians for their ongoing support in the lab. My sincere thanks go to the College of Science and Engineering for offering scholarship that made this PhD possible.

Finally, I would like to express my heartfelt gratitude to my dear friend Xinpei Zheng for her unwavering support and companionship throughout this lengthy PhD journey; our 25 years friendship continues to grow stronger despite the distance. I am also thankful to my friends Jue Wang and Weiqing Yi for their emotional support through endless cat photos and videos, and to Chen, Yujie Zeng for the delightful conversations over the years. Lastly, I with to thank my parents, Qiongtao Shi and Meimei Yang for their unconditional support.

Author's Declaration

I certify that the thesis presented here for examination for a PhD degree of the University of Glasgow is solely my own work other than where I have clearly indicated that it is the work of others (in which case the extent of any work carried out jointly by me and any other person is clearly identified in it) and that the thesis has not been edited by a third party beyond what is permitted by the University's PGR Code of Practice.

I declare that the thesis does not include work forming part of a thesis presented successfully for another degree.

I declare that this thesis has been produced in accordance with the University of Glasgow's Code of Good Practice in Research.

I acknowledge that if any issues are raised regarding good research practice based on review of the thesis, the examination may be postponed pending the outcome of any investigation of the issues.

Name: Linghui Shi

Registration number: xxxxxxx

Abbreviations

AMR	Antimicrobial Resistance
AR	Biodigester (anaerobic digester)
ARG	Antimicrobial Resistance Gene
ASV	Amplicon Sequencing Variants
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
Ct	Threshold Cycle
CW1	Subsurface horizontal constructed wetland
CW2	Subsurface vertical constructed wetland
DI	Deionized Water
DO	Dissolved Oxygen
EFF	Effluent
FCM	Flow Cytometer
HGT	Horizontal Gene Transfer
HRT	Hydraulic Retention Time
HT	Holding Tank
HT-qPCR	High-throughput real-time qPCR
ICC	Intact Cell Count
INF	Influent
LB	Lysogeny Broth
MDG	Millennium Development Goals
MDR	Multidrug Resistance
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentrations
MSC	Minimum Selective Concentrations
NTC	Non-Template Control
OD	Optical Density
ORP	Oxidation Reduction Potential
OUT	Operational taxonomic unit
PBS	Phosphate Buffered saline
qPCR	Real-time quantitative polymerase chain reaction
SDG	Sustainable Development Goals
TC	Total Carbon
TCC	Total Cell Count
TN	Total Nitrogen

TOC	Total Organic Carbon
TP	Total Phosphate
TSS	Total Suspended Solids
WASH	Water, Sanitation and Hygiene
WHO	World Health Organization
WS1	A constructed wetland system services for a household
WS2	A constructed wetland system services for a household
WS3	A constructed wetland system services for a school building
WWTP	Wastewater treatment plant

1 Introduction and literature review

1.1 Importance of water and water resource

Water is an essential element for all forms of life on the earth. In addition to basic biological needs of water, agriculture, transportation, food processing, cleaning as well as utilities and hydropower all require water. The earth surface is largely covered by water, and water cycles mostly through precipitation and evaporation (Figure 1-1), however, only less than 3% of all water is freshwater which can be used directly. Water demand is also increased with increasing population, together with unequally distributed freshwater, water quality and quantity issues are emerging.

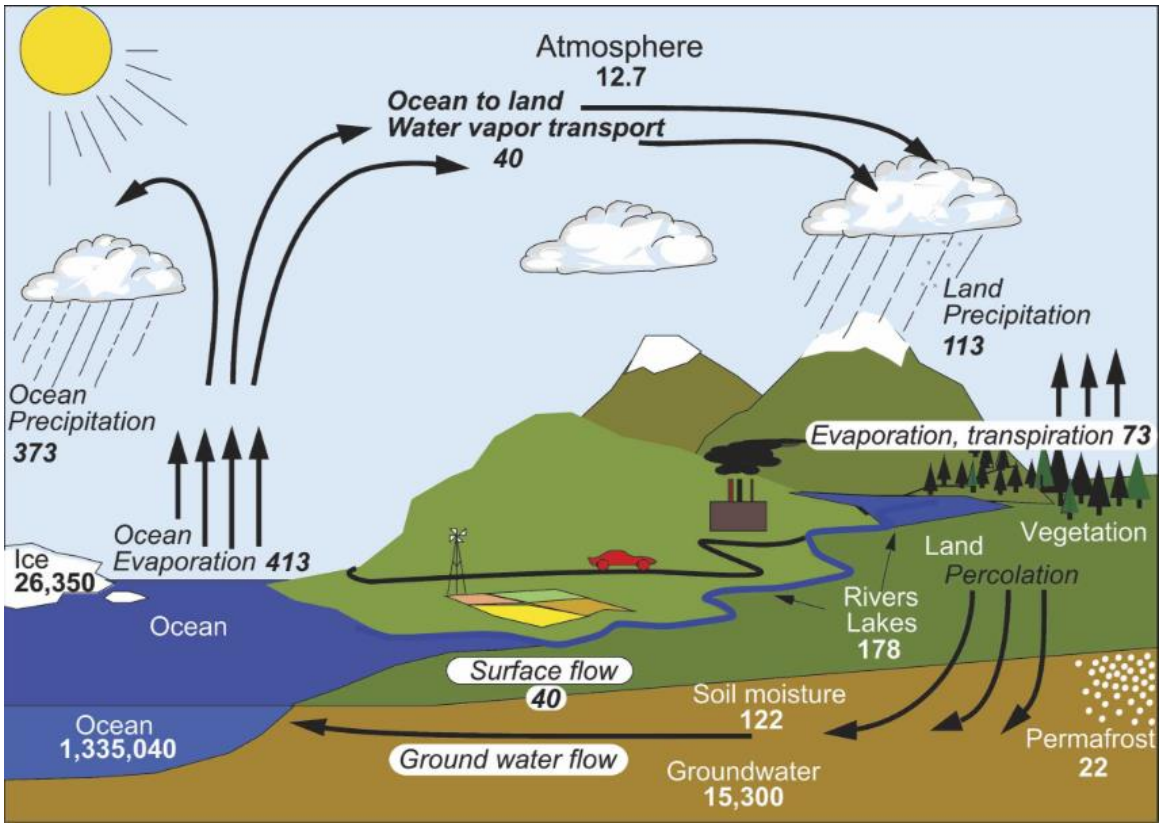

Figure 1-1 The global water cycle. Numbers for storages in 10^3/km^3, fluxes in 10^3 km^3 yr^{-1}. (Trenberth et al., 2007).

Potable water as a precious resource affects human health in many ways. Demand of drinking water increases with increasing population, whilst more human excrement also ends in freshwater which results in a poor water quality. Eutrophication, change of colour and odour are a few indicators of poor water quality caused by excess nutrient, where bacteria and other microorganisms facilitate the rich nutrient for their metabolisms and growth. Within the natural

mixed microbial communitas, pathogens can take advantage of poor water quality and claim of lives, both historically and in recent times in developing countries lacing appropriate sanitation. Cholera, hepatitis A and E, malaria, typhoid, legionellosis and schistosomiasis are some of the infamous ones. In 1854, John Snow identified that cholera was spreading through drinking water supply in London, via the communal use of a hand pump spreading disease, after which regulations for drinking water and sanitation came into enforcement (Tulchinsky, 2018). The progress of improving sanitation was slower than that of drinking water treatment. The first wastewater treatment plant was built in the late 19th century, but it was not widely used until the first standards for sewage discharges to rivers and tidal waters were introduced in 1912 (Whelan et al., 2022). Water quality improved in the late half of the 20th century where latest regulation required more advanced and effective treatment such as activated sludge process which further reduced the nutrients discharged into natural water. Although current technologies can treat water from any source to desired standard, the economic cost is often a main consideration.

Wastewater discharge standards are getting stricter in all countries around the world in the past century, water quality and intense wastewater treatment technology also improved as a result of the stricter water quality requirements. Cities with high population density benefit from centralised system where wastewater is collected through drainage network and treated in high efficiency large scale wastewater treatment plants. The high cost of construction and running centralised system makes it more preferred in developed countries. Although generally developed countries have better sanitation in rural areas than developing countries (United Nations Children's Fund (UNICEF) and World Health Organization (WHO), 2023, World Health Organization (WHO), 2023), wastewater treatment in less populated areas is still a challenge all over the world.

1.2 Improvement of water, sanitation and hygiene (WASH) globally

Water, sanitation and hygiene (WASH) have improved significantly in the last century, however improvements are not equal globally and huge difference between urban and rural areas cannot be ignored. The millennium development

goals (MDGs) set up in 2000 was the first international goal for water and sanitation. It targeted to reduce the proportion of the population without sustainable access to safe drinking water and basic sanitation by half by 2015 compared to 1990. Huge progress had been made ever since and the MDG is assessed as an overall success reaching its 2015 aims, whilst the progress of water and sanitation improvement is slightly different from other targets in MDG. 91% of the global population had access to improved drinking water in 2015 compared to 76% in 1990, meanwhile, 68% of the global population have access to improved sanitation in 2015 increased from 54% in 1990, which falls short of the 77% target (World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2015).

Figure 1-2 United Nations Sustainable Development Goals (SDG). Figure coped from United Nations (2015).

Improvement of water and sanitation are still on-going alongside the current United Nations Sustainable Development Goals (SDG) to ensure availability and sustainable management of water and sanitation for all by 2030 (Figure 1-2). It targets to increase drinking water quality from improved to safely managed. For this, previously shared community taps (improved) would need to be upgraded to be available to individual household (safely managed). The change of sanitation targets from improved to safely managed in SDG requires not only separating excreta from human contact but also safely manging the excreta. This

change in sanitation targets requires much more effort to achieve. According to World Health Organization (WHO) and United Nations Children's Fund (UNICEF) (2020), more than half of the global population still lack of access to safely managed sanitation in 2017. Despite the fact that the worldwide population with access to safely managed sanitation has doubled to 3.4 billion in 2017 compared to 2000, there is still much more work required to improve sanitation for the remaining 4.24 billion people in the world (World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2020).

The coverage of sanitation in rural areas is always lower than urban areas globally, where the improvements in rural sanitation are at a higher speed than those in urban areas between 2015 and 2020 (see Figure 1-3). In 2020, rural population with improved on-site sanitation facilities were greater than those with sewer connections, however, the improved on-site sanitation facilities could count as either safely managed or basic, depends on how wastewater was stored and managed. Septic tanks are by far the most widely applied on-site sanitation globally with a higher population using them than latrines and others combined globally. Although, the prevalence of septic tank varies from country to country, they service 24% of the global rural population and 20% of the global urban population. Meanwhile, it is estimated that in 2020, only 50% of wastewater produced by urban population was treated, but only 13% produced by rural populations was treated (by septic tanks or other treatment) (World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2021).

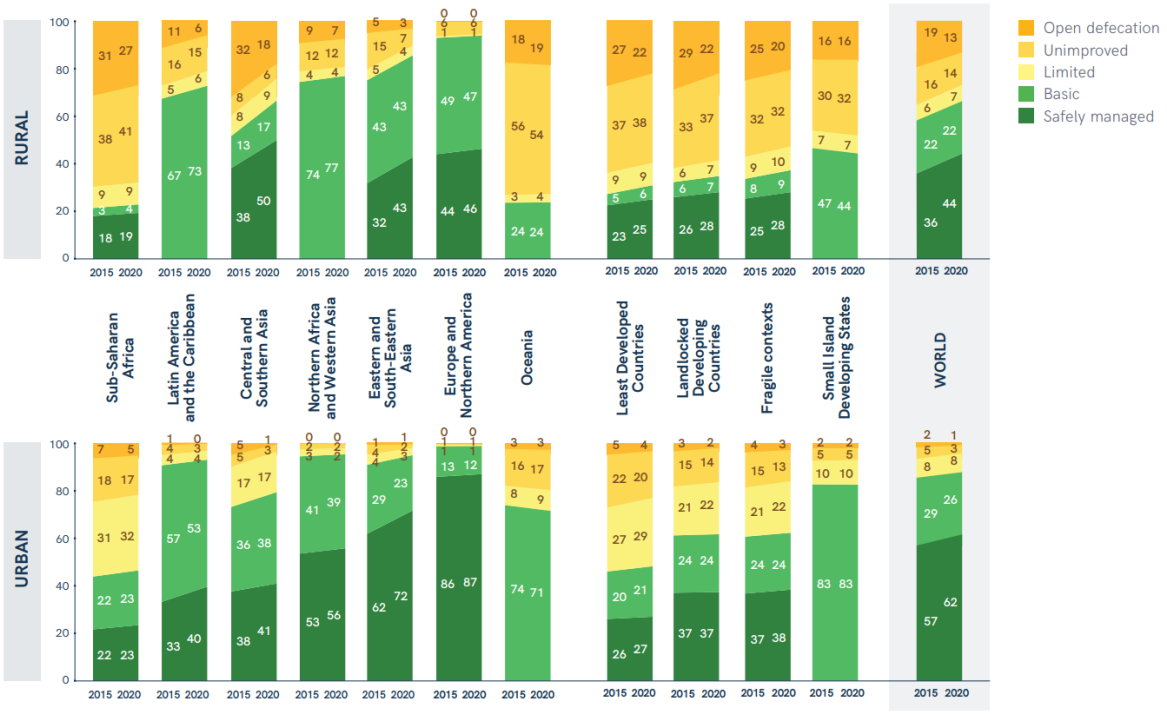

Figure 1-3 Rural and urban sanitation coverage by service level and SDG region, 2015-2020 (%) (World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2021).

1.3 Burden of disease attributable to unsafe drinking-water, sanitation and hygiene

The improvement of WASH reduces the burden of disease attributable to unsafe drinking water, sanitation and hygiene. Global burden of disease study assessed 84 risks revealing that the risk of expose to unsafe sanitation is estimated to have decreased by 47.8% between 1990 to 2017, which is the highest reduction in all 84 risks analysed. Meanwhile, the declining risks of unsafe water resource and unsafe sanitation reported are strongly correlated with increased development. However, the observed risk of exposure to unsafe drinking water is higher than the expected risk of exposure, this suggests that the improvement of drinking water falls behind the overall development between 1990 and 2017 (Stanaway et al., 2018).

It is estimated in 2019, unsafe WASH attribute to 100% of soil-transmitted helminthiases and 69% diarrhoea disease globally. The death attributes to diarrhoeal disease have seen decreasing with increasing safe WASH coverage in the past 30 years (Figure 1-4). Meanwhile over 1 million death attributes to unsafe WASH are estimated be preventable with safe WASH facilities. Among those deaths unsafe sanitation is estimated to have direct link to the most

diarrhoea related death (0.56 million) caused, followed by unsafe drinking water (0.50 million). Preventable death from soil-transmitted helminthiases is estimated to be 2000 which is much lower than diarrhoea disease, yet all of them are caused by unsafe WASH (World Health Organization (WHO), 2023).

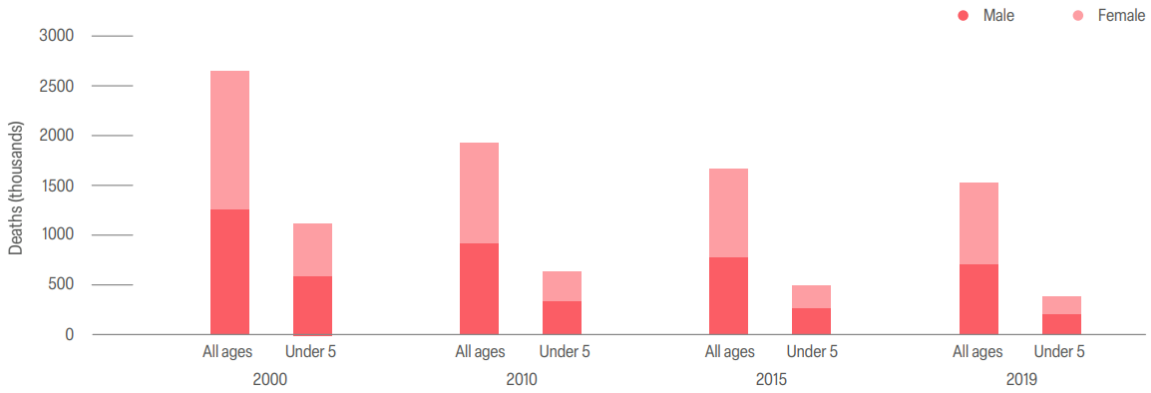

Figure 1-4 Diarrhoeal disease deaths by age and sex worldwide between 2000 and 2019. (World Health Organization (WHO), 2023).

1.4 Current WASH in Mexico

Mexico is one of the 80 countries in the world where in 2017 at least 99% of the population has access to at least basic drinking water and 96% of the population has access to a piped water supply (World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2019). Although the coverage of drinking water supply is high, water scarcity has long been the issue in Mexico. Groundwater is one of the main water resources in Mexico, in 2022, it provided 40.3% of the overall water consumption which increased from 39.4% in 2020 (CONAGUA, 2023, CONAGUA, 2022). As a result, treated wastewater reuse is common practise in Mexico. Approximately 30% of the total treated wastewater is reused, where irrigation accounts for up to 82% of the reused water including agriculture, green area, golf courses (Tabla-Vázquez et al., 2020).

The coverage of at least basic sanitation in Mexico has increased overtime, from 75% in 2000 to 89% in 2015, then further increased to 92% in 2020 (see Table 1-1). The proportion of the population with access to safely managed sanitation facilities also increased from 18% in 2000 to 57% in 2020, almost all of these improvements occurred in urban areas. Urbanisation is occurring, the proportion of urban population increased from 75% in 2000 to 81% in 2020, driving a higher demand for sanitation facilities in urban area (World Health Organization (WHO)

and United Nations Children's Fund (UNICEF), 2019, World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2021).

Table 1-1 Sanitation and population in Mexico (2000-2020). Data collected from World Health Organization (WHO) and United Nations Children's Fund (UNICEF) (2019) and World Health Organization (WHO) and United Nations Children's Fund (UNICEF) (2021).

	Population ($x10^3$)			Proportion of population (%)								
				At least basic sanitation			Septic tank			Sewer connection		
Year	2000	2015	2020	2000	2015	2020	2000	2015	2020	2000	2015	2020
Overall	101,720	121,858	128,933	75	89	92	13	15	16	64	77	81
Rural	25,430 *	25,590 **	24,497 ***	53	78	92	24	41	47	20	34	38
Urban	76,290 *	96,268 **	104,436 ***	83	86	94	10	9	8	79	89	91

*** Calculated from 75% urban population in 2000.**

**** Calculated from 79% urban population in 2015.**

***** Calculated from 81% urban population in 2020.**

Sanitation facilities in urban areas are different from rural areas. Sewer connection in urban areas increased from 79% in 2000 to 91% in 2017 and stays the same in 2020 (World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2019, World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2021). Total number of centralised wastewater treatment plants (WWTPs) and the proportion of wastewater receiving at least secondary treatment have increased in the past 30 years, however, the percentage of WWTPs out of operation was increasing in the past 10 years (de Anda and Shear, 2021). According to the national water commission (2023) 64.46% of the domestic wastewater was treated in 2021, which is the highest on the record. However, this still means a large percentage of wastewater is released directly to the environment untreated. Meanwhile, raw wastewater irrigation is a common practice in Mexico, where crops and maize at Mezquital Valley are irrigated with untreated wastewater for over 100 years and reported above average yields of crops and maize (Siebe et al., 2016).

In rural Mexico, sewer connection rate increased slowly from 34% in 2015 to 38% in 2020 despite the proportion of rural population decreased from 21% to 19% in the same period. Meanwhile, the proportion of rural population relying on septic tanks increased dramatically from 24% in 2000 to 47% in 2020 (World Health

Organization (WHO) and United Nations Children's Fund (UNICEF), 2019, World Health Organization (WHO) and United Nations Children's Fund (UNICEF), 2021). Septic tanks as well as other on-site sanitation are recognised as improved sanitation by WHO, as it prevents the direct contact of human excreta. Septic tank would be recognised as safely managed sanitation if further treatment is conducted to ensure the sludge is safely managed, however, there is no data available except some case studies (Kihila and Balengayabo, 2020).

1.5 Water and sanitation in Mexico City

Mexico City is the capital and the largest city in Mexico. 8.83 million people live in Mexico City, where 99.4% of the population lives in the urban area and only 0.6% in rural areas within the city limits (CONAGUA, 2017). The large urban population within the confines of the city, result in highest population density in Mexico, and worryingly the lowest renewable water resource per capita in Mexico (CONAGUA, 2017). Public water supply takes account of 97.15% of the total water usage in Mexico City, where 71.65% of the water comes from groundwater, 28.35% comes from surface water in 2016 (CONAGUA, 2017). However, surface water quality is not optimal in Mexico City, with 40% and 88.9% of surface water in Mexico City contaminated in terms of five-day biochemical oxygen demand (BOD_5) and chemical oxygen demand (COD) respectively in surface water quality monitoring in 2016 (CONAGUA, 2017).

Coverage of drinking water supply and improved sanitation have increased significantly in the past 30 years. Table 1-2 shows the coverage of household tap water and wastewater connects with public sewer or septic tanks in both Mexico and Mexico City in 2015. Mexico City has higher coverage than nationwide for both tap water and wastewater connection in urban area. Although the sewerage coverage is high in rural Mexico City, only **58.3% of the wastewater collected through public sewer is treated in Mexico 2016** (CONAGUA, 2017). Also, sewerage coverage in the report of CONAGUA (2017) takes account of either connecting with public sewer or septic tank, more than half of the effluent from both septic tank and public sewer is not treated and likely to be discharged into environment directly and post high risk to the environment.

Table 1-2 Tap water supply coverage and sewerage coverage in Mexico and Mexico City in 2015. Data collected from (CONAGUA, 2017).

	Tap water in homes or property			Wastewater connected with public sewer or septic tank		
	Overall	Urban	Rural	Overall	Urban	Rural
Mexico	94.4%	97.2%	85.0%	91.4%	96.6%	74.2%
Mexico City	98.6%	98.8%	59.3%	98.5%	98.5%	93.1%

According to the national inventory of wastewater treatment plants in operation published by national water commission in December 2020 (CONAGUA, 2020a), there are 29 registered WWTPs in Mexico City treating wastewater 2436.7L/s. The largest and latest Atotonilco WWTP operated from 2016 and it is capable of treating 60% of the wastewater from Mexico City, it has never operated at full capacity. This WWTP is not welcomed by downstream farmers at Mezquital Valley as irrigation with treated wastewater reduced their crop production compared with over 100 years practice of irrigating with untreated wastewater (Rosas-Baños and García-Salazar, 2024).

Growing population drives the demand for water and generates more wastewater, although sewage is widely available in Mexico City, a large proportion of wastewater is still not treated. Furthermore, increased water demand further stretches limited water resource, in the area the lack of sewage connections means that reuse of wastewater is preferred than discharge into environment directly.

1.6 Sanitation solutions for rural Mexico City

Septic tanks are welcomed in rural areas as they are easy to install, have low running cost and are low maintenance. They work as primary treatment, separating solids from wastewater and partially degrading organic compounds. They are considered as a ‘safely managed’ sanitation as it separates faeces from human contact. For them to work they must be emptied, and many households do not do this. Even when working optimally, the effluent from septic tanks does not meet the discharge standards and still poses a risk to contaminate water sources. Therefore, further treatment to improve the effluent quality from septic tanks is needed.

There are a number of nature-based solutions that are low cost and low maintenance that can improve wastewater treatment, based on biological activity of microbes to treat water. As the weather in Mexico City is warm all the year round (average annual temperature is 18℃ and there is no cold season (lowest average monthly temperature 15℃) (CONAGUA, 2020b). In addition, many areas in particular the rural areas within the city with low population densities have gardens and land space associated with homes. The warm weather and large land availability make it an ideal place to implement nature-based solution such as stabilisation pond or constructed wetland. Stabilisation ponds and aerated ponds have been used in treating wastewater in Mexico for years. In 2016, 17.6% of the collected wastewater was treated by ponds (CONAGUA, 2017). However, the proportion of wastewater treated by ponds reduced to 13.6% in 2020, the amount of wastewater treated by ponds reduced to around $2m^3/s$, which equivalents to 65 million m^3 per year (CONAGUA, 2022). Unpleasant odours and mosquitoes breeding may be the reason the ponds are treating less wastewater over the year (Zurita et al., 2012).

Constructed wetlands are becoming popular in Mexico in recent years. Total amount of constructed wetlands increased from 68 in 2015 to 230 by the end of 2020, operation flow rate also increased from 488.9L/s in 2015 to 1233.7L/s in 2020 (CONAGUA, 2015, CONAGUA, 2020a). The operation flowrate in those constructed wetlands various from 0.1L/s to 500L/s (CONAGUA, 2020a). Over half (116) of the constructed wetlands are built in the state of Sinaloa, but there is no registered constructed wetland in Mexico City. There are also some successful cases of using constructed wetlands as additional step to treat effluent from biological treatment unit (CONAGUA, 2015).

Reports of successful running constructed wetlands in the last 22 years are reviewed by Marín-Muñiz et al. (2023), where 18 full-scale constructed wetland treating domestic were found. This indicates constructed wetland is a solution for Mexico domestic wastewater treatment. The various size of constructed wetland summarised by Marín-Muñiz et al. (2023) also suggests that they have the potential to be a solution to treat wastewater in rural Mexico City. However, the information on the microorganisms within the constructed wetland is very limited.

1.7 Wastewater treatment - engineering enhanced natural process

The demand for sanitation increases with increasing global population, the role of sanitation changes from simply remove excreta from human contact to removal of pollutants from wastewater before release into environment (Chen et al., 2020). WWTPs are key elements in the water cycle reducing pollutants from wastewater before discharge into the natural environment. WWTPs treat wastewater from all sources such as domestic, industrial or hospital to meet the discharging standard. The scale of WWTPs varies depending on the need. Large WWTPs receive wastewater from thousands of people, while small ones only serve one to a few households. Despite huge size variations, two main principles of WWTPs are physical and biological processes, where microorganisms dominate key biological process. The pollutants (carbon, nitrogen, phosphate and others) in wastewater act as nutrients for bacteria, they grow rapidly in their favoured grow condition on wastewater contaminants therefore lowering the pollutants concentration. The technology of wastewater treatment improves with better knowledge of the underpinning microorganisms and microbial processes, where understanding their habitat and providing an optimised environment are the key points in improving treatment effectiveness. Activated sludge which employs microorganisms is one of the main breakthroughs in wastewater treatment technology, it is still main principle in most of the full-scale WWTP around the world despite developed in 1913 in the UK (Lofrano and Brown, 2010).

Full-scale WWTP always draws the most attention, as it treats large amounts of wastewater in a short period of time and takes relatively small footprint. Indeed, the risk associated with a failure WWTP is also greater due to its large capacity, as a result, research always starts from full-scale WWTP. Early research of full-scale WWTP starts from improve performance of treating wastewater from different resources to understanding the microbial communities within the WWTP and functional microorganisms nowadays (Kroiss et al., 2021, Wanner, 2021). Temporal studies analysed sludge samples from full-scale domestic WWTP over a 12 months period all found out that despite shift of microbial community composition was observed functional taxa were always relatively stable, and no significant change of seasonal pattern or other environmental factors was found (Ju and Zhang, 2015, Xue et al., 2019, Fan et

al., 2017, Jiang et al., 2018). Although the microbial communities within the WWTP are largely affected by the microbes in the influent, some core microbes were found in microbial community analysis with sequencing data from WWTP all around the world (Wu et al., 2019).

Microbial communities in other wastewater treatment are less known compared to full-scale WWTP, particularly in constructed wetland. Although publications including the underpinning microbiology of constructed wetland are increasing exponentially, the majority of them focus on short-term lab-based system feed with synthetic wastewater (Vymazal et al., 2021, Wang et al., 2022). Meanwhile, very few studies have paid attention to the change of microbial communities from the beginning of operation, with reports of samples taken at varied time after setting up, ranged from 30 days years or missing from literature (Liu et al., 2023b, Ayaz et al., 2015, Liu et al., 2023a). There are a few studies with long-term full-scale constructed wetland, however, their studies focus on the performance rather than the microorganisms (Gonzalez-Flo et al., 2023, Vymazal et al., 2021).

1.7.1 Main characteristics of constructed wetlands

Constructed wetland is one of the decentralised wastewater treatments widely used in rural area due to its relatively large land requirements compared to full-scale WWTP. Constructed wetland with or without free water surface are the two main categories often mentioned in wetland studies, where they have been further divided into horizontal flow and vertical flow based on the flow direction of water, meanwhile, vertical flow constructed wetland includes both up flow and downflow (see Figure 1-5). The hybrid system which includes more than one constructed wetland unit has been used in treating both sewage and industrial wastewater, it is the more preferred approach for sewage treatment due to its better nitrogen removal (Vymazal, 2011a, Vymazal, 2013). Vymazal (2013) reviewed 60 hybrid constructed wetlands and concluded that hybrid system with a vertical flow constructed wetland followed by a horizontal flow constructed wetland is the most efficient system in removing nitrogen from wastewater. However, the removal rate of other pollutants (BOD_5, COD, TSS and TP) is not substantially improved in hybrid system compared with single vertical constructed wetland or horizontal constructed wetland.

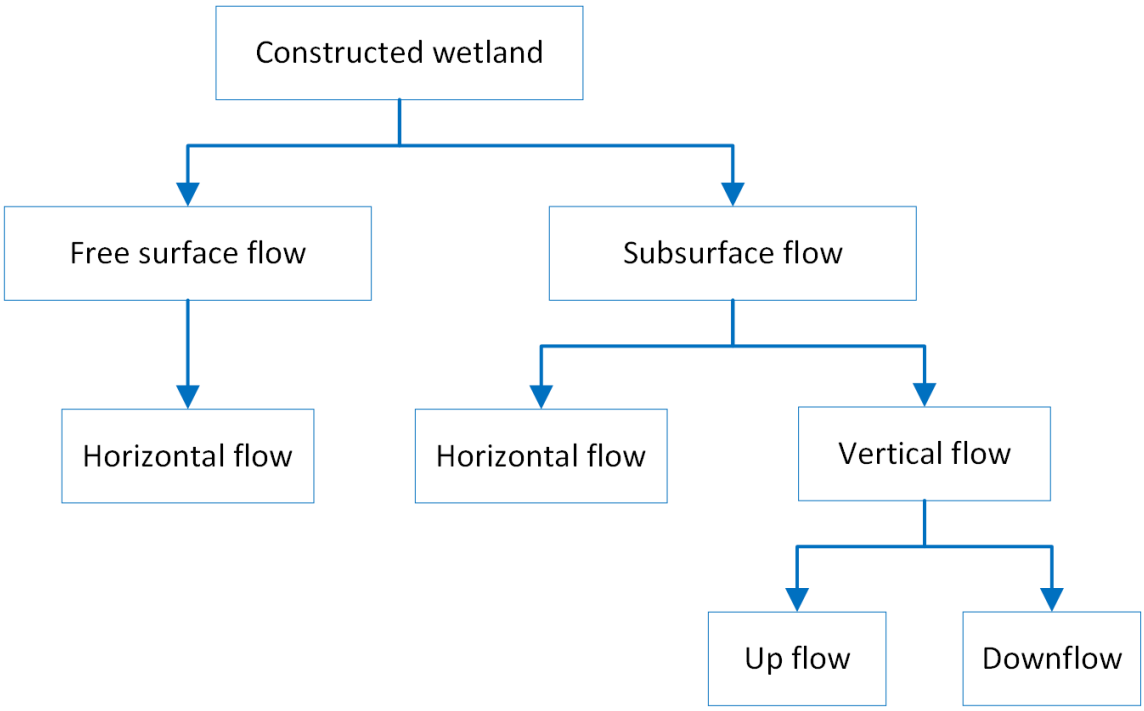

Figure 1-5 Types of constructed wetland.

Plants are optional for constructed wetlands, although Vymazal (2011b) argues that plants are the main feature that differentiates constructed wetlands from stabilization pond. However, there is no agreement that the presence of plant within constructed wetlands is beneficial for wastewater treatment. There are some studies that state that plants improve the treatment by providing surface for bacteria, stabilising the sediment, preventing medium clogging, increasing dissolved oxygen concentration, utilising the nutrients directly, as well as acting as physic barriers to remediate the impact of the weather (Vymazal, 2011b).

A surface flow constructed wetland without plants is sometimes considered as a pond or lagoon, where the performance of the pond compares with constructed wetland varies. There are some reports that ponds achieves a better performance compared with constructed wetland (Liu et al., 2014, Tanner et al., 2005). Mancuso et al. (2023) report that constructed wetlands are better at nitrogen removal while lagoons are better at phosphorus removal. Meanwhile, von Sperling et al. (2010) compared the performance of ponds, unplanted and planted subsurface constructed wetlands and concluded that the constructed wetlands have a better performance in removing organic matter and suspended solid, where the planted ones are better than unplanted ones. Nevertheless, a combined system of several constructed wetlands or lagoons are normally

required to get a better effluent quality. The odour and potential breeding area for mosquitoes and other insects make constructed wetland more preferred than ponds, also different types of constructed wetlands make it more adaptable to treat a variety of wastewaters.

1.7.2 Applications of constructed wetland

Domestic wastewater consists of greywater and blackwater, where blackwater refers to the wastewater from toilets, contributes 12-33% of the overall household wastewater, greywater refers to the rest of the household wastewater (Zhang et al., 2023b). Blackwater is low in total volume and contains less carbon but high in nitrogen compared with greywater, where greywater is the opposite to blackwater. COD in greywater ranges from 55-2000 mg/L and total nitrogen ranges from 6.44-52mg/L, they are generally lower in high income countries and higher in low income countries (Ghaitidak and Yadav, 2013). Blackwater has a COD concentration of 0.26-29.52mg/L and total nitrogen concentrations of 100-170mg/L (Zhang et al., 2023b).

There is a long history of treating domestic wastewater with constructed wetlands, the hybrid system combined vertical and horizontal wetlands improves nitrogen removal has made it favoured by rural and peri-rural area. Rodriguez-Dominguez et al. (2020) summarised the constructed wetlands in Latin America and the Caribbean in the last decade and found out that horizontal subsurface constructed wetland is the most common type and over half of this wetland type are operated in large scale with surface area over 1000m^2. The average removal rates of COD, total nitrogen (TN), total phosphate (TP) of full scale horizontal subsurface flow constructed wetlands are 65±31%, 47±30%, 41±28%, respectively. There are 18 full scale constructed wetland studies in Mexico published in the past 20 years, they treat domestic, community and municipal wastewater with surface area ranges from 31.6m^2 to 11600m^2 (Marín-Muñiz et al., 2023). They have achieved removal rates of 50-90%, 60-90%, 30-90%, and 30-70% for COD, BOD_5, TN and TP, respectively.

Industrial wastewater is always considered more challenging than domestic wastewater, where the characteristics of wastewater varied from industry to industry. Plants are considered one of the most important characteristics in

constructed wetland in 1970s, the concern of toxic industrial wastewater may poison the plant and biological system put the application of treating industrial wastewater with constructed wetland in a slow growth rate. In the past 30 years, more constructed wetlands have been used to treat various industrial wastewaters in at least 33 countries over the world (Vymazal, 2014). Full scale applications have seen in treating wastewater from oil refineries, pulp and paper industry, tannery industry, textile industry, agricultural industry, alcohol industry, food processing industry, laundry, chemical industry as well as landfill leachate and acid mine drainage and some mixed industries.

Vymazal (2014) summarised the characteristics of 138 constructed wetlands used to treat industrial wastewater, and found out that biochemical oxygen demand (BOD) concentrations of industrial wastewater vary from 10 to 150,000 mg/L and COD concentrations vary from 50 to 318,000 mg/L. Meanwhile, in most of the industries the ratio of BOD to COD is greater than 0.5, which indicates those wastewaters are easily biodegradable. In contrast to the pulp and paper industry wastewater which has a lower BOD to COD ratio (<0.5). Although pre-treatment is normally required due to high suspended solid and organic load (Sultana et al., 2015), widely application of constructed wetlands for industrial wastewater proves it is a reliable technology.

1.8 Antimicrobial resistance (AMR) -- an emerging issue in WASH

As the consumption of antibiotics increases with their availability, the amount of synthetic antibiotics released into environment is also increasing. Antibiotics have been detected in all kinds of aquatic environments (Kümmerer, 2009a, Kümmerer, 2009b), include drinking water (Watkinson et al., 2009) and treated wastewater (McArdell et al., 2003). Massé et al. (2014) state that 70% - 90% of antibiotics used in animal husbandry are excreted unchanged from the livestock. Kümmerer and Henninger (2003) observed that around 70% of the clinically used antibiotic are unmetabolized and excreted through urine and faeces maintaining their original chemical structure. Antibiotic concentrations in hospital wastewater are reported higher than domestic wastewater (Paulus et al., 2019). The intact antibiotics from both human and animal origin enter wastewater system and subsequently circulate in the environment (see Figure 1-6). The total

consumption of antibiotic in the UK was 705 tonnes in 2019, in which 68% consumed by human and 32% consumed by animals. Despite the total consumption of antibiotic has dropped 28% compared with 2014, the amount of antibiotic released into environment is still huge compared with pre-antibiotic era (Veterinary Medicines Directorate, 2023).

The only available overall clinical antibiotic consumption data in Mexico is decades old (Amábile-Cuevas, 2010), while some studies report antibiotic consumption data from sales of pharmacy (Sánchez-Huesca et al., 2020). Mandatory prescription for antibiotics only started from 2010, but it can be by-passed easily through phone ordering or home delivery (Amabile-Cuevas, 2021). Meanwhile, generic antibiotics are widely available in Mexico, while the quality control of oral generic antibiotic via bioequivalence approval only required from 2013, where generic antibiotics pass the test by demonstrating 80-125% effectiveness (Amabile-Cuevas, 2021). Isolated clinically pathogenic bacteria summarised by Amábile-Cuevas (2010) and Amábile-Cuevas (2010) show that penicillin and ampicillin resistant bacteria have the higher prevalence in Mexico. Since both penicillin and ampicillin are both beta lactam antibiotic, it suggests that beta lactam antibiotic may be the most consumed antibiotic by human in Mexico. Meanwhile, board-spectrum penicillin is also the highest consumed antibiotic in the world, where the consumption keeps increasing from 2000 to 2015 globally (Klein et al., 2018).

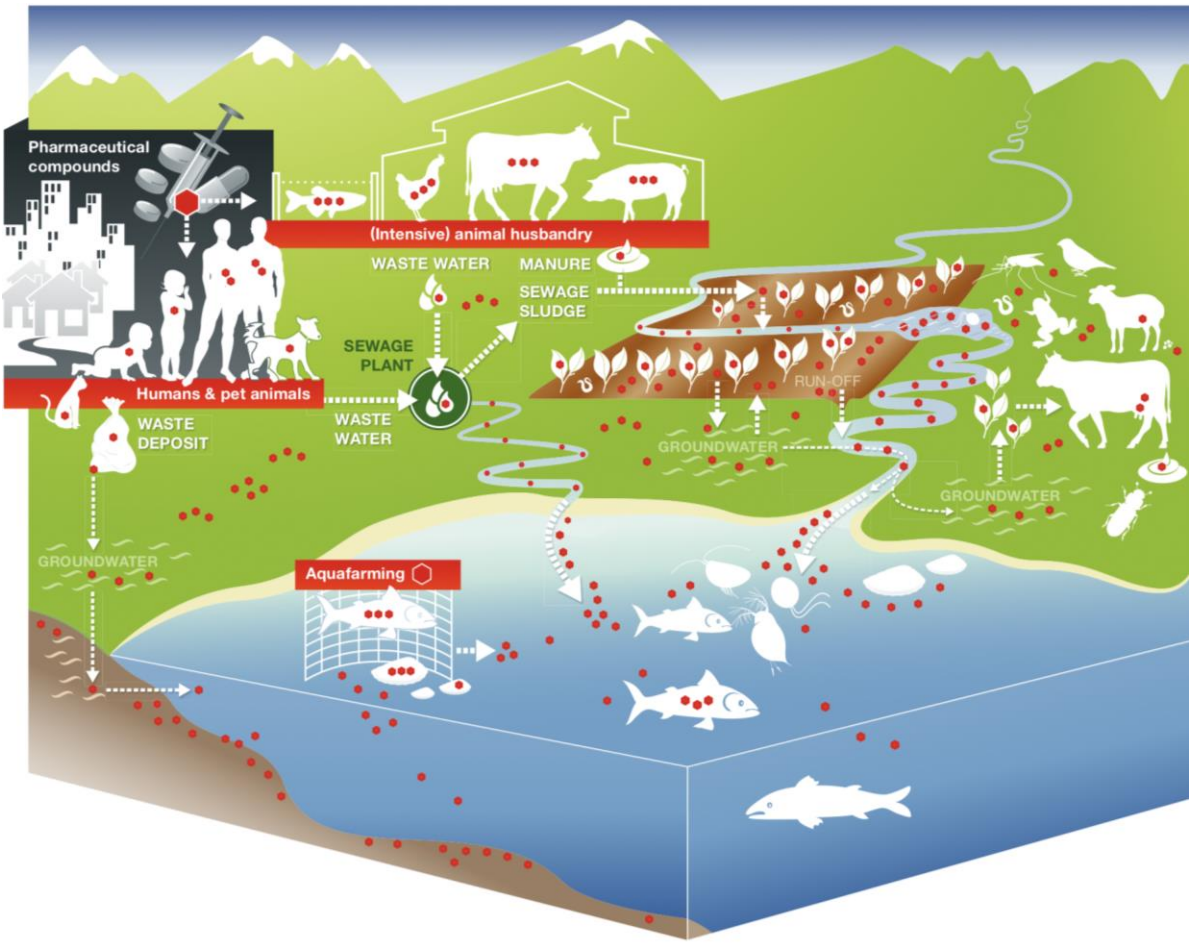

Figure 1-6 The pathway of antibiotic in the environment. (Berkner et al., 2014)

Antibiotics are crucial in treating infection at the same time they are a major source of AMR. It has been reported that minimum selective concentrations (MSCs) for antimicrobial resistance genes (ARGs) are lower than minimum inhibitory concentrations (MICs) (Gullberg et al., 2011, Gullberg et al., 2014, Stanton et al., 2020). Along with antibiotics, human excreta and wastewater as well as the AMR bacteria and their ARGs are considered as hotspots of AMR in the environment (World Health Organization (WHO), 2014). Improve WASH would benefit from both reduced risk of infection and limited chance of direct exposure to AMR in human waste. The speed of improving WASH globally falls behind the evolution of AMR bacteria. Also worth mentioning, there is no strict regulation on antibiotic concentrations in the environment globally (Polianciuc et al., 2020).

1.9 AMR transmission within wastewater treatment plant

While extensive literature on AMR and decentralised approaches have yet to be published, there is a reasonable body of evidence on AMR in WWTP. WWTP has

been considered as a source of AMR, where antibiotic residual in wastewater mixed up with high bacteria density is the concern (see Figure 1-7). Biological treatment is one of the main processes in removing pollutants from wastewater, where bacteria reproduce rapidly and consume large amount of carbon and nitrogen from wastewater. WWTPs are not designed to degrade antibiotics, despite this around 80% removal of antibiotics has been reported in full scale WWTPs, rivers receiving effluent from WWTPs are detected at significantly higher antibiotic concentrations than the ones without (Watkinson et al., 2009). Antibiotic residue in the wastewater along with high bacteria could potentially select AMR bacteria and ARG could increase with increasing AMR bacteria. Co-selection of AMR from heavy metals or other antimicrobial agents is also possible to happen in the WWTP (Manaia et al., 2018). In addition, to develop AMR by exposure to antimicrobial agents, high bacterial density within WWTPs facilitates the chance of horizontal gene transfer (HGT), which results to a mixture of AMR bacteria, ARG and antibiotic in treated wastewater (see Figure 1-7). In addition, the co-localisation of ARGs and mobile genetic elements (MGEs) are observed in WWTPs (Ju et al., 2019). 90% of the ARGs are reported been removed from wastewater after WWTP (Burgmann et al., 2018), but the relative abundance of ARGs in effluents are reported higher than the influents (Ju et al., 2019), this suggests WWTP enriches AMR hosts. Also, the change of microbial communities and ARG hosts in wastewater after the wastewater treatment are reported (Hultman et al., 2018), which suggests the WWTP may affect the AMR in downstream water.

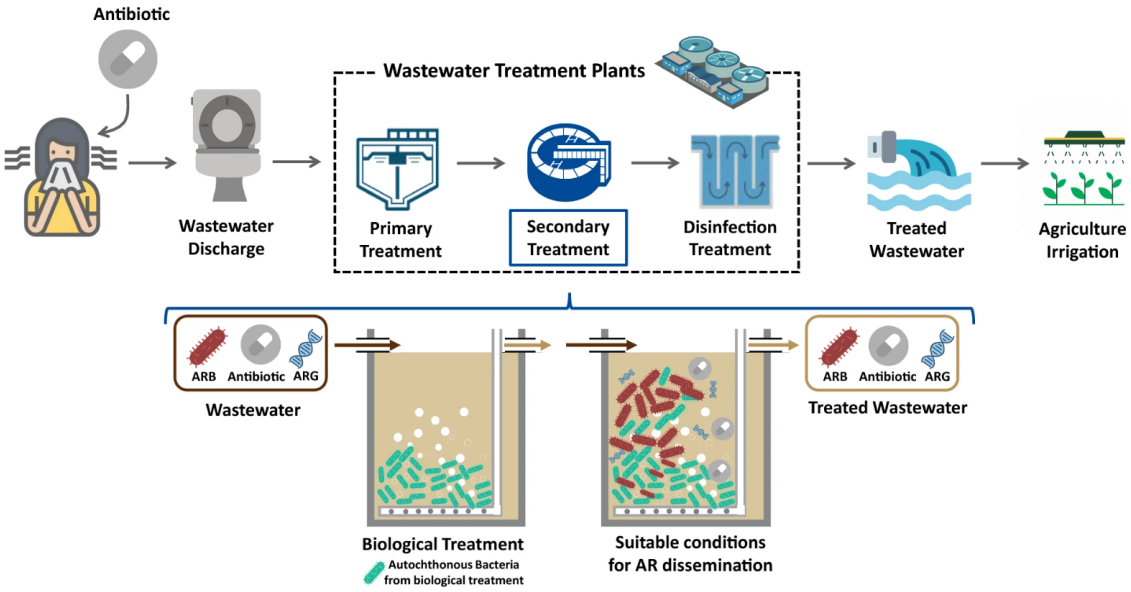

Figure 1-7 Wastewater treatment plant as a source of antibiotic resistance. (Leiva et al., 2021).

1.10 AMR in on-site sanitation and reclaimed water

Similar to full-scale WWTP, on-site sanitation systems are designed to remove pollutants from wastewater but AMR is not the target. Most of the pollutants in decentralised wastewater treatment are removed biologically, where high bacterial population brings up the risk of ARB and ARG dissemination. Meanwhile, decentralised wastewater treatment is normally associated with reclaimed water for irrigation or discharge into the nearby environment, which has more potential risk than centralised WWTP, particularly if the ARG carrier pathogens are present in the treated wastewater. It is reported that irrigation with untreated wastewater resulted in an accumulation of ARGs in soil with over 100 years of practice in Mexico (Jechalke et al., 2015). Also, ARGs in soil were also seen increasing after long-term application of using sludge or chicken manure as fertilizer (Chen et al., 2016b). However, ARGs in soil after irrigation with treated wastewater has mixed results, where half of the literature reviewed by Slobodiuk et al. (2021) reported a positive association and the other reported negative or mixed associations. Although a short study conducted by Gentile et al. (2024) reported that ARG were not found in lettuce leaves from lettuce grown with domestic wastewater treated by a pilot-scale constructed wetland. However, it is unknown if this persists after long-term application of wastewater on the lettuce.

1.11 AMR Detection

Since AMR was observed soon after the discovery of penicillin, testing AMR on isolated pathogen has always been the first option and it is still standard practise in clinical settings (International Organization for Standardization, 2019). Testing for AMR bacteria from environmental samples based on isolation have also been reported (Ekakoro et al., 2023, Kim et al., 2024, Appling et al., 2023), but this approach is time consuming and highly selective dependent on culturing medium. The occurrence frequences of clinical and environmental AMR for major pathogens are not the same (Hua et al., 2020). Non-pathogenic bacteria species develop the ability to host AMR during their evolution from exposure, and potential are a risk for the spread of AMR into the environment (Larsson and Flach, 2022). However, the vast majority of the bacteria in the environment are non-culturable which makes culture-based methods less favourable in environmental AMR studies.

Culture-independent methods test ARGs in the genetic material, which is more sensitive than culture-dependant method in detecting the potential resistance in environmental studies. Real-time quantitative polymerase chain reaction (qPCR) quantifies gene copy numbers of specific ARG is one of the most common molecular methods applied in recent studies. For example it had been used in various studies to detect ARGs from wastewater, surface water, WWTP, sludge (Paulus et al., 2019, Osińska et al., 2020, Cacace et al., 2019, Rodriguez-Mozaz et al., 2015, Sabri et al., 2020a). Although, gene targets of each qPCR test can be customised to detect the desired gene, it is normally limited to a few ARGs due to relative high cost and labour demand.

High-throughput real-time qPCR (HT-qPCR) which is a qPCR-based method utilises small amounts of DNA and simultaneously tests numerous ARGs. Low DNA requirement is a major advantage for surface water samples as they tend to have much lower DNA yield compared to wastewater or sludge (Franklin et al., 2021). Waseem et al. (2019) reviewed that HT-qPCR has been employed in 51 studies tested ARGs in around 1000 environmental samples over the previous 7 years. Surface water, wastewater, animal faecal are some examples of samples been tested (Liu et al., 2018, Quintela-Baluja et al., 2019, Liu et al., 2021).

Metagenomic is a molecular based sequencing method provides a high resolution of all the genetic material. As it is a non-target method, it has a potential to discover new ARGs, especially in environmental samples (Franklin et al., 2021). Despite the high cost and high DNA quaintly requirements, lots of environmental ARG studies are taking the advantage of it, such as Zhu et al. (2023), Guo et al. (2017) and (Zhang et al., 2023a).

1.12 Research Gap

There is an urgent need to develop decentralised wastewater treatment that is low cost, low energy and low maintenance. Nature based solutions, where treatment is driven by the biological activity of microbes within the systems offer potential exciting solutions to global challenges. However, they are often characterised by large footprints, making them unsuitable as small-scale sanitation solutions. Full scale constructed wetlands have been reported to treat community wastewater, but it is unknown how their performance would be on a small-scale, but have many positive attributes highlight above, plus the potential to supply water for re-use. We propose that household scale constructed wetlands are a potential solution to wastewater treatment in Mexico City. We also consider the global challenge of AMR in wastewater treatment and decentralised approaches and water reuse. There are currently significant challenges associated with monitoring AMR within complex mixed microbial communities.

The hypotheses of this thesis are:

Constructed wetland system could be minimised to treat wastewater from single household or a school building and the treated wastewater has potential to be reused for irrigation or toilet flushing. Humans have indirect contact with treated wastewater or the constructed wetland system, but the risk of antimicrobial resistance within the wastewater is unknown. A rapid and low-cost method to detect antimicrobial resistance within off-grid wastewater treatment system would provide in time risk assessment of antimicrobial resistance to the user, which hopefully reduces the risk of user acquires antimicrobial resistance from wastewater.

a) The aims of this thesis are:Monitor the performance and the changes of microbial community in operational small-scale constructed wetlands treating domestic wastewater from start-up (first 3 months) and long term (10 months) in Mexico City.

b) Develop a rapid approach to detect the antimicrobial resistance in mixed microbial community from wastewater.

c) Analysis the change of microbial communities and ARGs in response to a range of amoxicillin concentrations.

1.13 Thesis outline

Chapter 1 of this thesis provides background information on the challenges in water and sanitation globally with emphasise the AMR in wastewater. Current research focuses on full scale WWTPs, little is known in decentralised wastewater treatment with low energy input, particularly the microbial community and AMR inside.

Chapter 2 of this thesis introduces three small-scale constructed wetland systems treating domestic wastewater in Mexico City. The system consists of a biodigester, a horizontal constructed, a vertical constructed wetland and a holding tank with potential to reuse the treated wastewater. Two identical systems receive wastewater from one household each, and another system receives wastewater from the toilet of a school building. Water quality of before and after the wetland treatments and microbial community of all three systems were monitored in the first three months' operation.

Chapter 3 continues the monitoring of the two household constructed wetland systems for another 10 months. Additional sample points within both constructed wetlands in each system were added to the analysis. Despite influent wastewaters are different in two systems, pollutants were removed consistently by both systems and achieved stable removal rate at the same time point. Microbial community composition shifted through the system and stabilised two months after performance became stable.

Chapter 4 aims to develop a method to measure the AMR of a community by measuring the community growth rate. Preliminary experiments revealed that the community growth rates measured by optical density (OD) reflect the change of microbial community in responding to a range of amoxicillin concentrations. Secondly, in order to check how the OD reflects the 'true' growth rate of a mixed community, a more precise method of counting cells by flow cytometer (FCM) was validated. Last, a comparison of growth rate measured by both OD and FCM with selected amoxicillin concentrations was conducted.

Chapter 5 analyses the genetic material in the last experiment with selected amoxicillin concentrations in Chapter 4. The change of microbial community composition before and after exposure to amoxicillin of each concentration were analysed through both DNA and RNA. The ARGs and ARGs expression within the microbial communities before and after amoxicillin exposure were first screening through HT-qPCR and then tested with selected gene from the screening test.

Chapter 6 reflects on the thesis findings and gives suggestion for further study.

2 Design and performance monitoring of three small-scale constructed wetland systems for treating wastewater in Mexico City

2.1 Introduction

Wetlands as a natural solution have been employed in treating wastewater for over a century, where report shows United Kingdom and North America are the pioneers (Vymazal, 2011a). Dr. Käthe Seidel carried out the first experiment on plants in wastewater treatment wetlands in 1950s, which eventually led to full scale operation systems in 1960s. Since then, constructed wetlands have been applied all over the world, where reports shows both industrial and domestic wastewater have been treated. Research about improving the performance of constructed wetlands draws more attention, where constructed wetlands have been adapted to treating wastewater with various characteristics.

2.1.1 Current research about constructed wetlands

Constructed wetlands have been used in treating wastewater for over a century, a lot of research focuses on processes inside the wetlands, outcome of the wetland's treatment with various types and sizes of constructed wetlands has been done in the past 60 years (Vymazal, 2011a). The surface area of constructed wetlands varied from less than $1m^3$ lab scale to over $5000m^3$ full scale, where they have been generally classified as microcosm, mesocosm, pilot scale and full scale (Marín-Muñiz et al., 2023). The wastewaters used in those studies include synthetic wastewater, domestic wastewater and industrial wastewater. The medium within the constructed wetlands have also been studied, where they can be adapted to improve the efficiency of specific pollutants (Parde et al., 2021). The concentration of pollutants and the loading rates in research also varied in large ranges (Shukla et al., 2022). Types of plant used in constructed wetlands have drawn a lot of attention, where the species of the plants and the role of plants in the treatment have been studied (Vymazal, 2011b). Studies about microbial composition within constructed wetlands are increasing in recent years, since the microorganisms are the ones eliminating the pollutants, but little is known about them. The greenhouse gas emissions and emerging pollutants have also been targeted in recent studies (Vymazal et al.,

2021). However, current studies about constructed wetlands mainly focus on short term lab studies with synthetic wastewater, studies around full-scale constructed wetlands treating actual wastewater in long-term operation are much less (Vymazal et al., 2021).

2.1.2 Research gap

Currently research about using constructed wetland as the main treatment for single household wastewater is very limited, meanwhile the microbiomes in the system are largely unknown. Furthermore, research around the setting up and stabilisation of constructed wetland mainly focuses on lab-scale study. Therefore, the research question for this study is: can constructed wetlands be miniaturised and used as the primary treatment of grey and black wastewater at household scale within Mexico City?

2.1.3 Hypothesis and aims

The hypothesis is that our small-scale constructed wetland systems are able to remove pollutants from wastewater.

The aim of this study is to monitor the performance of the start-up period of three operational decentralised small scale constructed wetland systems treating wastewater for two households and one school in Mexico City.

2.1.4 Objectives

- To monitor the performance of three small scale constructed wetland systems as primary treatment for the black and grey water over the starting up-period (first three months).
- To characterise the changes in the microbial community, and pathogens across the wetlands from the influent and effluent of the wetland system.

2.2 Materials and methods

2.2.1 Introduction of three decentralised wastewater treatment plants in Mexico City

Three small scale constructed wetlands for decentralised wastewater treatment (WS1, WS2 and WS3) were designed and constructed in the peri-urban area of

Cuajimalpa de Morelos municipality, Mexico City in late 2019 (Figure 2-1). Water supply in this area is inconsistent, therefore all three systems were designed to reuse the treated wastewater. WS1 and WS2 are household systems located in the garden of the household, while WS3 serves a school building and is located next to the building. There are eight adults and two children in WS1 household whilst two adults lived with 15 dogs in WS2 household, the number of students and staffs using the building changes every day during the school opening time. For each system, wastewater flows into the equalization tank and is then pumped into a biodigester. In the household systems, effluent from the biodigester subsequently flows into a subsurface horizontal constructed wetland (CW1) and then into a subsurface vertical constructed wetland (CW2), from which the treated water then flows into a holding tank via gravity. The treated wastewater is stored in the holding tank, which is then either pumped out for the system for use, e.g. irrigations, or is recirculated within the system. The main process units of two household systems are identical (Figure 2-2) while the WS3 (school) includes an extra aeration tank after the biodigester in Figure 2-3 c). The aeration works in line with the pump operating time during the day, and the water in the holding tank is pumped back to flush the toilets. Photos of each system are shown in Figure 2-3. Detailed process units and characteristics are shown in Table 2-1.

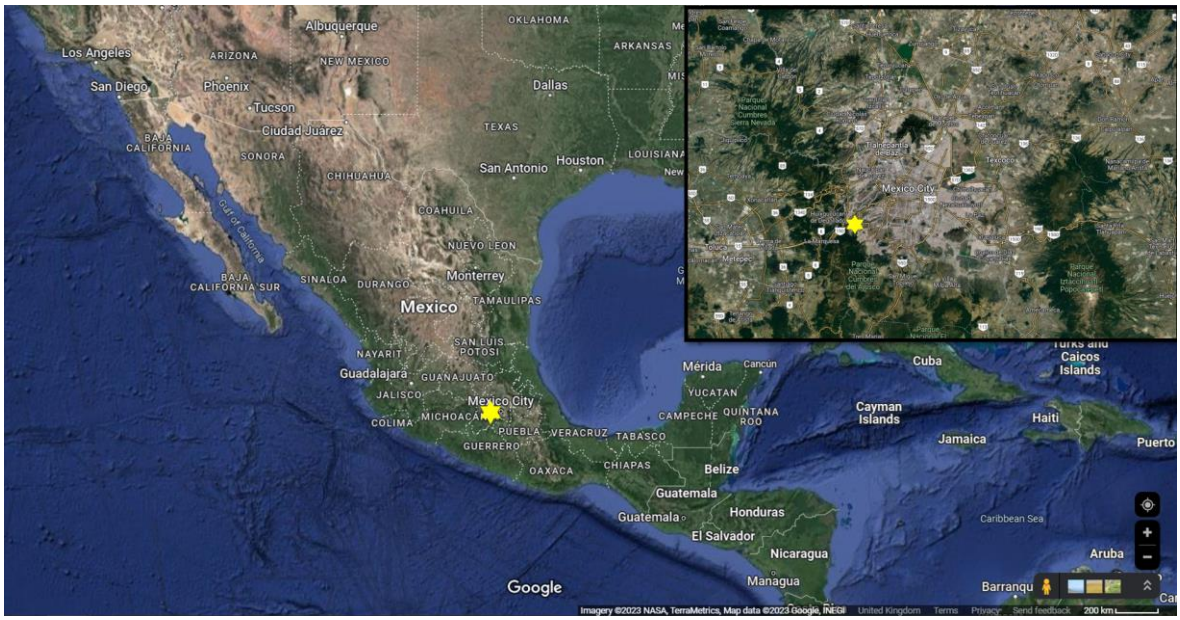

Figure 2-1 Location of the three decentralised wastewater treatment plants in peri-urban Mexico City in this study.

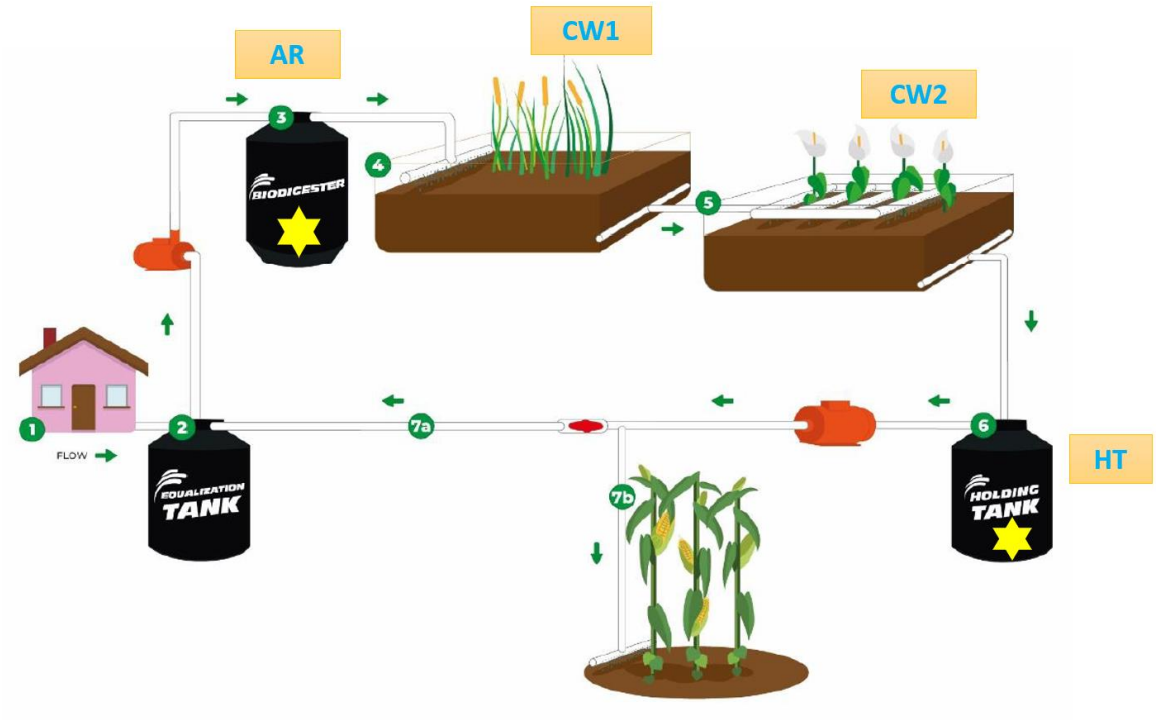

Figure 2-2 Diagram of a household wastewater treatment plant. Household wastewater flows into an equalization tank and is then pumped into a biodigester (AR). The effluent from the biodigesters subsequently flows into a subsurface horizontal constructed wetland (CW 1) and a subsurface vertical constructed wetland (CW 2) via gravity. A holding tank (HT) collects the effluent from the vertical wetland with the potential of reuse the water for irrigation or recirculation. Yellow asterisks on biodigester and holding tank indicate where the samples were taken from.

a) WS1 (Household system)

b) WS2 (Household system)

c) WS3 (School system)

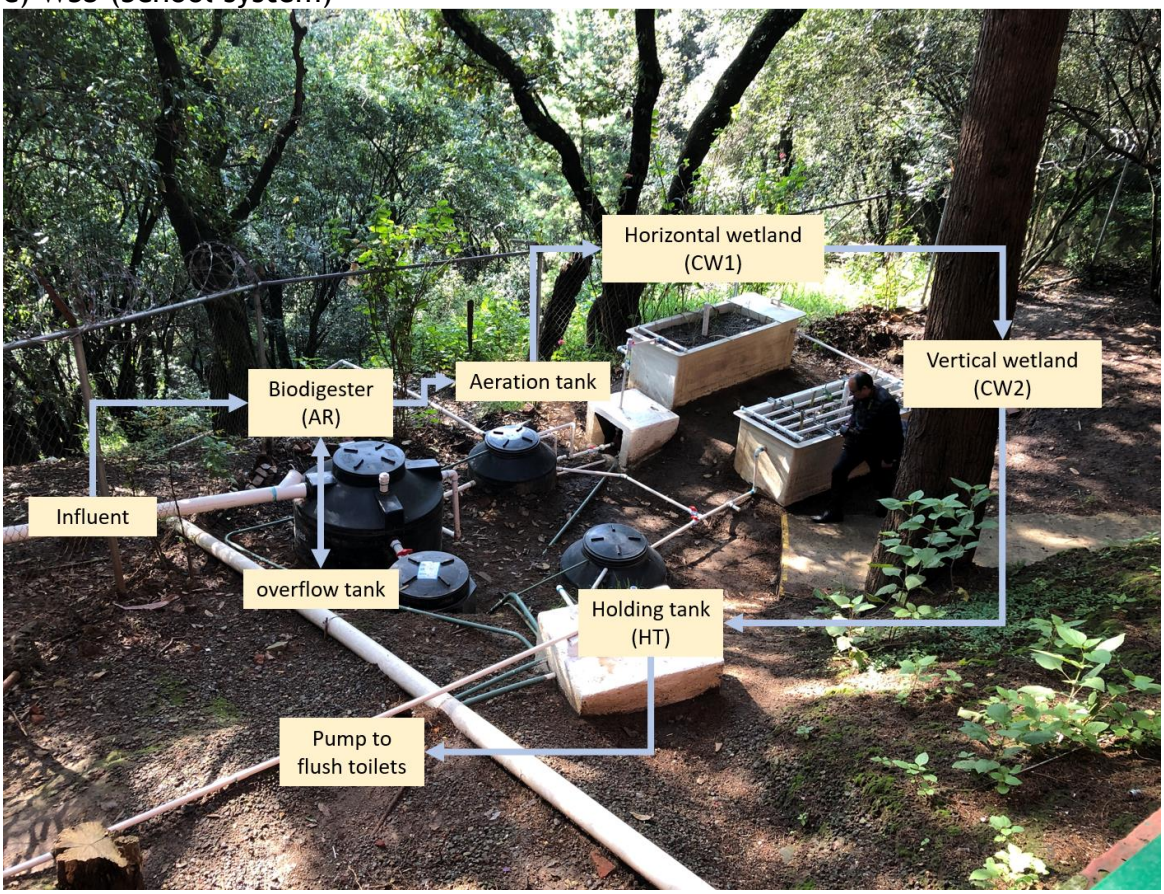

Figure 2-3 Photos of the three operational WWTPs in this study. All photos were taken on 4th November 2019. a) WS1 (household system) just finished construction at the time this photo was taken, it started recirculation mode soon afterwards. b) WS2 (household system) was about to finish at the time this photo was taken. c) WS3 (school system) is constructed and in recirculation mode when this photo was taken. Detailed process units are labelled in this photo.

2.2.2 System design and operation

The WS1, WS2 and WS3 were constructed in November, November and September, respectively. All three systems were designed to treat 700L of wastewater every week. There was an overflow pipe connected to equalization tank in each system, where if the wastewater excessed the treatment capacity would flow into the public drainage system connected nearby.

All systems were operated in recirculation mode until January 2020, and then operated in feeding and resting mode from January to March 2020. Detailed operational information is shown in Table 2-2, where the pumps in all three systems were operated 4 times a day for 30s, 40s and 40s each time for WS1, WS2 and WS3, respectively from January to February, while in March pumps were run 8 times a day for 30s each time in WS1, 8 times a day for 10s each time in WS2 and 4 times a day for 40s each time in WS3. During the operation, hydraulic retention time (HRT) of WS1, WS2 were 7 days, 15 days, respectively.

Table 2-1 Summary of process units and characteristics of all three decentralised wastewater systems.

Process unit	Characteristic	WS1	WS2	WS3
1. Equalization tank	Volume	200 L	500 L	500 L
2. Biodigester (AR)	Volume	1300 L	600 L	1200L
3. Aeration tank	Volume	--	--	500L
3. Subsurface horizontal constructed wetland (CW 1)	Material	1 mm geomembrane	Fiberglass	Fiberglass
	Size (m^3) (trapezoidal prism) (b1/b2; w; h)	0.90 m^3 (0.40/0.95; 2.31; 0.60)	0.84 m^3 (2;0.70; 0.60)	0.84 m^3 (2;0.70; 0.60)
	Area (rectangle)	2.2 m^2	1.4 m^2	1.4 m^2
	Depth and media	0.6 mm (20 cm) and 1.2 mm (40 cm) gravel	0.6 mm (20 cm) and 1.2 mm (40 cm) gravel	0.6 mm (20 cm) and 1.2 mm (40 cm) gravel
	Plant	*A. donax, and Typha sp*	*Scirpus sp., A. donax and Typha sp*	*Thypa sp., Scirpus sp., Phragmites sp. australis.*
4. Subsurface vertical constructed wetland (CW 2)	Material	1mm geomembrane	1mm geomembrane	Fiberglass
	Size (m^3) (trapezoidal prism) (b1/b2; w; h)	0.94 m^3 (0.40/0.95; 2.35; 0.60)	0.90 m^3 (0.40/0.95; 2.31; 0.60)	0.84 m^3 (2;0.70; 0.60)
	Area (rectangle)	2.23 m^2	2.2 m^2	1.4 m^2
	Depth and media	0.6 mm (20 cm) and 1.2 mm (40 cm) gravel	0.6 mm (20 cm) and 1.2 mm (40 cm) gravel	0.6 mm (20 cm) and 1.2 mm (40 cm) gravel
	Plant	*P. australis and Z. aethiopica*	*Z. aethiopica*	*Z. aethiopica*
5. Holding tank (HT)	Volume	1200 L	500 L	500L

Table 2-2 Operation information of all three systems. The recirculation pumps of each system only run during the feeding period.

Time	Parameter	WS1	WS2	WS3
January-February 2020	Number of recirculation	4	4	4
	Feeding period duration (s)	30	40	40
	Estimated feedwater flow (L/d)	467	578	240
March 2020	Number of recirculation	8	8	4
	Feeding period duration (s)	30	10	40
	Estimated feedwater flow (L/d)	933	289	240

2.2.3 Sampling, water quality analysis and DNA extraction

Water sampling, and water quality analysis were conducted by our collaborators Mario Alberto Salinas-Toledano and Tania Lizet Gómez-Borraz from the Universidad Autónoma Metropolitana-Cuajimalpa (Metropolitan Autonomous University-Cuajimalpa), Mexico City.

1L of water sample was taken from inside the biodigester and the holding tank of each system on 13th January, 17th February and 9th March 2020 while systems were at resting mode. Samples were stored at 4℃ and analysed within 24 hours. COD (HI93754B-25), phosphate (HI93713-01), nitrate (HI93728-01), nitrite (HI93708-01) from all the samples were analysed following Standard Methods for the examination of Water and Wastewater (Baird et al., 2017) with kit from Hanna Instruments. Samples were diluted with deionized water (DI) when necessary, no further pretreatment for COD, phosphate, nitrate and nitrite analysis.

DNA extraction was conducted by Tania Lizet Gómez-Borraz in the laboratory at Universidad Autónoma Metropolitana-Cuajimalpa (Metropolitan Autonomous University-Cuajimalpa), Mexico City. 500ml of the biodigester or holding tank water was centrifuged at 4000 xg (Beckman Avanti® JCN-26) for 10mins immediately after sampling and the pellets were stored at -20℃ until extraction. 0.2g of each pellet was extracted with DNeasy® PowerSoil® Kit (Qiagen, 12888) and eluted with 40µl of 10 mM Tris (provided by the kit) followed the

manufacturer's manual. DNA samples were stored at -20°C before shipping to Glasgow.

Table 2-3 Samples for this study. Influent samples were taken from biodigester, effluent samples were taken from holding tank of each system.

			Sample name	COD	NO_2^-	NO_3^-	P	DNA
Jan	WS1	Influent	WS1_Inf_Jan	✓	✓	✓	✓	✓
		Effluent	WS1_Eff_Jan	✓	✓	✓	✓	✓
	WS2	Influent	WS2_Inf_Jan	✓	✓	✓	✓	✓
		Effluent	WS2_Eff_Jan	✓	✓	✓	✓	✓
	WS3	Influent	WS3_Inf_Jan	✓	✓	✓	✓	✓
		Effluent	WS3_Eff_Jan	✓	✓	✓	✓	✓
Feb	WS1	Influent	WS1_Inf_Feb	✓	✓	✓	✓	✓
		Effluent	WS1_Eff_Feb	✓	✓	✓	✓	✓
	WS2	Influent	WS2_Inf_Feb	✓	✓	✓	✓	✓
		Effluent	WS2_Eff_Feb	✓	✓	✓	✓	✓
	WS3	Influent	WS3_Inf_Feb	✓	✓	✓	✓	✓
		Effluent	WS3_Eff_Feb	✓	✓	✓	✓	✓
Mar	WS1	Influent	WS1_Inf_Mar	✓	✓	✓	✓	*
		Effluent	WS1_Eff_Mar	✓	✓	✓	✓	✓
	WS2	Influent	WS2_Inf_Mar	✓	✓	✓	✓	✓
		Effluent	WS2_Eff_Mar	✓	✓	✓	✓	✓
	WS3	Influent	WS3_Inf_Mar	✓	✓	✓	✓	✓
		Effluent	WS3_Eff_Mar	✓	✓	✓	✓	✓

Note: * in the table means that DNA sample is missing from DNA study.

2.2.4 *16S rRNA* gene PCR amplification and library pooling for Illumina MiSeq sequencing preparation

DNA concentration was measured with Qubit™ Broad Range (BR) kits (Invitrogen™ Q33266) at Glasgow University and then stored at -80°C until further use. DNA samples (template) were diluted to 1ng/µl with DNase/RNase free water (Invitrogen, 10977035) for template for each PCR reaction.

The PCR reaction followed the *16S rRNA* Illumina amplicon protocol of Earth Microbiome Project, one step PCR with primer 515F and 926R (Quince et al., 2011, Parada et al., 2016) targeting V4-V5 region, and the annealing temperature optimised prior to library construction.

Two different DNA samples (an influent sample from WS1 and a sediment sample from WS2, they are samples used in the next chapter) were selected for the annealing temperature optimisation test. KAPA HiFi HotStart PCR kit (KAPA, KK2502) was used for the PCR reaction, the reaction volume was 15µl, and contained either 3.75µl of DNA template or water (NTC) (see Table 2-4), alongside primers, dNTPs and buffer. The annealing temperature of 50℃, 52℃, 54℃, 56℃, 58℃ and 60℃ were applied to the PCR reaction with two tubes each and one for negative control. The PCR condition was 5 minutes denaturation at 95℃, 25 cycles of 20 seconds at 98℃ and 30 seconds at annealing temperatures and 40 seconds at 72℃, plus 1 minute extension at 72℃ with Thermal Cycler (Applied Biosystems™ 4375786).

Table 2-4 PCR reaction conditions for optimisation and amplicon PCR.

Item	PCR tube (µl) (optimisation)	PCR tube (µl) (amplicon PCR)	Per Sample (µl) (amplicon PCR)
Water	6.6	11	33
KAPA HiFi Fid buffer (5x)	3	5	15
KAPA HiFi dNTP's (10mM)	0.45	0.75	2.25
Reverse Primer (10µM)	0.45	0.75	2.25
KAPA HiFi HotStart Taq (1U/µl)	0.3	0.5	1.5
Forward Primer (10µM)	0.45	0.75	2.25
Template 1ng/µl(or NTC)	3.75	6.25	13 (or 6.25)
TOTAL	**15**	**25**	**75**

After amplification, the PCR products and a 1Kb plus DNA ladder (Invitrogen, 10787026) were mixed with 1% (v/v) loading dye respectively and loaded onto 1% (w/v) agarose gel containing SYBR™ Safe DNA gel stain (Invitrogen, S33102), and run at 120V for 50 minutes. The gel was visualised with Gel Doc™ XR+ imager (Bio-rad, 1708195) shown on Figure 2-4. The brightest band under the highest annealing temperature is considered as the optimised annealing temperature, therefore 58℃ was selected as the optimised temperature for the following PCR amplification.

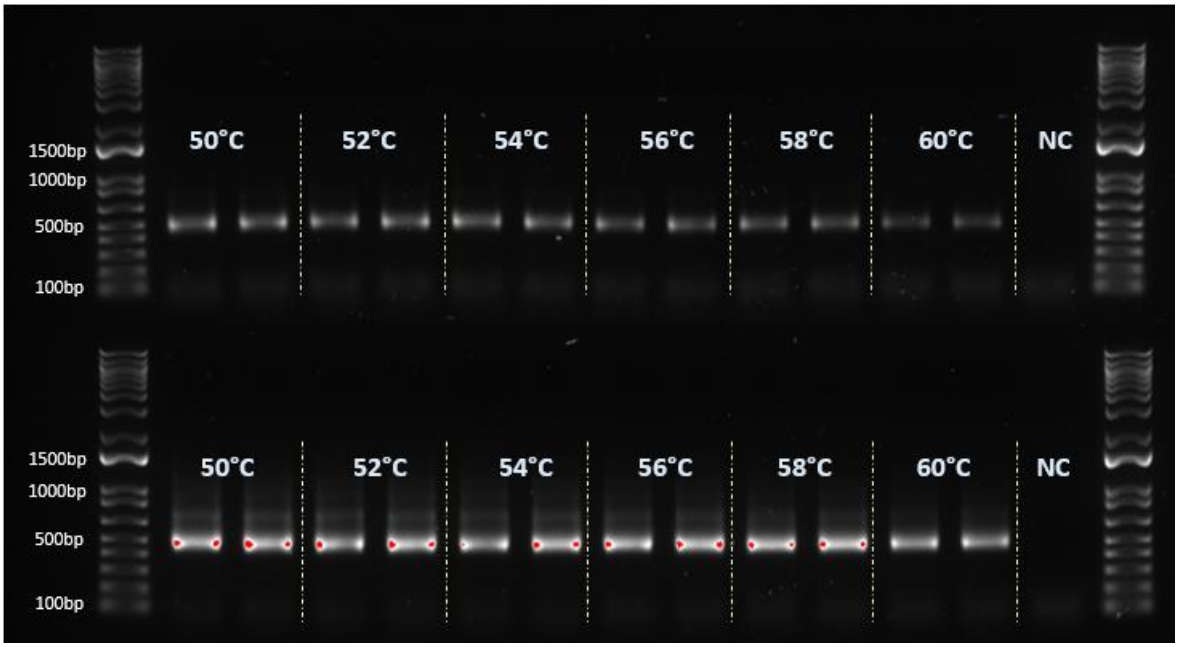

Figure 2-4 Gel image of PCR annealing temperature gradient of 2 samples. Two samples were tested with annealing temperature of 50℃, 52℃, 54℃, 56℃, 58℃ and 60℃. The PCR products of 15µl each was mixed with 1.5µl loading dye and loaded onto the gel against the 1kb plus ladder respectively. The brightness of the bands decreases with the increasing temperature. The brightest band under the highest annealing temperature is considered as the optimised annealing temperature, therefore 58℃ is selected as the optimised temperature for the following PCR amplification.

One step PCR was performed with different barcoded forward primers for different samples, each sample was amplified in duplicate alongside a negative control. PCR reaction was prepared as detailed in Table 2-4, and PCR conditions listed above with an annealing temperature of 58℃. Each PCR product was mixed with 2.5µl of loading dye and loaded onto the gel to size against a 1kb plus ladder. Amplicons of the correct size were gel excised in a 2ml Eppendorf tube with Zymoclean™ Gel DNA Recovery Kit (D4002) following the manufacturer's protocol. The recovered DNA was eluted with 10µl of DNA free water and measured with Qubit™ High Sensitivity (HS) kits (Invitrogen™ Q33231) and then stored at -20℃ until further use. The recovered PCR product from each sample was diluted to around 5ng/µl with DNA free water and then 5ng of each sample was added to make the library pool for sequencing.

2.2.5 Sequencing and bioinformatics

Bioinformatics were performed under the supervision and guidance of Dr. Umer Ijaz.

The pooled library was sequenced by Earlham Institute (Norwich, UK) via Illumina MiSeq v3 300bp. The Qiime2 workflow (V2019.7) (Caporaso et al., 2010) and DADA2 pipeline (Callahan et al., 2016) (https://github.com/umerijaz/tutorials/blob/master/qiime2_tutorial.md) was used to create Amplicon Sequencing Variants (ASVs). Abundance tables were generated by constructing ASVs using only the forward reads in the QIIME2 workflow. We have used Deblur algorithm (Amir et al., 2017) with a read length of 240bp and using SILVA SSU Ref NR database release v.138 (Quast et al., 2013) as a reference database in `qiime deblur denoise-other` plugin. Prior to using Deblur algorithm, the reads were quality trimmed using Phred quality score of 20. This gave us 21,386 ASVs for n=305 samples. Then combined the taxonomic information with the abundance table to generate a BIOM file. The rooted phylogenetic tree, also generated using the QIIME2 framework, along with the above BIOM file as well as the functional tables from PICRUSt2 were then used in the downstream statistical analyses in R.

2.2.6 Statistics

Statistics were performed under the supervision and guidance of Dr. Umer Ijaz.

As a pre-processing step, we removed typical contaminants such as *Mitochondria*, and *Chloroplasts*, as well as any ASVs that were unassigned at all levels, as per recommendations given at https://docs.qiime2.org/2022.8/tutorials/filtering/ and also filtered out samples that were not relevant to this study (or are <5,000 reads) giving abundance tables of: *n*=305 samples x P=21,040 ASVs with summary statistics of ASVs per sample as follows: [Minimum: 9,756; 1st Quartile: 36,518; Median: 43,319; Mean: 46,624; 3rd Quartile: 53,179; Maximum: 113,796]. In this study, we have used the first 17 samples corresponding to the start-up phase of the three wetland systems (see the next chapter for long term operation of WS1 and WS2), with the final abundance table of: n=17 samples x P=3,775 ASVs.

The R's vegan package (Oksanen et al., 2007) was used for alpha and beta diversity analyses. For alpha diversity measures we used: (i) *rarefied richness* – the estimated number of species/features in a rarefied sample (to minimum library size); and (ii) *Pielou's eveness* – the relative abundance of the microbial

community, which is constrained between 0 and 1 where value approaching 0 suggests less evenness in communities between the species (i.e. the presence of a dominant species). We have used R's aov() function to calculate the pair-wise analysis of variance (ANOVA) p-values which were then drawn on top of alpha diversity figures.

To visualise beta diversity, Principal Coordinate Analysis (PCoA) was used with the R' Vegan's package. We have used two different distance measures in PCoA: (i) *Bray-Curtis distance* on the ASV abundance table to see if there is compositional changes; and (ii) *Unweighted UniFrac distance* (proportion of branch length shared between samples) estimated using R's Phyloseq package (McMurdie and Holmes, 2013) to see if there is any change in the phylogeny. To find taxa/function that were at least 2 log fold different, we have used the DESeq2 package (Love et al., 2014). DESeq2 uses negative binomial GLM to obtain maximum likelihood estimates for log fold change of features between the two conditions and then applies Bayesian to obtain shrunken log fold changes subsequently employing the Wald test for obtaining significances. We have used a p-value cut-off of 0.05 in the procedure.

Additionally, the Vegan package was also used to perform PERMANOVA analyses to see if the microbial or functional community structures can be explained by different sources of variability. We have further used two more approaches: a) a constrained ordination approach, Canonical Correspondence Analysis (CCA) using cca() function from R's vegan package where the Chi-square transformed abundance data is subjected to weighted linear regression on constraining physico-chemical parameters, and then ANOVA (anova.cca() function with parameter `by="terms"`) to suggest whether there is a relationship; and b) an environmental fitting approach, where we fitted smooth surfaces of the covariates on ordination plot (PCoA in this case; *Bray-curtis distance*) using penalised splines by employing ordisurf() function from R's Vegan package. The latter method uses generalised additive model by regressing the covariate as y ~ S(Dim1,Dim2), where Dim1 and Dim2 are the ordination scores extracted from PCoA and S() is a spline function. We have only shown those covariates where the model fits i.e., $p < 0.05$.

A list of potential pathogens was developed based on the WHO priority pathogens list (2017) and amended with relevant pathogens from Bartlett et al. (2022). Sequencing result was cross checked with potential pathogens' list to reveal the relative abundance of potential pathogens in all samples.

2.2.7 Quantitative PCR

Multiplex quantitative PCR method was developed and tested by Dr Fabien Cholet.

Two multiplex real-time PCR assays were performed to quantify total Bacteria (*16S rRNA*), *Escherichia coli* and *Pseudomonas aeruginosa*, and *Legionella*, *Legionella pneumophila* and *Legionella pneumophilla* serogroup 1, respectively. Each assay had a reaction volume of 25µl containing 12.5µl of QuantiTect Probe PCR Master Mix (Qiagen, Cat. No.204343), 1µl of each forward and reverse primer (10nM), 0.2µl of each probe (10nM), 2µl of template and 4.9µl of DNA free water. Detailed primer sets used in the assay are listed in Table 2-5. The reaction condition was 15 minutes denaturation at 95℃ followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute by QuantStudio 3 (Thermo Fisher Scientific).

Table 2-5 Target organism, gene and primer and probe sets used in quantitative PCR reaction.

	Target organism	Target gene (length bp)	Orientation	Sequence	Reference
Assay 1	Total Bacteria	*16S rRNA* (124)	Forward	CGGTGAATACGTTCYCGG	Suzuki et al. (2000)
			Reverse	CGGTGAATACGTTCYCGG	
			Probe	CGGTGAATACGTTCYCGG	
	Escherichia coli	*rodA* (195)	Forward	GCAAACCACCTTTGG TCG	Chern et al. (2011)
			Reverse	CTGTGGGTGTGGATT GACAT	
			Probe	AACCCCTACAACCGG CAGAATACC	
	Pseudomonas aeruginosa	*gyrB* (220)	Forward	CCTGACCATCCGTCGCCACAAC	Anuj et al. (2009)
			Reverse	CGCAGCAGGATGCCGACGCC	
			Probe	CCGTGGTGGTAGACCTGTTCCCAGACC	
Assay 2	*Legionella* spp.	*ssrA* (101)	Forward	GGCGACCTGGCTTC	Benitez and Winchell (2013)
			Reverse	GGTCATCGTTTGCATTTATATTTA	
			Probe	ACGTGGGTTGCAA	
	Legionella pneumophila	*mip* (115)	Forward	TTGTCTTATAGCATTGGTGCCG	Benitez and Winchell (2013)
			Reverse	CCAATTGAGCGCCACTCATAG	
			Probe	CGGAAGCAATGGCTAAAGGCATGCA	
	Legionella pneumophila serogroup 1	*wzm* (70)	Forward	TGCCTCTGGCTTTGCAGTTA	Benitez and Winchell (2013)
			Reverse	CACACAGGCACAGCAGAAACA	
			Probe	TTTATTACTCCACTCCAGCGAT	

2.3 Results

2.3.1 Water quality and pollutant removal efficiency

The concentrations of COD, NO_2^-, NO_3^- and P from both influent and effluent of every system are shown in Figure 2-5. The effluent concentrations of COD, NO_2^-, NO_3^- and P of each system were much lower than their respective influent, indicating effective operation of the wetlands. WS2 had the highest COD, NO_2^-, NO_3^- and P concentrations in influent among all systems at every sample time, whist WS3 had the lowest.

COD concentration in the influent of WS1 varied from 753.0mg/L to 1462.0mg/L, whereas in the effluent they were reduced to 422.0-432.0 mg/L irrespective of starting concentration. In WS2, the influent COD concentration varied from 1635.0 mg/L to 2410.0 mg/L, whist the effluent concentration ranged from 106.0 to 482.0 mg/L. In WS3, the influent COD concentration ranged from 153.0 to 587.0 mg/L, whilst the effluent concentration varied from 57.0 to 147.0 mg/L.

The ranges of NO_2^- concentration in influent of WS1, WS2 and WS3 were 33.0-65.2mg/L, 69.7-104.2mg/L and 3.0-54.4mg/L, respectively. The effluent concentrations of NO_2^- were 16.6-31.0mg/L, 5.0-27.2mg/L and 1.8-29.2mg/L for WS1, WS2 and WS3, respectively. The ranges of NO_3^- concentration in influent of WS1, WS2 and WS3 were 106.9-157.9mg/L, 223.2-247.4mg/L and 13.7-65.4mg/L, respectively. The effluent concentrations of NO_3^- were 62.9-67.9mg/L, 17.4-76.2mg/L and 9.5-40.9mg/L for WS1, WS2 and WS3, respectively.

The influent concentrations of P were 1.2-12.1mg/L, 3.1-14.7mg/L and 0.5-8.5mg/L for WS1, WS2 and WS3, respectively. And the range of P concentrations in the effluent were 1.1-6.6mg/L, 0-7.2mg/L and 0.01-6.1mg/L for WS1, WS2 and WS3, respectively.

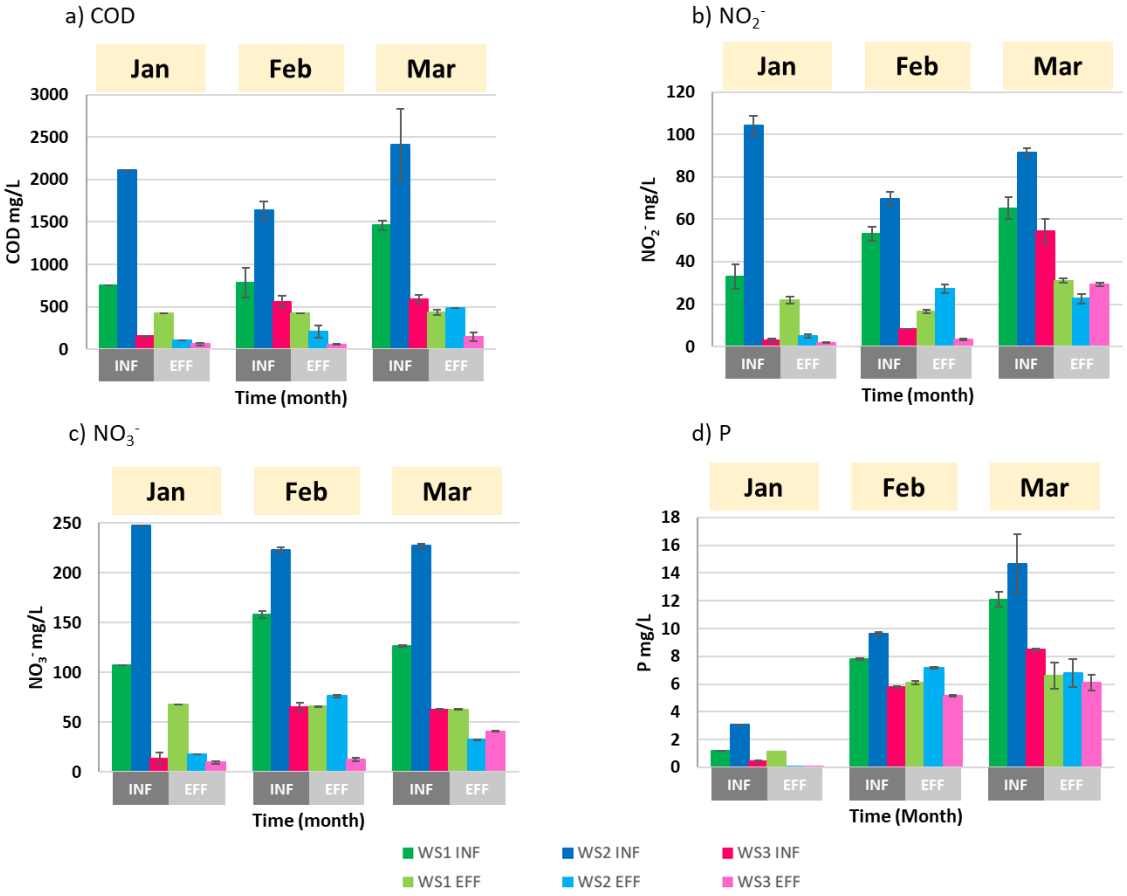

Figure 2-5 Concentrations of COD, NO_2^-, NO_3^- and P in the influent and effluent of all three systems over three months. Error bars stand for standard deviation, which calculated from two measurements from each sample. (n=18)

$$\boldsymbol{Removal\ rate} = \frac{\boldsymbol{C_{inf}} - \boldsymbol{C_{eff}}}{\boldsymbol{C_{inf}}} \times \mathbf{100\%} \qquad \textbf{Equation 1}$$

Where, $\boldsymbol{C_{inf}}$ means the influent concentration (mg/L), $\boldsymbol{C_{eff}}$ means the effluent concentration (mg/L).

The removal rate of COD, NO_2^-, NO_3^- and P were calculated by *Equation 1*, the removal rate of COD, NO_2^-, NO_3^- and P of all three systems was shown in Figure 2-6. The removal rates of COD from all three systems ranged 43.8-70.5%, 80.0-95.0%, 58.8-89.9% for WS1, WS2 and WS3, respectively. The removal rates of NO_2^- from all three systems ranged 33.9-68.9%, 61.0-95.2%, 39.3-58.8% for WS1, WS2 and WS3, respectively. The removal rates of NO_3^- from all three systems ranged 36.5-58.4%, 65.9-93.0%, 30.7-80.7% for WS1, WS2 and WS3, respectively. The removal rates of P from all three systems ranged 8.3-45.5%, 25.7-100.0%, 10.5-98.8% for WS1, WS2 and WS3, respectively.

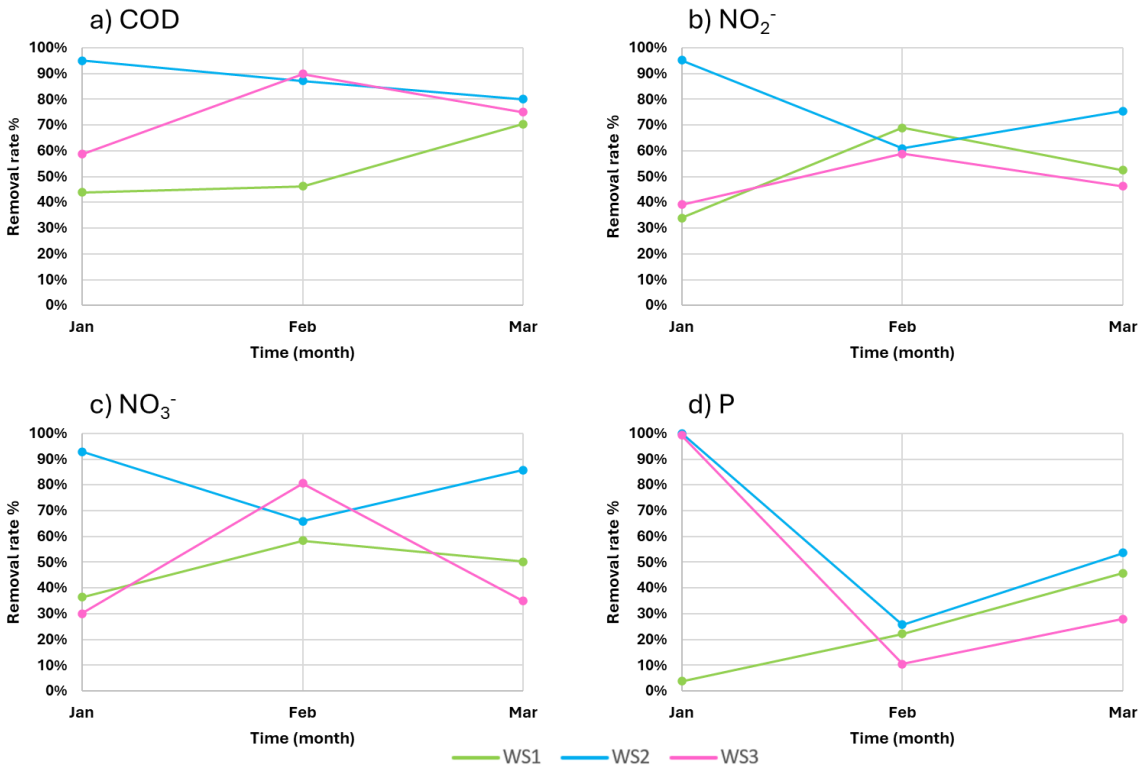

Figure 2-6 Calculated removal rate of COD, NO_2^-, NO_3^- and P of all three systems over three months. (n=9, removal rate calculated from the average concentration (of two measurements from one sample) before and after treatment.)

In WS2, the removal of NO_2^-, NO_3^- and P showed a similar trend with highest removal in January then lowest in February before increasing again in March. The change of removal of NO_2^- and NO_3^- in both WS1 and WS3 as well as COD in

WS3, also showed the same trend, where the lowest removal was in January followed by highest removal in February and then lower in March.

In general, COD had the highest removal in all three systems, but the amount of COD removed differed between systems, as huge variations of COD concentrations among the systems were seen. In WS1, COD removal increased slightly from 43.8% in January to 46.2% in February and then hiked substantially to 70.5% COD removal in March. In WS2, COD removal was high from the start of operation, decreasing slightly over time, from 95.0% in January to 80.0% in March. In WS3, COD removal increased from 58.8% in January to 90.0% in February then dropped to 75.0% in March.

The removal rate of P showed the most variation, having both the highest and the lowest removal among the measured parameters. However, P concentrations were much lower compared to other parameters. In January, P had the lowest concentration whilst highest removal rate except WS. Meanwhile, P concentrations increased with time, but the removal rate also increased with time in all three systems.

2.3.2 Microbial community analysis

2.3.2.1 Quantification of total bacteria via *16S rRNA* gene amplification

Total bacteria were quantified by *16S rRNA* gene amplification, although it is not a single copy gene in most bacteria, it is positively associated with the number of bacteria in mixed microbial community (Větrovský and Baldrian, 2013). *16S rRNA* gene copies of each sample was normalised with per gram of biomass (see Table 2-6). The gene copies per gram of biomass varied from 6.24 $x10^7$ to 7.02 $x10^{10}$ with no significant difference between influent and effluent of each system (all INF vs EFF, P=0.772, T-test (paired two sample for means), indicating that the wetlands did not overall reduce bacterial numbers in per gram of biomass in the effluent.

Table 2-6 *16S rRNA* gene copies per gram biomass. n=17, single measurement of each sample.

		Jan	Feb	Mar
WS1	INF	1.69 X 10^{10}	1.41 X 10^{10}	
	EFF	8.35 X 10^{9}	2.79 X 10^{10}	2.46 X 10^{9}
WS2	INF	8.76 X10^{9}	4.83 X 10^{9}	1.33 X 10^{10}
	EFF	1.22 X 10^{10}	1.79 X 10^{10}	2.19 X 10^{9}
WS3	INF	5.39 X 10^{9}	7.02 X 10^{10}	2.35 X 10^{9}
	EFF	6.24 X 10^{7}	3.56 X 10^{8}	5.03 X 10^{8}

* indicates missing sample.

2.3.2.2 Alpha diversity of Pielou's evenness and species richness

Diversity within microbial communities was analysed through species richness and Pielou's evenness of the *16S rRNA* sequencing data, where species richness measures the presence of different species, Pielou's evenness measures the relative abundance of species. The microbial communities of the biodigester (influent) and holding tank (effluent) of the three wetlands were characterised over the three-month start-up period. Species richness ranged from 260 ASVs in influent of WS3 to 659 ASVs in the influent of WS1. Species richness varied in the influent of all three systems, where influent from WS1 was significantly higher than WS2 (P=0.028) and WS3 (P=0.0071). In WS3, there was a statistically significant increase in species richness from the influent (maximum 310 ASVs) to the effluent (maximum 449 ASVs, P=0.02).

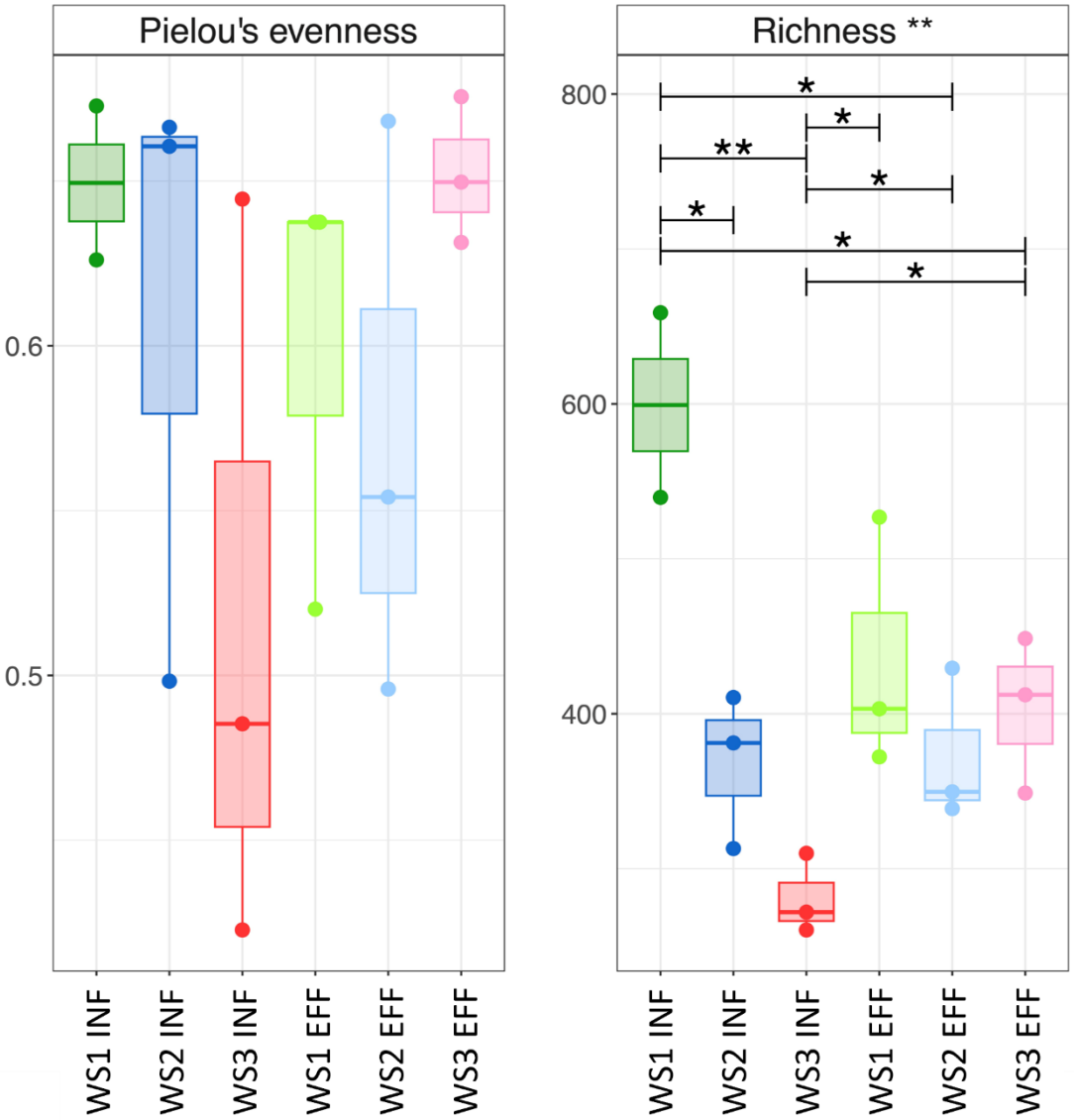

Figure 2-7 Pielou's evenness and richness value of the influent and effluent of the wetlands in three WWTPs analysed by ANOVA. n=17, one sample from each time point was taken as listed in Table 2-3. Asterisk represents P value, where "*" p≤0.001, "**" 0.001 < p ≤ 0.01, "*" 0.01< p ≤ 0.05. Boxplot for visualisation purpose and easy compassion with Figure 3-11.**

The relative abundance of species within the groups were measured by Pielou's evenness, where the trends were similar as species richness. In the influent, evenness was highest for WS1 although WS2 had a higher medium and upper quartile value. In the effluent, the evenness was lower in the effluent of WS1 and WS2 compared to their influent in terms of upper quartile value, while in WS3 the evenness in the effluent increase, however there was no significant difference in Pielou's evenness among all groups.

2.3.2.3 Microbial community composition of the effluent from two different household converged after three months of operation.

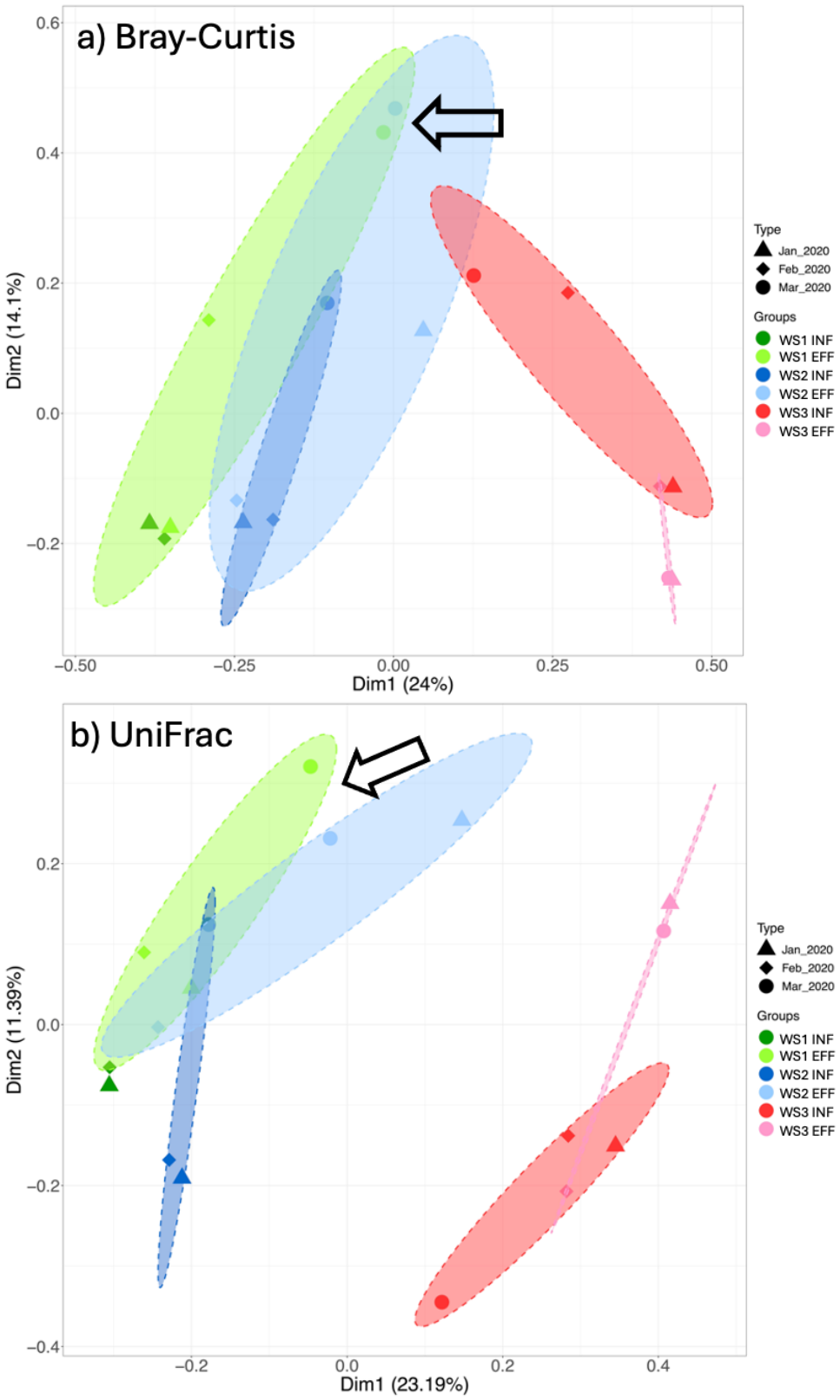

Figure 2-8 Beta diversity plotted in PCOA with a) Bray-Curtis dissimilarity and b) unweighted UniFrac distance of the influent and effluent from all three WWTPs. Ellipses generated with standard errors of influent or effluent from each system. Sample months were indicated by different shapes (triangle, diamond and circle for January, February and March, respectively) and systems by colour with influent a darker shade than effluent. Arrows point to the effluent samples from WS1 and WS2 in March, where they converged in the third month. One sample from each time point was taken as listed in Table 2-3 (n=17).

Microbial community composition of every sample was compared through Bray-Curtis and unweighted Unifrac of the beta diversity analysis, where Bray-Curtis measures the relative abundance of the microbial species and unweighted Unifrac measurement focuses on the phylogenetic presence/absence of microbial species. The microbial community composition of all three systems were different (P=0.001 for both Bray-Curtis R^2 =0.282 and unweighted Unifrac R^2=0.285), and changed from the influent to the effluent within each system (P=0.042, R^2=0.079, Bray-Curtis; P=0.026, R^2=0.086, unweighted Unifrac) (Figure 2-8). The two household systems, WS1 and WS2 were more similar to each other than the school system, WS3. For WS3, despite the variation in the influent community, from the first month of operation, produced a stable effluent microbial community that remained the same over the first three months of operation (Figure 2-8, a) and b)). In contrast for the two household systems, while the influent varied after three months of operation, the effluent from the two household systems became similar and in March clustered together, on both the Bray Curtis (relative abundance) and the Unifrac (Phylogeny) plots, indicating that the microbial communities in the effluent of the two independent wetland systems converged after three months of operation.

Canonical correspondence analysis (CCA) reflects the relationship between microbial community structure and the concentrations of N and P in the influent and effluent (see Figure 2-9). The reduction in concentration of wastewater COD, N and P as across the wetland is also associated with a change in the community composition in influent and effluent. The arrows point to the higher concentration of each parameter, where COD, NO_2^-, NO_3^- were positively related to higher concentrations (influent samples) (P=0.011, COD; P=0.012, NO_3^-). The arrow of P was not strongly associated with influent samples, this is because the concentration differences of P between influent and effluent are much smaller than other parameters.

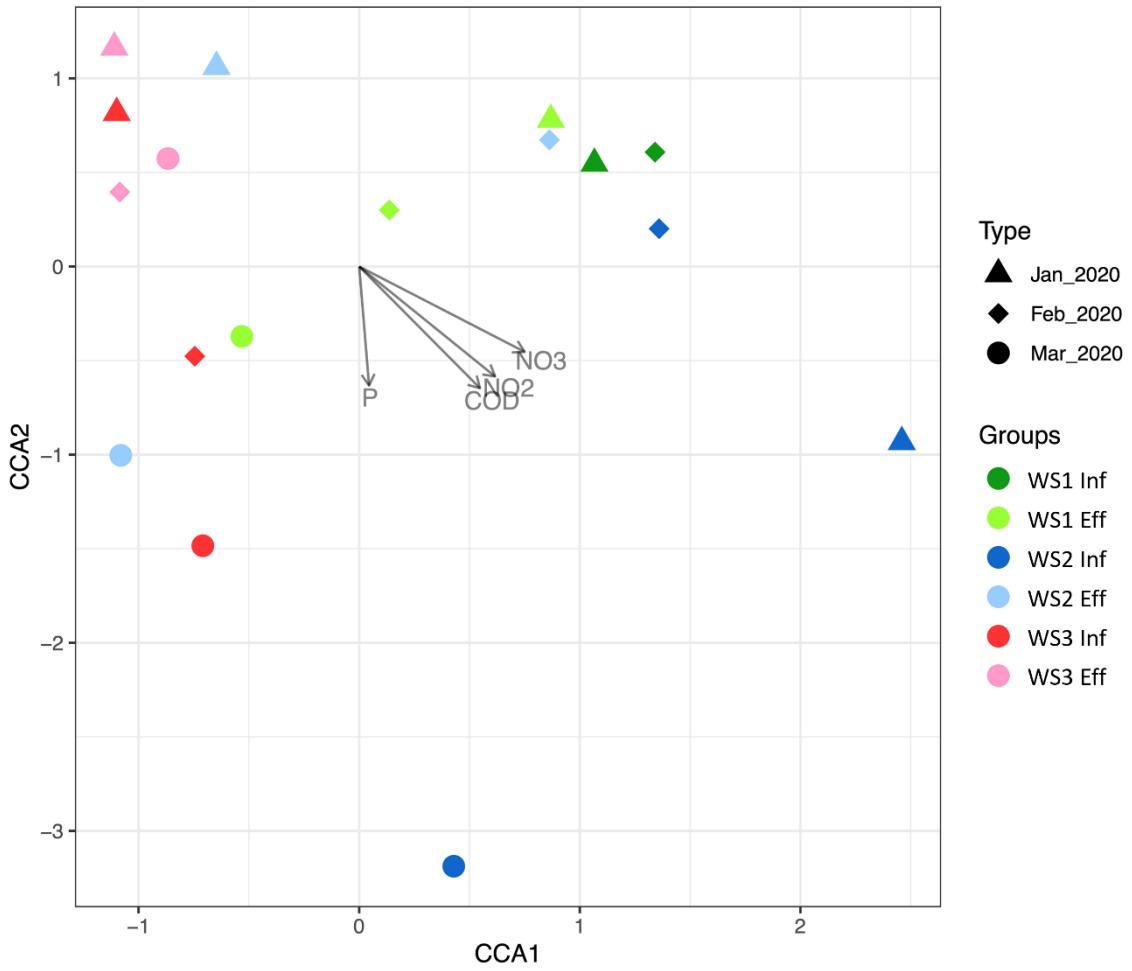

Figure 2-9 The canonical correspondence analysis (CCA) analysis. COD, NO_2^-, NO_3^- and P concentrations of each sample are plotted against microbial community structure. Sample months were indicated by different shapes (triangle, diamond and circle for January, February and March, respectively) and systems by colour with influent a darker shade than effluent. One sample from each time point was taken as listed in Table 2-3 (n=17).

2.3.2.4 Top 25 most abundant taxa

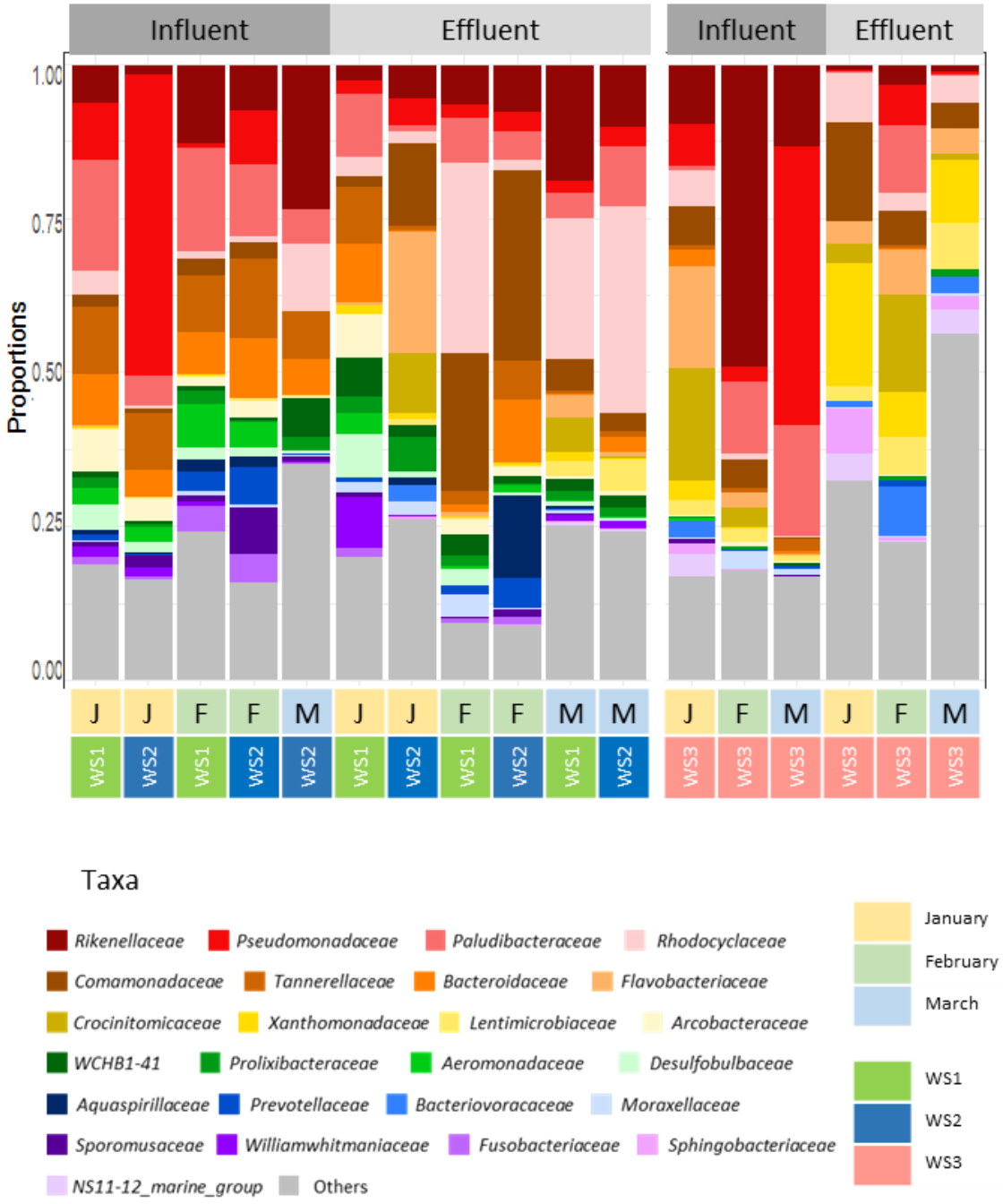

Figure 2-10 Top 25 most abundant taxa at family level. Taxa bars were grouped by influent and effluent and separated as household systems (WS1 and WS2) and school system (WS3). One sample from each time point was taken as listed in Table 2-3 (n=17).

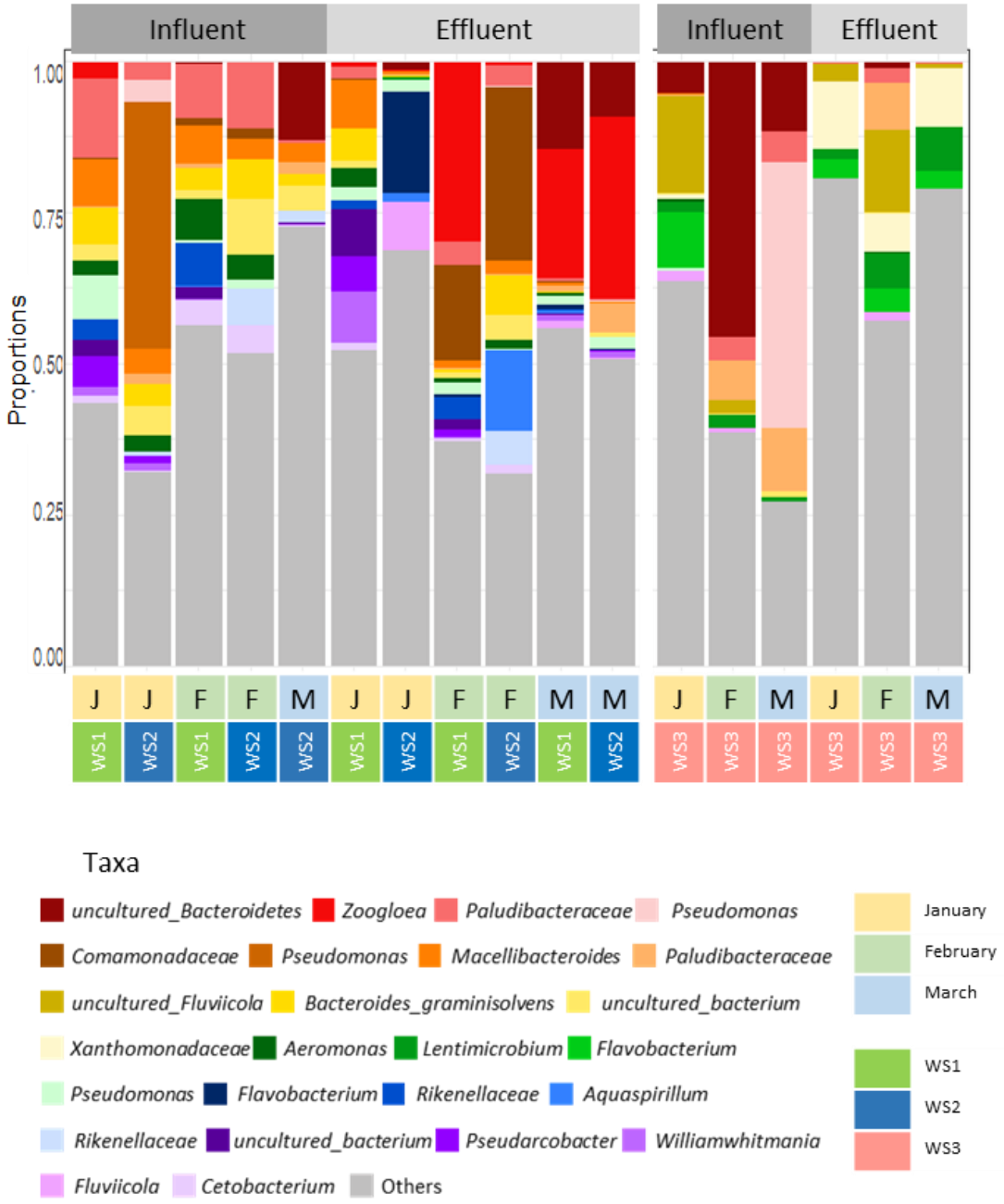

Figure 2-11 Top 25 most abundant taxa as ASV level. Taxa bars were grouped by influent and effluent and separated as household systems (WS1 and WS2) and school system (WS3). One sample from each time point was taken as listed in Table 2-3 (n=17).

As indicated in the beta diversity analysis, the top 25 taxa show how the household systems (WS1 and WS2) were different from the school system (WS3). In household systems, the influent microbial communities were variable over the time, and changed over the wetland, resulting in different effluent microbial communities unique to each wetland. These became more similar over the operational time of the wetlands, so much so that the effluent samples from March, after three months of operation, were highly similar to each other despite different influent. In contrast the school system effluent was different and with the top 25 most abundant taxa varying in the influent, yet the wetland

effluent microbial communities were highly similar to each other from the start of operation.

Top 25 most abundant ASVs accounted for 19.31-72.76% of the total taxa in the influent of all three systems, whilst 27.23-68.05% in the two household systems and both highest and lowest appeared in the school system (see Figure 2-11). Top 25 most abundant families had a coverage of 43.76-91.03% of the total taxa in all three system, while the effluent from school in March had the lowest coverage, the coverage of the rest of the samples was similar to each other.

2.3.2.5 Potential pathogens

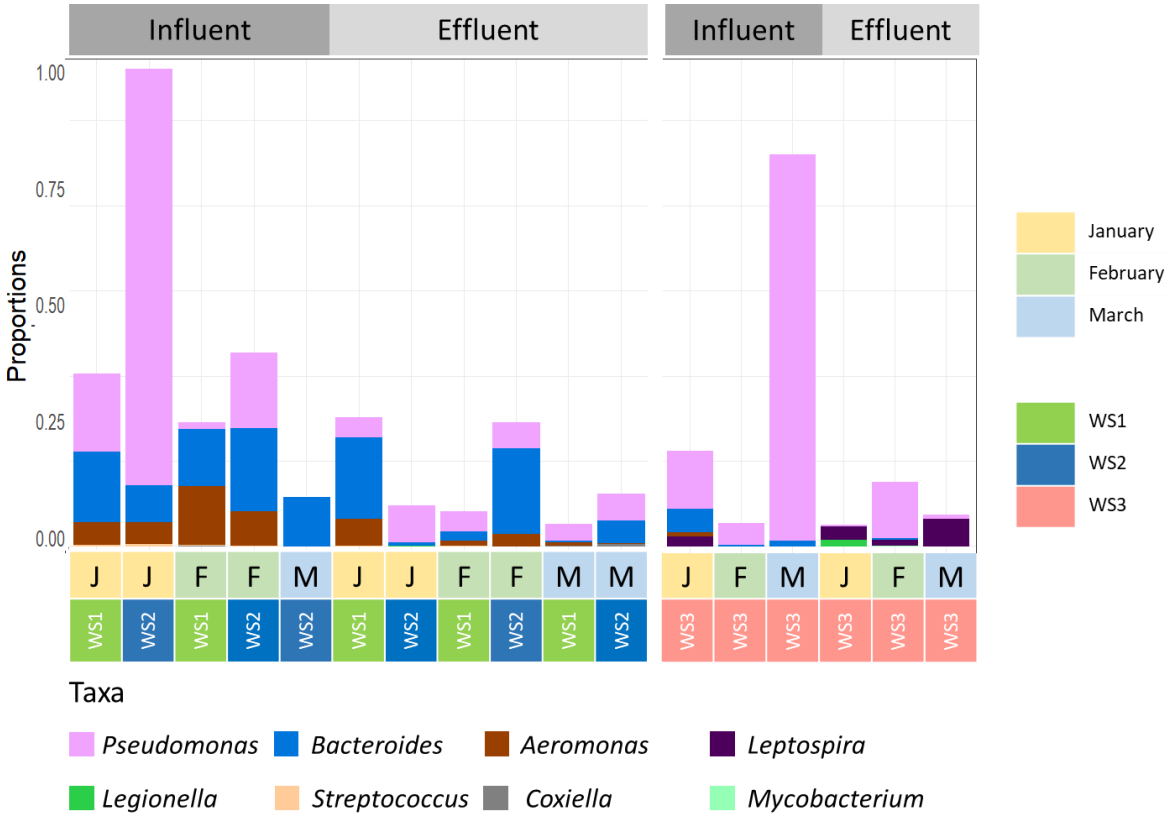

Figure 2-12 Relative abundance of potential pathogens in influent and effluent of all three systems at genus level. Samples were grouped as influent and effluent, school system was separated from household system. One sample from each time point was taken as listed in Table 2-3 (n=17).

As all three systems were designed to re-use the treated wastewater, particularly for the household systems, we looked within the datasets for potential pathogens and examined changes in their relative abundances across the wetlands over the start-up period. In the household systems (WS1 and WS2), *Pseudomonas*, *Bacteroides* and *Aeromonas* were the dominant potential pathogens in both influent and effluent, while in WS3, *Leptospira* was also

observed in most of the samples (Figure 2-12). There was a decline in the abundance of *Pseudomonas* in effluent across WS1 and WS2 in January, however, a slight increase of *Pseudomonas* was seen in the effluent of WS1 in February and WS2 in March. There was no influent data for WS1 in March, but the proportion of *Pseudomonas* in the effluent was low. In the school system (WS3), the abundance of *Pseudomonas* decreased in both January and March but increased slightly in February. The proportions of *Leptospira* increased in the effluent of WS3, in all three months, and Legionella was only observed in the effluent in the first month's operation.

Faecal indicator *E. coli* was quantified via single copy gene *rodA* (see Figure 2-13), where the overall average effluent *E. coli* gene copies were significant lower (P=0.007) than the overall influent. The effluent *E. coli* gene copies in WS1 decreased overtime, whilst the other two systems saw an increase in February and dropped to zero in the last month. The ratios of *E. coli* to total bacteria (*16S rRNA*) were shown in Table 2-7, where the overall average ratios were significantly lower (P=0.112) than the overall average influent. Those results indicate that all three systems worked well in removing *E. coli* from the wastewater. However, as replicates were not taken, the robustness of the removal by individual systems could not be determined.

Pseudomonas aeruginosa was detected only at one time point in both influent and effluent from WS2, where their gene copies were very low and similar values in both influent and effluent (see Figure 2-14), indicating that when *Pseudomonas aeruginosa* presented it was not removed by the wetlands. The ratios of *Pseudomonas aeruginosa* to *16S rRNA* gene (Table 2-7) were much lower than the relative abundance of *Pseudomonas* genus identified in Figure 2-12. This indicates that non-pathogenic *Pseudomonas* species were the main contributors to the high relative abundance of *Pseudomonas* genus.

Three *Legionella* species were quantified through PCR (*Legionella spp*, *Legionella pneumophila*, and *Legionella pneumophila* serogroup 1) results were shown in Figure 2-15 and Figure 2-16. *Legionella spp* was found in all samples and ranged from 5.66 $x10^2$ to 6.44 $x10^6$ in the influent and 0 to 1.72$x10^5$ the effluent. *Legionella pneumophila* was also quantified in all samples, ranged from 3.73 $x10^3$ to 6.84 $x10^6$. However, *Legionella pneumophila* serogroup 1 was not

found in any of the samples. No statistical difference between the average gene copies in all influents and effluents (P=0.346, *Legionella spp*; P=0.347, *Legionella pneumophila)*. Those results indicate that only WS1 worked in removing *Legionella spp*. The ratio of *Legionella spp* to *16S rRNA* were shown in * indicates missing sample.

Table 2-8, there was no difference between the average ratio of overall influent and effluent (P=0.371, *Legionella spp*; P=0.615, *Legionella pneumophila)*. This result indicates that the wetland systems were not removing *Legionella* from wastewater. *Legionella* genus was found in the effluent of school system from the sequencing data set in January, but *Legionella* was not detected by qPCR.

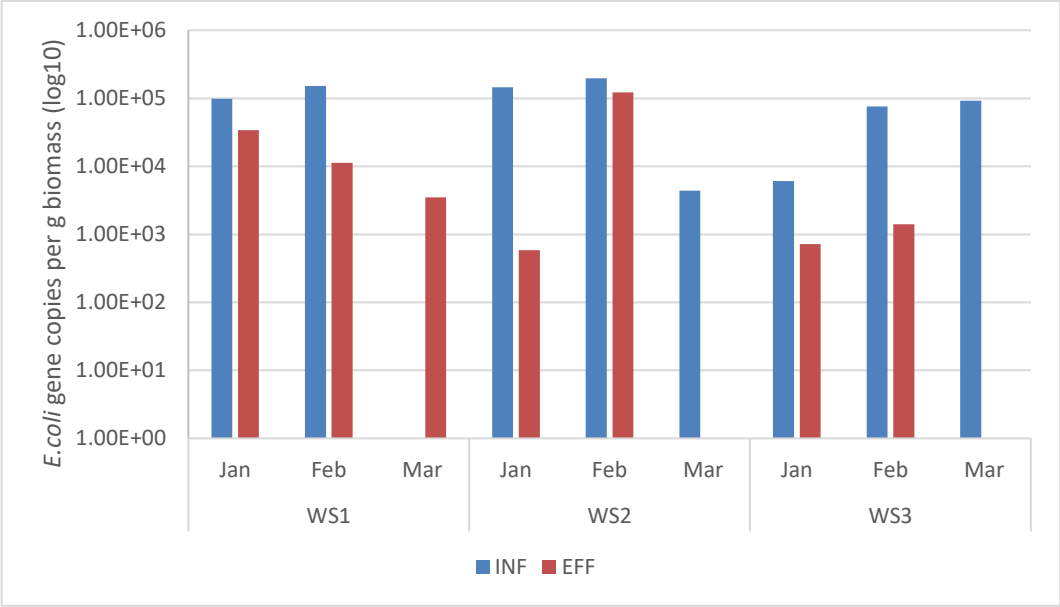

Figure 2-13 *E. coli* gene copies per gram of biomass of all influent and effluent samples from each system in log_{10} scale. (n=17, one measurement of one sample from each time point was taken as listed in Table 2-3).

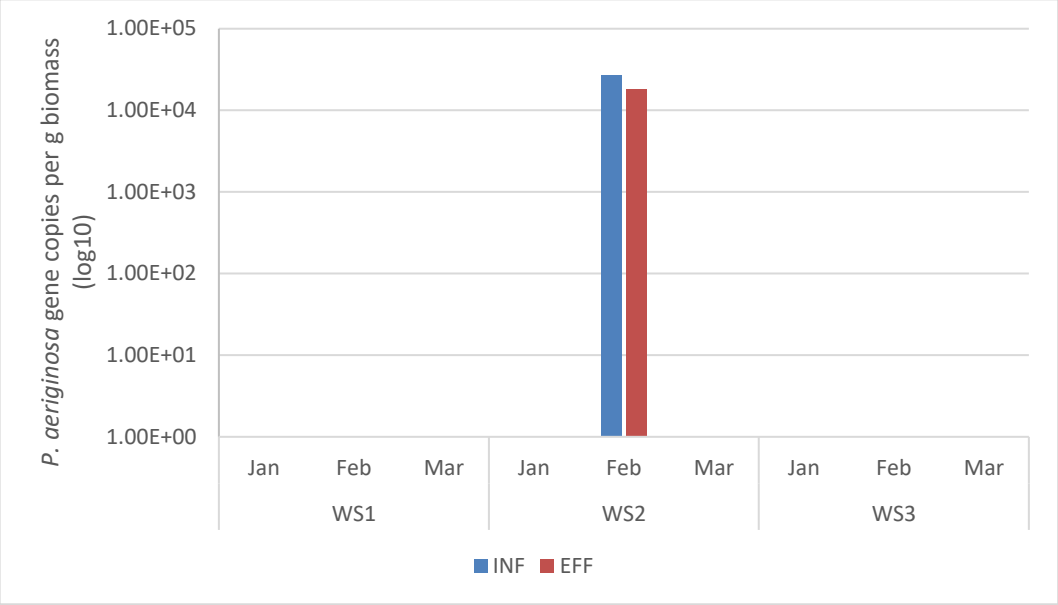

Figure 2-14 *P. aeruginosa* gene copies per gram of biomass of all influent and effluent samples from each system in log10 scale. (n=17, one measurement of one sample from each time point was taken as listed in Table 2-3).

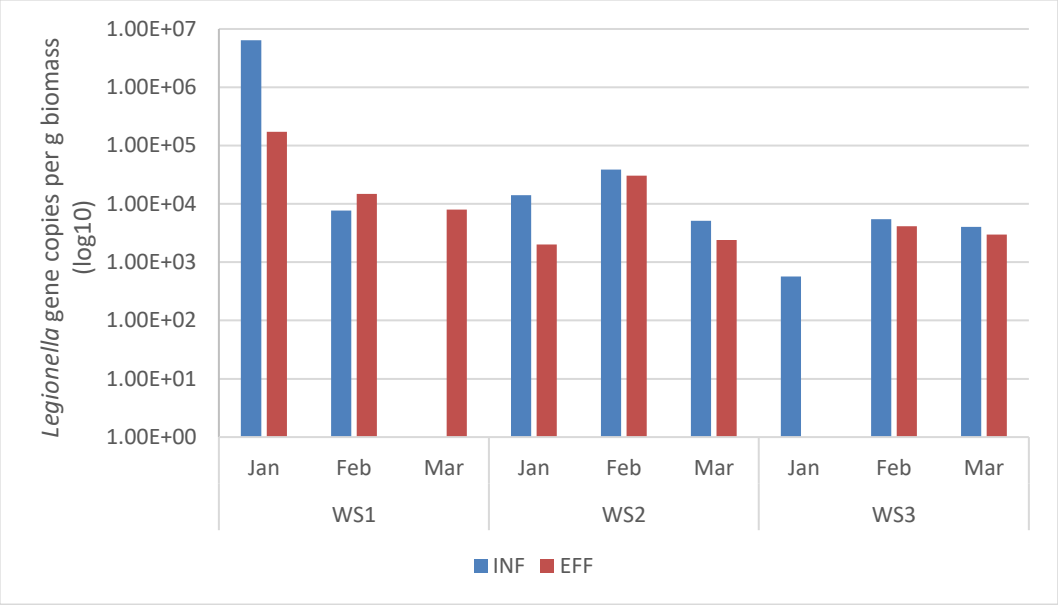

Figure 2-15 *Legionella* gene copies per gram of biomass of all influent and effluent samples from each system in log10 scale. (n=17, one measurement of one sample from each time point was taken as listed in Table 2-3).

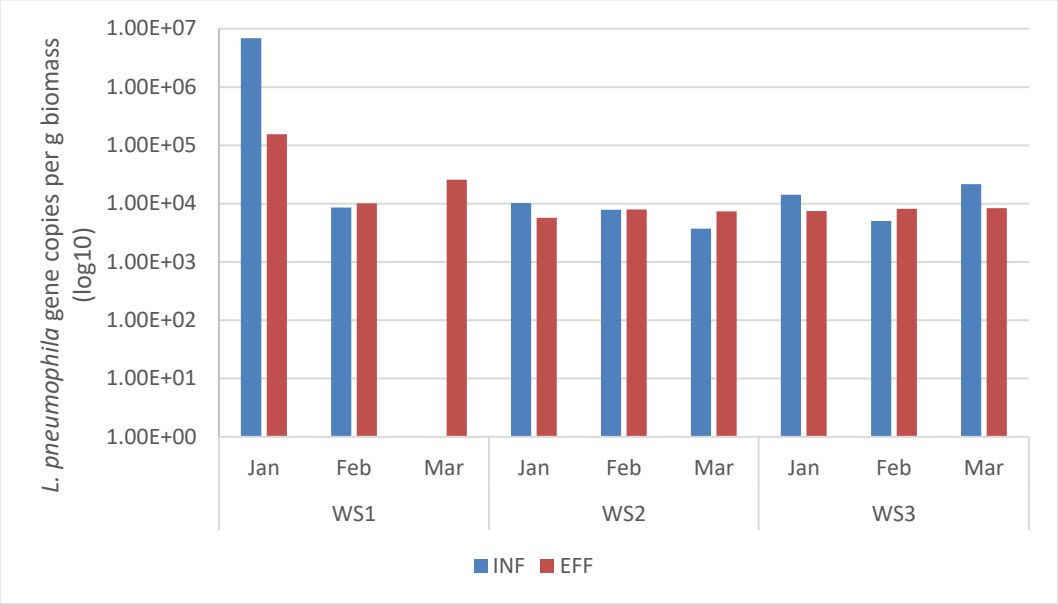

Figure 2-16 *L. pneumophila* gene copies per gram of biomass of all influent and effluent samples from each system in log10 scale. (n=17, one measurement of one sample from each time point was taken as listed in Table 2-3).

Table 2-7 Ratio of *E. coli* to *16S rRNA* and *P. aeruginosa* to *16S rRNA* of all influent and effluent samples from every month.(n=17, one measurement of one sample from each time point was taken as listed in Table 2-3).

		E. coli			*P. aeruginosa*		
		Jan	Feb	Mar	Jan	Feb	Mar
INF	WS1	5.79×10^{-06}	1.07×10^{-05}	*	0	0	*
	WS2	1.74×10^{-05}	7.07×10^{-06}	1.78×10^{-06}	0	9.69×10^{-07}	0
	WS3	6.92×10^{-07}	1.57×10^{-05}	6.97×10^{-06}	0	0	0
EFF	WS1	2.78×10^{-06}	6.31×10^{-07}	1.60×10^{-06}	0	0	0
	WS2	1.09×10^{-07}	1.74×10^{-06}	0	0	2.60×10^{-07}	0
	WS3	1.15×10^{-05}	3.95×10^{-06}	0	0	0	0

* indicates missing sample.

Table 2-8 Ratio of *Legionella* to *16S rRNA* and *L. pneumophila* to *16S rRNA* of all influent and effluent samples from every month. (n=17, one sample from each time point was taken as listed in Table 2-3).

		Legionella			*L. pneumophila*		
		Jan	Feb	Mar	Jan	Feb	Mar
INF	WS1	3.80×10^{-04}	5.46×10^{-07}	*	4.04×10^{-04}	6.08×10^{-07}	*
	WS2	1.68×10^{-06}	1.39×10^{-06}	2.08×10^{-06}	1.22×10^{-06}	2.81×10^{-07}	1.51×10^{-06}
	WS3	6.45×10^{-08}	1.14×10^{-06}	3.05×10^{-07}	1.62×10^{-06}	1.04×10^{-06}	1.63×10^{-06}
EFF	WS1	1.41×10^{-05}	8.25×10^{-07}	3.65×10^{-06}	1.26×10^{-05}	5.66×10^{-07}	1.17×10^{-05}
	WS2	3.74×10^{-07}	4.36×10^{-07}	1.02×10^{-06}	1.06×10^{-06}	1.12×10^{-07}	3.16×10^{-06}
	WS3	0	1.16×10^{-05}	5.96×10^{-06}	1.20×10^{-04}	2.28×10^{-05}	1.66×10^{-05}

* indicates missing sample.

2.4 Discussion

2.4.1 Water quality and pollutants removal efficiency

We set out to monitor if small scale constructed wetland systems were capable of treating domestic and school wastewater. Over the first three months of operation, each constructed wetland performed well in removing organic load and nitrogen from wastewater. Influent COD concentrations in the two household systems were much higher than the school system, especially in the WS2. Although that is expected as grey water has a high organic load while black water contains higher nitrogen, the difference between households and school is wider than predicted. However, De Anda et al. (2018) and Belmont et al. (2004) observed domestic wastewater with average COD concentration of 988mg/L and 1569mg/L in Mexico, which are comparable to our data. Also, the family using WS2 has 15 dogs and they put dog faeces into the biodigester, this would contribute the high pollutant concentration in the biodigester in WS2.

According to Mexican standards for reuse treated wastewater (NOM-003-SEMARNAT-1997), monthly average BOD_5 concentration should be no more than 30mg/L for indirect contact (Gutiérrez, 2008). Since BOD_5 was not measured in this study, we assume that half of the COD in the influent was biodegradable, which is within the common range of 0.3-0.8 BOD_5/COD in domestic wastewater. There is only one time point sample at WS3 in February meets the standard after conversion, but this sampling campaign only obtained one sample from each system every month, there is no monthly average data to compare against the standards. Also, since the treated wastewater in WS1 and WS2 are used for irrigation, the extra organic carbon and nitrogen provide extra nutrients for the soil and plants. Nitrogen and P concentrations are not listed in the standard for reuse treated wastewater, meanwhile, the plants would benefit from up taking the residual nitrogen and P in the effluent as fertilizer.

COD removal rate in this study is comparable to the matured full scale constructed wetlands in Mexico. Marín-Muñiz et al. (2023) summaries average COD removal rates ranged 64-93% from 18 full scale constructed wetlands in Mexico. In this study, the average COD removal rates are 53.51%, 87.38% and 74.55% for WS1, WS2 and WS3 respectively, which are within the range of full

scale constructed wetlands. Also, the COD removal rates varied in the first two months of monitoring and reached 70% in all three systems in the third month, which indicate the systems are improving over time in terms of COD removal.

Méndez-Mendoza et al. (2015) monitored COD removal rate in subsurface horizontal constructed wetlands treating domestic wastewater with different plants in the first six months of operation, the removal rates increase overtime in the first four months, which are similar to WS1 and WS3. Although the removal rate in WS2 drops overtime, but they maintain a high removal rate of over 80%. The COD removal rates in the first month of Méndez-Mendoza et al. (2015) study vary from 15% to 35%, where in our study, the COD removal rates in first month are higher than 43%. This difference may be caused by the hydraulic retention time, where it is 5 days in our study but only 1 day (24h) (Méndez-Mendoza et al., 2015).

Phosphate removal rate in all kinds of wetlands is low (40-60%), unless the wetland is built with high absorption material (Vymazal, 2007). This also explains the high phosphate removal rate in the first month that then dropped in our study. Meanwhile, phosphate concentrations in all the effluent are lower than the latest Mexican standards for wastewater discharges directly into surface or groundwater of no more than 10mg/L daily average or 13mg/L at any time point (SECRETARIA DE MEDIO AMBIENTE Y RECURSOS NATURALES, 2022).

The trends of NO_2^- and NO_3^- of all three systems remained the same during those three months, WS1 and WS2 had the lowest removal rate in January then peaked in February, whilst in WS2 it peaked in January but plummeted in February. Sirivedhin and Gray (2006) found out that higher nitrate and carbon concentrations promote denitrification in wetlands both natural and laboratory scale. In this study, WS2 had the highest concentrations of NO_2^-, NO_3^- and COD among all the systems, and highest removal rates of all them were observed in the same system (WS2), which meets the finding by Sirivedhin and Gray (2006).

Plants in the constructed wetlands are generally considered to bring oxygen down to the sediment inside the constructed (Truu et al., 2009). However, in this study plants were planted in November and due to low temperature and low rainfall, the plants were still small at the end of this study.

2.4.2 Microbial community analysis

We set out to monitor if these small-scale constructed wetlands were capable of treating domestic and school wastewater and examining what was happening to the microbial communities in the wastewater. The microbial community composition of beta diversity results from both Bray-Curtis and UniFrac shown that the effluent samples of the two household from the third month converged, which illustrates the microbial communities in the household effluent become similar in the third month. Similar trends were observed on both sediment and water samples in microcosm constructed wetland. Fernandes et al. (2015) observed sediment samples were clustered by week in Bray-Curtis beta diversity, despite the influents were mixed up with different antibiotics. Weber and Legge (2011) found that microbial community activities reached a steady state at day 75 observed by carbon resources utilisation on BIOLOG™ ECO plates in mesocosms study. Those suggest that time is related to the structure of microbial community, however, it is unknown how the results would be affected by the different research methods used in those studies. Meanwhile, *Zoogloea* is the most abundant genus at ASV level for both household effluents in March. *Zoogloea* is reported to be a heterotrophic nitrification and aerobic denitrification bacteria (Wang et al., 2022), which suggests that the nitrogen concentration reduced through the system is more likely to be removed by microbes rather than adsorbed by the wetland media.

We have observed that in household systems, species richness is higher in influent and lower effluent, while the school system is opposite to household systems. Wang et al. (2022) state that species richness is reduced when nitrogen and phosphorus concentrations are high, meanwhile C/N ratio and wastewater type also affect the species richness. In this study, household systems receive both black and grey water while school system receives blackwater only, which explains why school system has the lowest species richness in the influent among all three systems. Meanwhile, WS2 with higher pollutant concentrations and lower species richness than WS1 can also be explained by this statement. In the school system, the species richness is higher in the effluent, this may be caused by lower pollutant concentrations, where the school effluent is always the lowest among all three systems. However, total nitrogen was not measured in

this study, and therefore it is not known how the C/N ratio affects species richness.

School effluent had the highest evenness in all sample groups, whilst lowest coverage in top 25 most abundant taxa and constant ASVs and families in all three months' observation. Higher evenness value means the species within the microbial community are more equal distributed, therefore, the top 25 most abundant taxa cover the least of overall taxa. In the school system, both influent and effluent pollutant concentrations are the lowest among all three system, low pollutant concentrations may be favoured by microbes which results in a more diverse and equally distrusted microbial community with less dominant species (Wang et al., 2022). Through the statistical analysis of bacterial community structure analysed by Fernandes et al. (2015), reveals that time of exposure has the highest coefficient with bacterial community structure. This probably explains why the taxa of the effluents from school system (WS3) were consistent, as this system was built and operated in recirculation mode two months earlier than the household systems. Two different *Xanthomonadaceae* have been found high in abundance in the effluent, meanwhile Torrentó et al. (2011) state that *Xanthomonadaceae* may be a good candidate for autotrophic denitrification. This indicates that the school system also works in removing nitrogen by microbes, probably because of the different influent, the main nitrogen removal microbe is different from household systems.

The potential pathogens in all samples were quantified through single copy genes via qPCR, where there was a significant decrease in average *E. coli* gene copies per gram of biomass in effluent samples. This indicates those systems are capable of removing *E. coli* from the wastewater. There is no data about how much biomass has been removed by the system, since the TSS is generally lower in the effluent than the influent, the actual *E. coli* removal rate of those systems could potentially be higher. Due to lack replicate samples, the removal rate of single system is not available, also qPCR may over quantify *E. coli* compares to culture-based method. As qPCR tests *rodA* gene, where dead *E.coli* with intact *rodA* gene can be detected, but culture-based method only tests for live *E.coli*. The faecal coliforms in Mexican stands for reuse treated wastewater should be less than 1000 MPN/100ml (Gutiérrez, 2008), since we lack biomass

information from the wastewater, the result cannot be compared with the standard.

Pseudomonas is considered as one of the potential pathogens, and it has been detected in almost every sample through microbial community analysis, however, the pathogenic qPCR results reveal that most of the *Pseudomonas* species are not pathogenic species. Also, Szekeres et al. (2002) found that *Pseudomonas* is also linked with denitrification activities, which suggests that those no pathogenic *Pseudomonas* species may be the potential denitrifier.

2.5 Conclusion

Small scale constructed wetland systems worked in removing pollutants from wastewater in two households and a school in this study. The removal of COD, NO_2^-, NO_3^- from the first month are comparable to long-term running full scale constructed wetland in Mexico. The microbial community composition reveals that two household effluents converged in the third month despite different influent. The school system microbial community is very different from households, probably caused by different wastewater resource and characteristics. *E. coli* has been removed significantly from biomass in all three systems, with potentially meet the standards of reuse treated wastewater in Mexico. Those systems were not capable to remove *Legionella* species from biomass. All those results from the first three months' operation and monitoring are promising, however, it is not known how their performances would be in long-term operation. Next step, the systems will be monitored for longer time period, more physical and chemical parameters and samples within constructed wetlands would provide more information about the performance of the systems and the change of microbial community composition.

3 Long-term performance monitoring and microbial community analysis of two small-scale constructed wetland systems treating household wastewater in Mexico City

3.1 Introduction

Our study of the first three months' operation demonstrated that our small-scale constructed wetland systems worked in reducing pollutants from the first month. Long-term performance of full-scale constructed wetland treating community wastewater has been reported since the first constructed wetland went into operation in Yorkshire UK in 1903, it was monitored with good performance until the end of service at 1992 (Hiley, 1995, Hiley, 1990).

3.1.1 Current research of full-scale constructed wetlands

Full-scale constructed wetlands have been employed to treat wastewater from different resources, where domestic wastewater (Masharqa et al., 2023, Belmont et al., 2004), industrial wastewater (Vymazal, 2014), mixture of domestic and industrial wastewater (A et al., 2023, Wu et al., 2017), landfill leachate (Arliyani et al., 2021), agricultural wastewater (Vymazal, 2009), river water (Pu et al., 2023) and so on. Long terms performance of full-scale constructed wetlands from 12 months to almost 90 years are reported by Benvenuti et al. (2018), Gunes et al. (2012), A et al. (2023), Pérez et al. (2024), Wu et al. (2017), Pu et al. (2023) and Hiley (1995). All those studies reported steady performance during the monitoring period, whilst average pollutant removal rates were reported mostly, the change of performance overtime was less common to see. Meanwhile, studies of microbial communities in full-scale constructed wetlands are only seen in a few recent years, in which one time point microbial community analysis is more common than temporal studies (Vymazal et al., 2021).

3.1.2 Current research of small-scale constructed wetlands

The size of constructed wetlands from less than 0.2m^2 surface area lab-scale studies to larger than 10,000m^2 surface area full-scale are being reported by multiple review literatures (Vymazal, 2014, Rodriguez-Dominguez et al., 2020,

Marín-Muñiz et al., 2023, Chen et al., 2024). It is worth mentioning that there is no clear definition of small-scale constructed wetland, surface area of less than 20m^2 are classified as small-scale by Rodriguez-Dominguez et al. (2020) and Marín-Muñiz et al. (2023), whereas surface area of less than 80,000m^2 are defined as small-scale by Chen et al. (2024). It is generally considered that pilot-scale, mesocosm-scale, and lab-scale constructed wetlands are small-scale as opposite to full-scale. Vymazal et al. (2021) summarised that more than 90% of recent constructed wetland studies are small-scale constructed wetlands.

Within those smaller scale constructed wetland studies, pilot and mesocosm scale are more likely to have long-term studies with real wastewater. Hijosa-Valsero et al. (2012) conducted a 39-month study of mesocosm-scale constructed wetlands treating primary treated domestic wastewater from a full-scale WWTP. Primary treated wastewater from full-scale WWTP was also used in a two-year mesocosm-scale study conducted by Hench et al. (2003). Belmont et al. (2004) demonstrated that a pilot-scale constructed wetland system was capable of treating high strength wastewater equivalent to five households in around two years. Although Hijosa-Valsero et al. (2012) and Hench et al. (2003) reported the change of performance by year, reports of average performance during the monitoring time are more common. Also, these studies included changes in microbial communities over the monitoring time, reporting stable microbial communities.

In contrast, lab-scale studies are more likely to report microbial communities within the system, but they normally operate for less than 100 days. For example, a 48 hours batch study was reported by Huang et al. (2019), a 30 days continuous study was conducted by Si et al. (2018), an electrochemically constructed wetland coupled system with 56 days (Wang et al., 2023a), 120 days and 220 days (Fan et al., 2016) lab-scale constructed wetland studies were also found. Although all those studies included the analysis of microbial communities, it is unknown how these reflect real systems since synthetic wastewater were used.

Our constructed wetland systems, treating real domestic wastewater from single households, are barely reported in the literature, with only three studies with constructed wetland systems similar to ours found. 21 constructed wetland

systems were monitored for seven years by in America Steer et al. (2002), whereas the change of performance overtime was missing since it was only reported as average of each system. 20 constructed wetland systems were built and monitored for 1.5 years in Israel by Alfiya et al. (2013), the performance of systems was reported with average by region. The only report with yearly performance comparison was conducted by Obarska-Pempkowiak et al. (2015) in Poland, where nine systems with three configurations were monitored for four years and COD and nitrogen removal were improved after three years' operation. However, the activities of microbial community within those systems were unknown as they were not reported.

3.1.3 Research gap

Current long-term full-scale constructed wetland studies normally lack microbial community analysis, meanwhile studies include microbial communities are commonly only have samples from one time point. Lab-scale constructed studies are more likely to report microbial community within the system, but they are normally run over short-time periods with synthesis wastewater. While there have been some pilot and mesocosm-scale constructed wetlands of a similar size to our systems and run over longer time periods with real wastewater, but their performance is commonly reported as average during the monitoring period and without analysis the microbial communities. Research about the change of performance and microbial community within constructed wetland overtime is barely seen, and urgently needed.

Three decentralised constructed wetland systems were built and operated successfully in Mexico City, Mexico in 2020. The first three months of operation has shown promising results of pollutants removal, whereas the long-term performance of the wetlands and the microbial community behaviours are largely unknown. Therefore, we continued to monitor the operation of the two household systems for 10 months. Monitoring of the third system, a school, was discontinued due to closure of the school during the COVID-19 pandemic.

3.1.4 Hypothesis and aims

The results from Chapter 2 showed that all three constructed wetland systems were able to remove pollutants from the beginning of the operation, we hypothesise that two household constructed wetland systems will continually remove pollutants, and achieve stable removal rates and microbial community composition at the same time.

The aim of this study is to monitor the performance and characterise the microbial communities of two identical small scale constructed wetland systems treating household wastewater for 10 months from July 2020 to April 2021.

3.1.5 Objectives

- To monitor and compare the performance of two identical small scale constructed wetland systems treating household wastewater for 10 months.

- To characterise and compare any changes in the microbial community within the systems. Including changes from influent to effluent but also within the wetland themselves.

- Introduce replication to the monitoring to make it more robust.

3.2 Materials and methods

3.2.1 Sampling campaign and physical chemical data and analysis

Water sampling, and water quality analysis were conducted by our collaborators Mario Alberto Salinas-Toledano and Tania Lizet Gómez-Borraz from the Universidad Autónoma Metropolitana-Cuajimalpa (Metropolitan Autonomous University-Cuajimalpa), Mexico City.

Samples were taken from the two household systems (WS1 and WS2) in Chapter 2, from 2nd July 2020 to 19th April 2021. In 2020, samples were taken monthly from 2nd July to 24th November, and in 2021 samples were taken weekly from

24th February to 19th April. Water samples of influent and effluent were taken in the same way as in 2.2.3, whilst from 24th October 2020 additional water and sediment samples from within both wetlands of each system were collected. Wetland samples water samples (500ml) were collected near the outlet from water near the surface while sediment samples (500ml slurry) were collected with a manual water pump.

One sample at each location was taken for the first 3 time points (2nd July to 27th August, 2 replicate samples were taken from September 2020 until the end of this sampling campaign. All samples were stored at 4℃ and analysed within 24h after collection.

Water samples from influent (AR) and effluent (HT) in 2020 were analysed for COD, NO_2^-, NO_3^-, P, coliforms, and TSS following Standard Methods for the examination of Water and Wastewater (Baird et al., 2017). Water samples in 2021 were analysed with additional NH_4^+-N, TOC, TC, TN following Standard Methods for the examination of Water and Wastewater. Sensors had been installed in both systems in 2021, where temperature, pH, dissolved oxygen (DO) and (oxidation reduction potential) ORP were measured through those sensors.

Removal rate was calculated through *Equation 1* (see 2.3.1), where the overall removal rate was calculated with the influent and effluent concentration of the whole system. The CW1 removal rate was calculated with the influent concentration and the concentration in CW1, and the CW2 removal rate was calculated with the influent concentration and concentration in CW2 then substrate the removal rate of CW1.

DNA extraction of both water and sediment samples were performed as in 2.2.3. DNA samples were shipped to Glasgow with samples from 2.2.3, the following *16S rRNA* PCR amplification, library pooling and sequencing were the same as 2.2.4. Detailed sampling date, location, physicochemical analysis, and DNA extraction were shown in Figure 3-2.

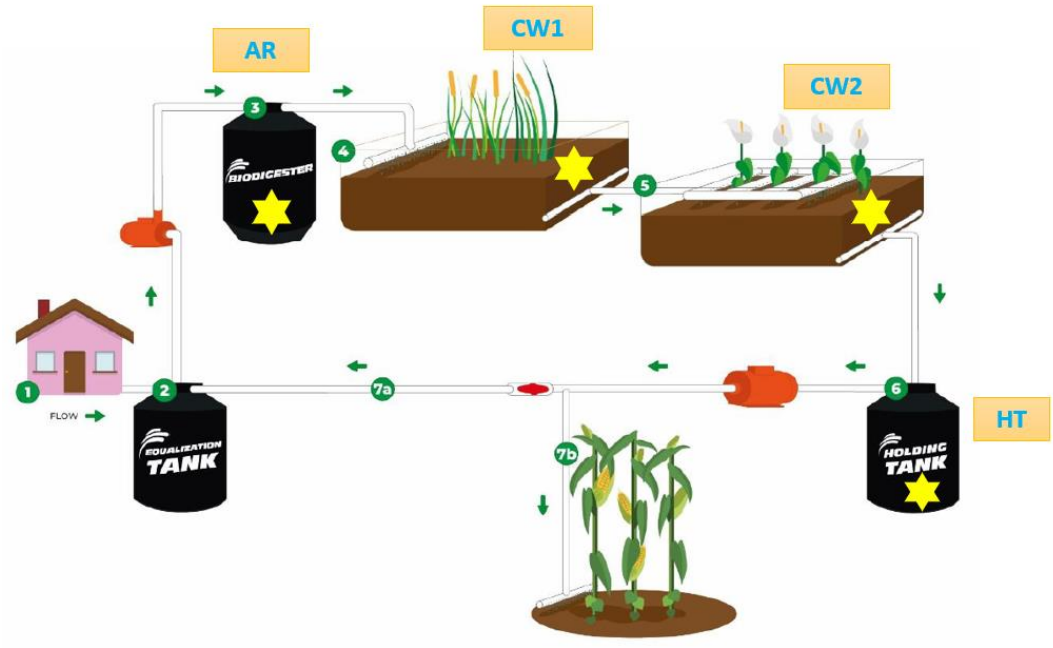

Figure 3-1 Wastewater treatment system diagram. AR means the anaerobic reactor, where it is considered as the influent in this study. CW1 means the horizontal constructed wetland, CW2 means the vertical constructed wetland. HT means the holding tank, where it is considered as effluent of the system. Yellow asterisks indicate where the samples were taken.

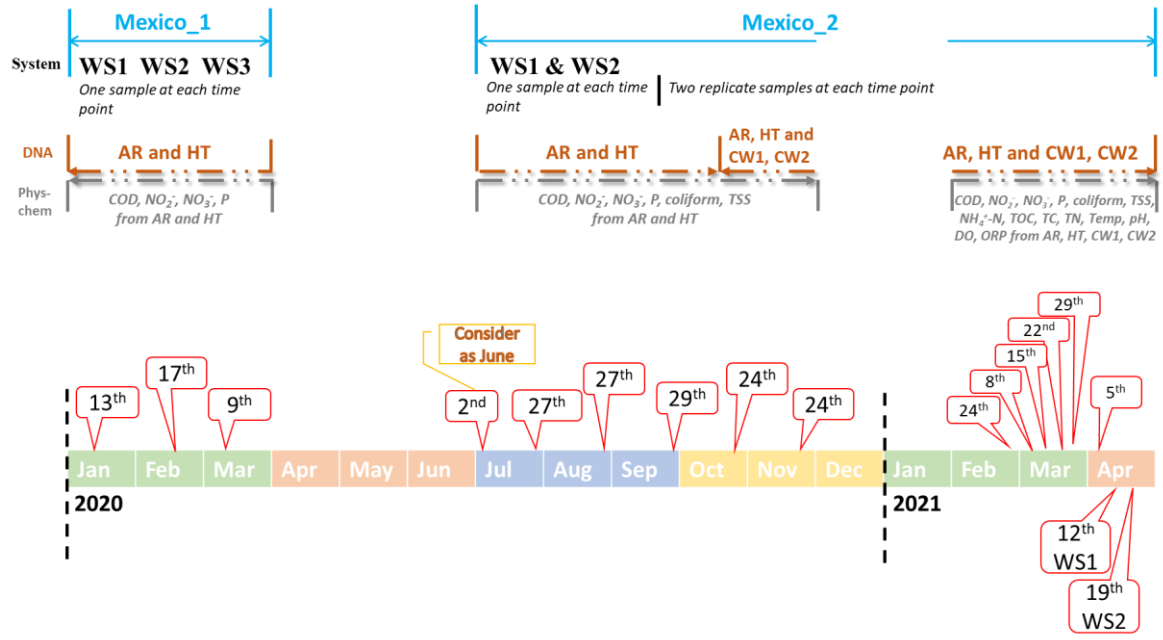

Figure 3-2 Detailed time scale and parameters have been measured and DNA sampled during the whole sampling campaign from 2020 to 2021. AR means the anaerobic reactor, where it is considered as the influent in this study. CW1 means the horizontal constructed wetland, CW2 means the vertical constructed wetland. HT means the holding tank, where it is considered as effluent of the system.

3.2.2 Molecular work

Samples were prepared and sequenced as detailed in 2.2.4 and 2.2.5.

3.2.3 Statistics

In this study, we have used 211 samples from WS1 and WS2 corresponding to the long-term operation, with the final abundance table of: n=211 samples x P=17,224 ASVs.

3.3 Results

3.3.1 Wastewater treatment performance

The monitoring results of the main wastewater treatment performance indicators are shown in Figure 3-3 to **Error! Reference source not found.**. COD, P, NO_2^-, NO_3^- in the influent and effluent were monitored throughout the whole sampling campaign, results of those parameters included the data from the first three months, which was previously shown in 2.3.1. NH_4^+, total nitrogen and all the other parameters within both constructed wetlands were measured in the short interval sampling in 2021. The results of coliform, total suspended solids (TSS), total organic carbon (TOC), total carbon (TC), TN, temperature, pH, DO and ORP were shown in Table 7-2 to Table 7-10 of the appendix.

3.3.1.1 COD

Both systems worked well in removing COD from wastewater, with effluent COD concentration much lower than influent at all time points (see Figure 3-3). Influent COD concentrations in WS2 were constantly higher than WS1, with the highest concentration in WS2 almost double the value of the highest in WS1. The COD concentrations within both wetlands of each system were measured in 2021. In WS1 there was a decline of COD concentration from influent to CW1 and a further reduction in CW2, however, the effluent COD concentration was always higher than CW2. Similar trends were observed in WS2, COD concentrations decreased in CW1 and further decreased in CW2 (except 15th March), in the last four time points there was an increase of COD concentration in effluent, but the same trend was not observed in the first three time point in 2021.

COD removal rates are shown in Figure 3-4, where a) showed the overall removal rates of both systems and b) the removal rate within each constructed wetland in both systems. Overall removal rate measures the whole system performance

of removing organic carbon from wastewater by comparing influent and effluent concentrations, where the removal rate in WS1 ranged from 41.22-95.84% in WS1 and 72.66–96.63% in WS2. WS2 had a higher overall removal rate than WS1 at almost all time points. For both systems, the overall removal rate fluctuated in the first nine months, then they both stabilised from October 2020. The change of the removal rates from October 2020 in both systems shows the same trend with both lowest in February 2021 and increased over time then both dropped in the last time point.

The removal rates of single constructed wetland shown that CW1 (horizontal constructed wetland) had a higher COD removal rate than CW2 at almost all time points. In WS1, the COD removal rate in CW1 and CW2 ranged from 41.53-73.28%, 6.47-28.41%. respectively. In WS2, the removal rates were 28.87-77.21% in CW1 and 4.94-49.98% in CW2, where the CW2 showed a much higher removal rate when CW failed to remove any COD in the wastewater. In WS2, the vertical constructed wetland (CW2) had higher removal rates at all time points in March 2021, which were close to removal rates in CW1. However, this was not observed in February and April in WS2 nor WS1.

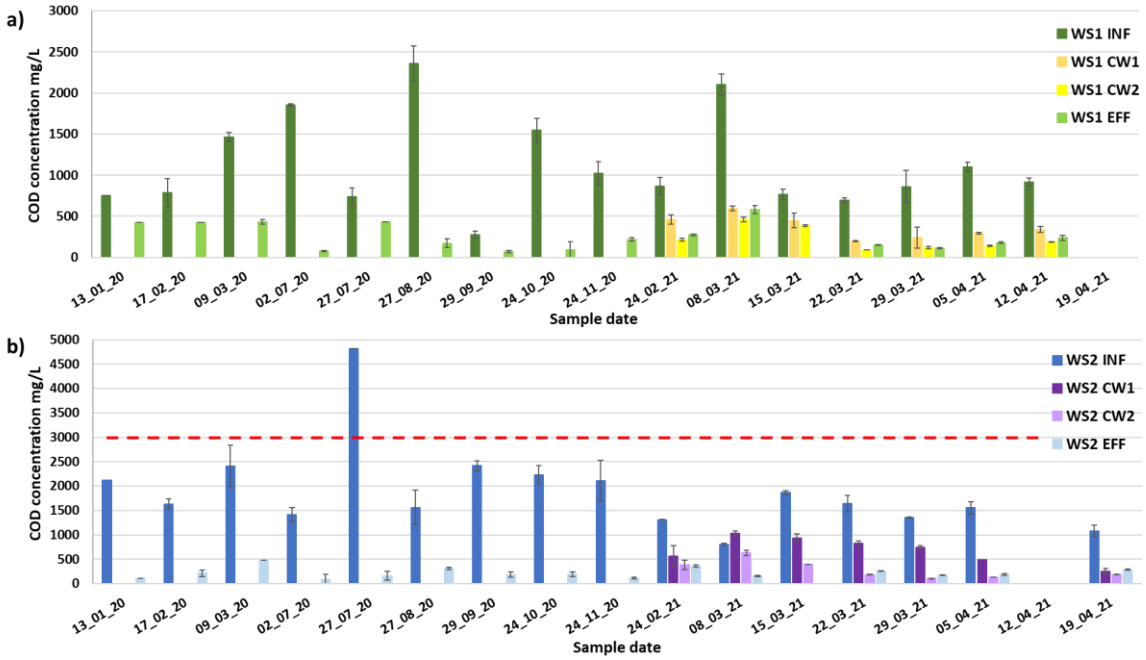

Figure 3-3 COD concentrations in influent, horizontal constructed wetland (CW1), vertical constructed wetland (CW2) and effluent overtime from both systems. a) WS1. b) WS2. Red dash line in b) indicates the COD concentration of 3000mg/l. (n=160, 12 samples (6 each system) from the first three months were also included. Error bars stand for standard deviation, which was calculated from two measurements of one sample from January to August 2020, one measurement from each sample was used for standard from September 2020 as two samples were taken at each time point.)

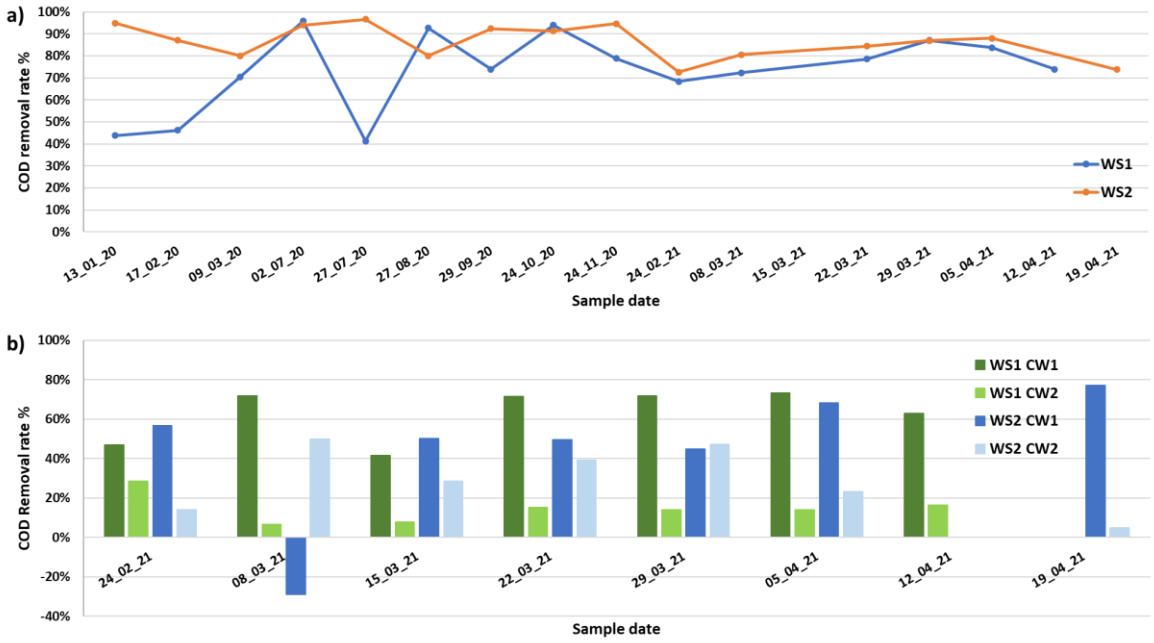

Figure 3-4 COD removal rates in both systems. a) Overall COD removal rates of both systems. b) COD removal rates of each constructed wetland from both systems. (Removal rate was calculated by *Equation 1* with average COD concentration before and after treatment unit.)

Figure 3-4 shows that COD removal rates of both systems were fluctuating at the beginning of the monitoring period but became more stable from October 2020. NO_2^- and NO_3^- removal rates showed similar trends in both systems (see Figure 3-9), where less fluctuation was observed from September 2020. Meanwhile, all those stabilised removal rates were higher than the fluctuating period, which suggests a better performance in a more matured system. Those changes of removal rates also suggest that our small scale constructed wetland system require nine months to acclimate in order to achieve a stable COD, NO_2^- and NO_3^- removal rate.

3.3.1.2 Phosphorus

Phosphorus concentrations in the effluent were lower than their influent concentrations in both systems at most time points (see Figure 3-5), where influent concentrations varied from 1.2 to 47.2mg/L in WS1 and 3.05 to 43.6mg/L in WS2, and effluent concentrations varied from 1.1 to 23.23mg/L in WS1 and 0 to 41.7mg/L in WS2. However, phosphorus concentrations increased overtime in both influent and effluent in both systems, which suggests that phosphorus was accumulated within the system. In WS1, phosphorus concentration in CW1 was lower than influent, meanwhile there was a dip of phosphorus concentration in CW2 and the effluent concentration was almost the

same as CW2. This trend was not observed in WS2, where the phosphorus concentrations were very similar at all sample locations.

The overall phosphorus removal rates were shown in a) in Figure 3-6 and the removal rate within both systems were shown in b) Figure 3-6. There was an increase of overall phosphorus removal rate in WS1, whereas the overall phosphorus removal rate decreased overtime in WS2, and the removal rates in the last four time points were close to 0. Phosphorus was mostly removed by CW2 in WS1, while in WS2 it was removed more in CW1 than CW2.

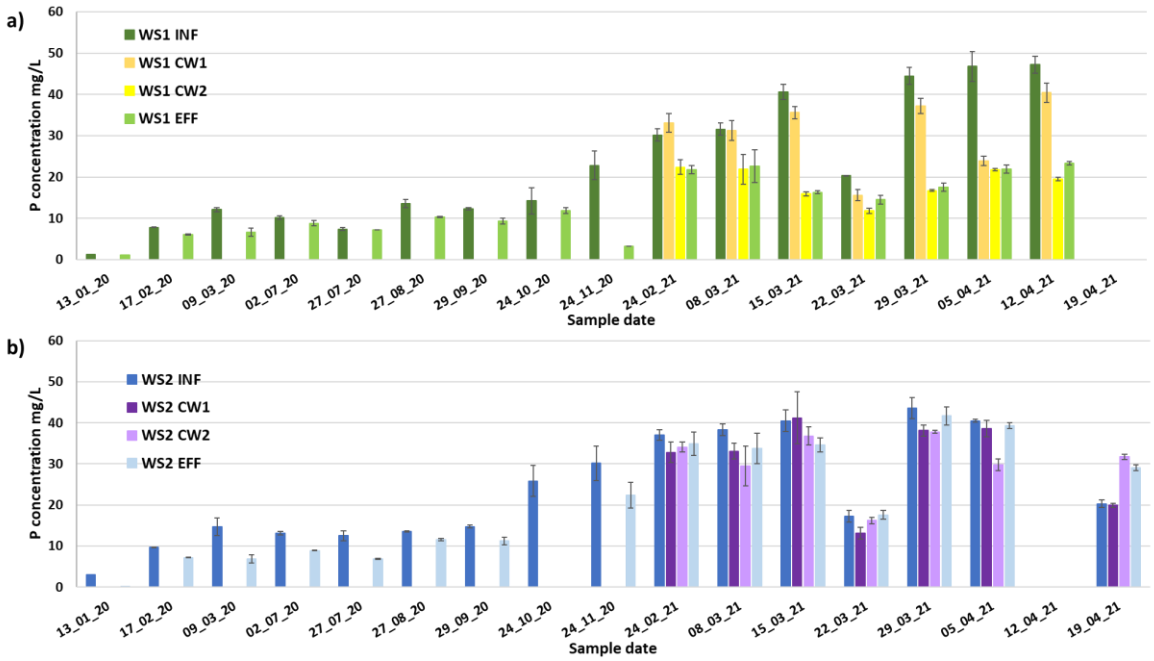

Figure 3-5 Phosphorus concentrations in both systems. a) WS1. b) WS2. (n=80 for both WS1 and WS2, where they both include 6 samples from the first three months.) Error bars stand for standard deviation, which was calculated from two measurements of one sample from January to August 2020, one measurement from each sample was used for standard deviation from September 2020 as two samples were taken at each time point.

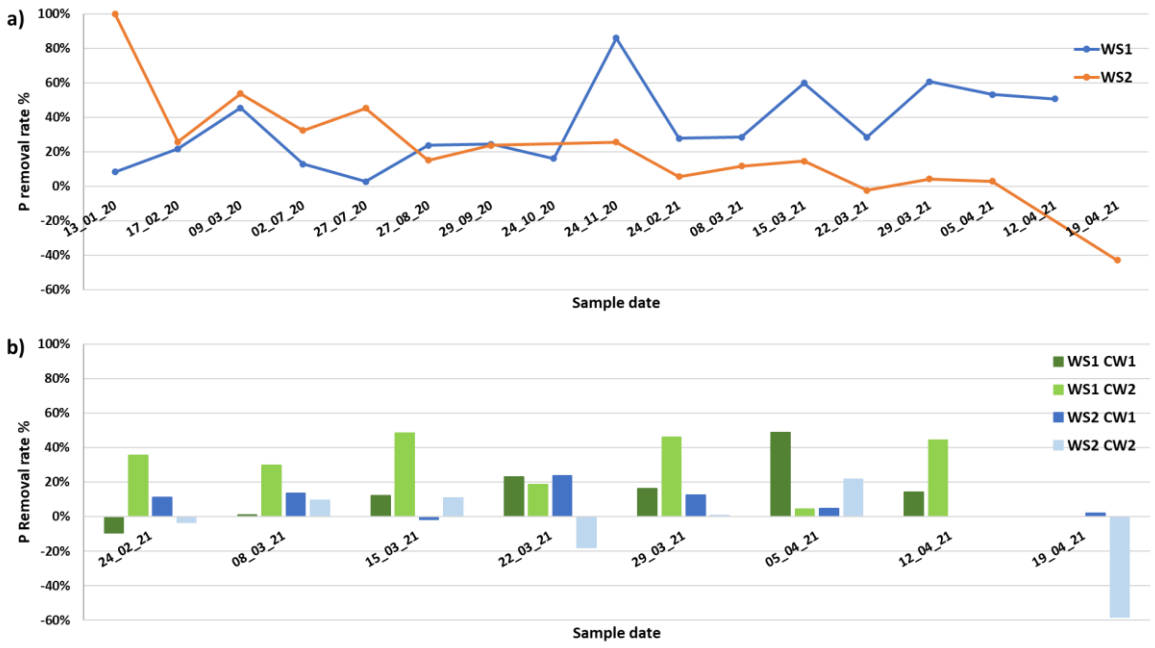

Figure 3-6 Phosphorus removal rates in both systems. a) Overall phosphorus removal rates of both systems, n=32. b) Phosphorus removal rates of each constructed wetland from both systems, n=28. (Removal rate was calculated by *Equation 1* with average phosphate concentration before and after treatment.)

3.3.1.3 Nitrogen

Nitrogen concentrations were monitored through NO_2^-, NO_3^- and NH_4^+, where NO_2^- and NO_3^- concentrations were monitored from January 2020 to April 2021, NH_4^+ was only monitored from February 2021 to April 2021 on a weekly basis.

NO_2^- and NO_3^- concentrations were showed in Figure 3-7 and Figure 3-8, and their remove rates were shown in Figure 3-9. In both NO_2^- and NO_3^-, the effluent concentrations were much lower than their influent concentrations for both systems, indicating both systems worked in removing NO_2^- and NO_3^- from wastewater. Meanwhile, in both systems, NO_3^- concentrations were higher than NO_2^-, and both NO_2^- and NO_3^- concentrations in WS2 were higher than WS1. In WS1 NO_2^- and NO_3^- concentrations ranged from 13.5 to 96.4mg/L in influent and 0.9 to 31.0mg/L in effluent and 49.8 to 157.9 mg/L in influent and 5.4 to 67.9mg/L in effluent respectively. In WS2 NO_2^- and NO_3^- concentrations ranged from 26.8 to 113.8mg/L in influent and 0.04 to 27.2mg/L in effluent and 124.1 to 355.8mg/L in influent and 9.2 to 76.2mg/L in effluent respectively.

Both systems had high overall removal rates for both NO_2^- and NO_3^-, where 33.94 to 100% of NO_2^- and 36.48 to 92.59% of NO_3^- were removed from WS1, and 60.98 to 100% of NO_2^- and 65.86 to 100% of NO_3^- were removed from WS2. WS2 had a

higher removal rate at almost all time points for both NO_2^- and NO_3^-. NO_2^- and NO_3^- removal rates fluctuated in the first 6 time points in both systems, and then became relatively stable from September 2020. Within the system, horizontal constructed wetland (CW1) removed more NO_2^- and NO_3^- than the vertical constructed wetland (CW2) at almost all time point.

The results of the short time intensive monitoring of NH_4^+ concentrations and removal rates were shown in **Error! Reference source not found.**. NH_4^+ concentrations ranged from 107 to 176mg/L in WS1 and 99.5 to 162mg/L in WS2. The highest overall NH_4^+ removal rate was 23.83% in WS1 and 21.91% in WS2, whilst the highest removal rate in single constructed wetland in both systems was similar to the overall removal rate at 18.38% in WS1 and 24.92% in WS2. Both NH_4^+ concentrations and removal rates indicated that those systems were not removing NH_4^+.

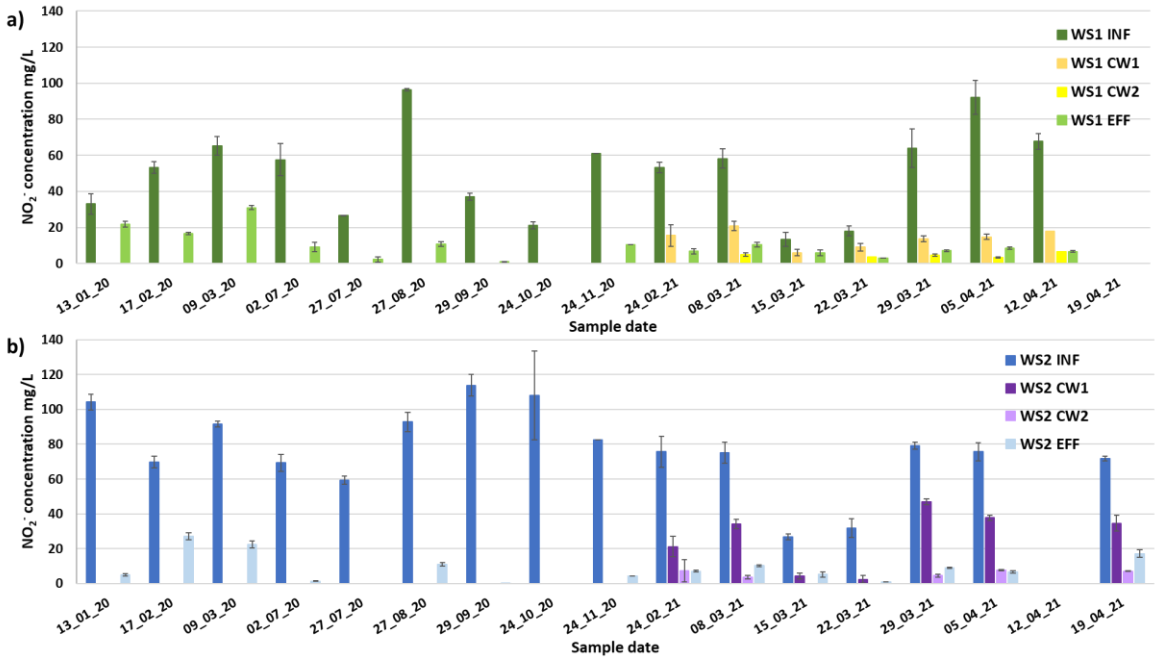

Figure 3-7 NO_2^- concentrations in influent, horizontal constructed wetland (CW1), vertical constructed wetland (CW2) and effluent overtime from both systems. a) WS1 b) WS2. (n=80 for both WS1 and WS2, where they both include 6 samples from the first three months.) Error bars stand for standard deviation, which was calculated from two measurements of one sample from January to August 2020, one measurement from each sample was used for standard deviation from September 2020 as two samples were taken at each time point.

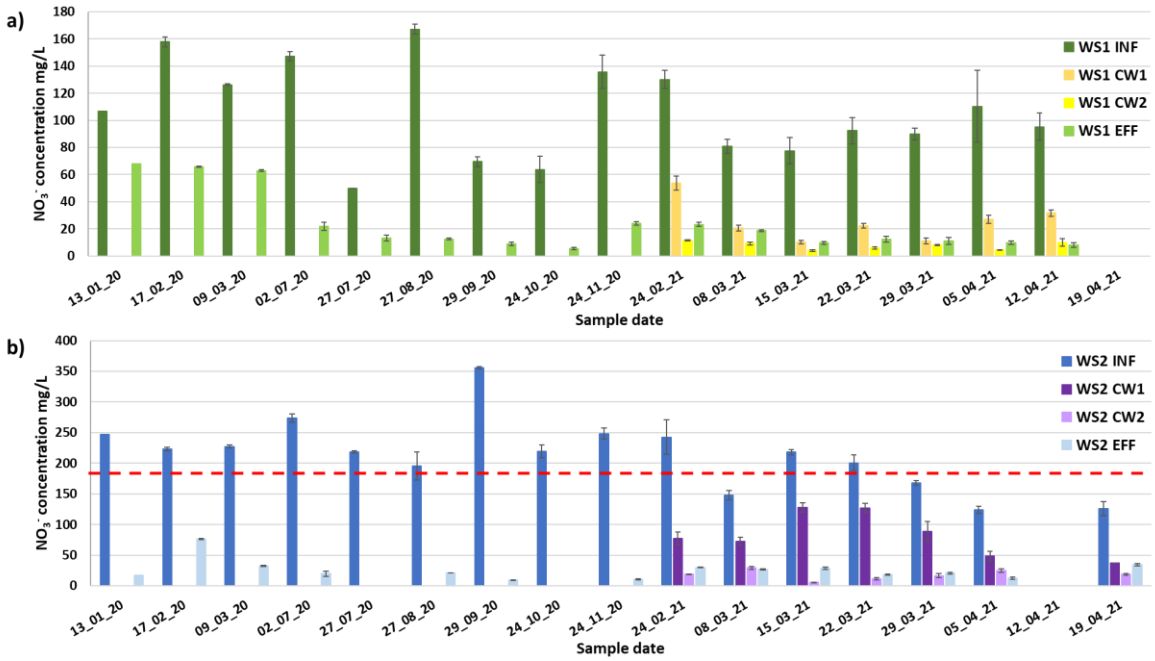

Figure 3-8 NO_3^- concentrations in influent, horizontal constructed wetland (CW1), vertical constructed wetland (CW2) and effluent overtime from both system overtime. a) WS1 b) WS2. Red dash line in b) indicates the NO_3^- concentration of 180mg/L. (n=80 for both WS1 and WS2, where they both include 6 samples from the first three months.) Error bars stand for standard deviation, which was calculated from two measurements of one sample from January to August 2020, one measurement from each sample was used for standard deviation from September 2020 as two samples were taken at each time point.

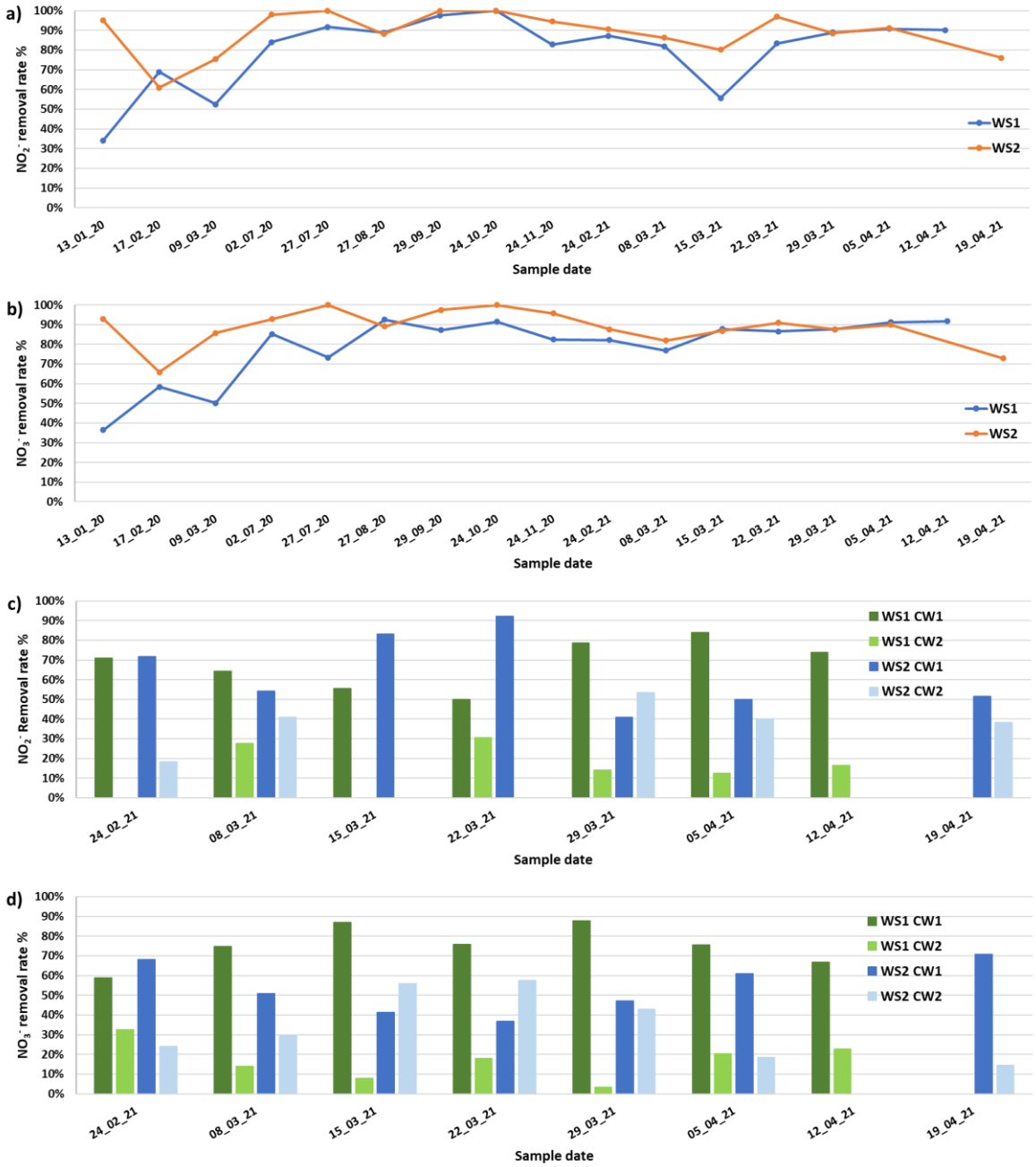

Figure 3-9 NO_2^- and NO_3^- removal rates of both systems. a) overall NO_2^- removal rate from both systems, n=32. b) overall NO_3^- removal rate from both systems, n=32. c) NO_2^- removal rate in each constructed wetland of both systems, n=24. d) NO_3^- removal rate in each constructed wetland of both systems, n=28. (Removal rate was calculated by *Equation 1* with average NO_2^- or NO_3^- concentration before and after treatment unit.)

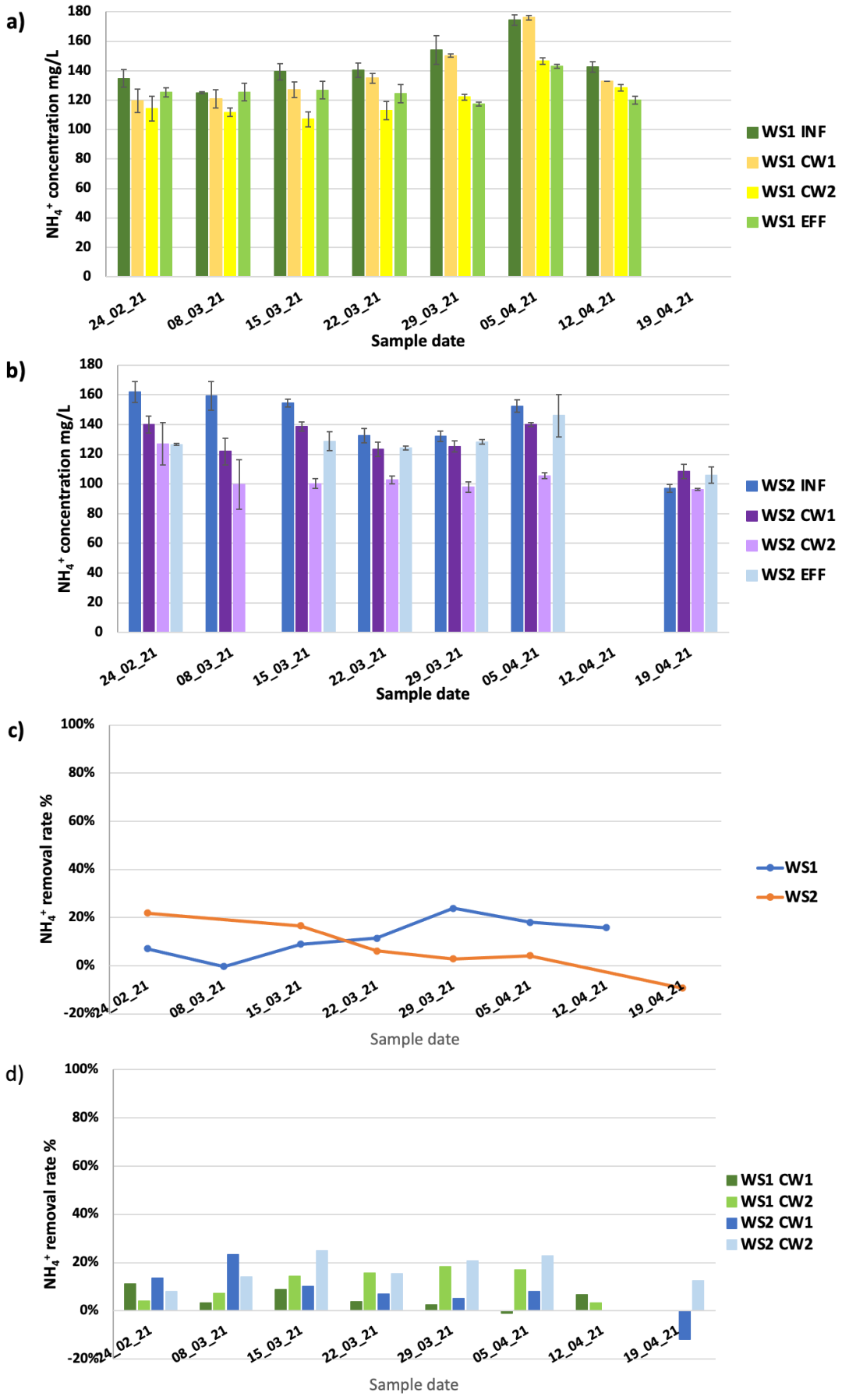

Figure 3-10 NH_4^+ concentrations and removal rates. a) NH_4^+ concentration in WS1, n=56. b) NH_4^+ concentration in WS2, n=56. c) Overall removal rates of NH_4^+ in both systems, n=13. d) NH_4^+ removal rates in CW1 and CW2 of both systems, n=28. (Removal rate in c) and d) was calculated by *Equation 1* with average NH_4^+ concentration before and after treatment unit.)

3.3.2 Microbial community analysis – alpha diversity

The pollutant removal results have shown the performance maturation of the systems over a year's operation, it is worth to examine the dynamics of microbial community driving nutrient removal within and across the system on top of the performance data. Therefore, an in depth *16S rRNA* analysis was conducted to reveal the changes in the microbial community in the monitoring time.

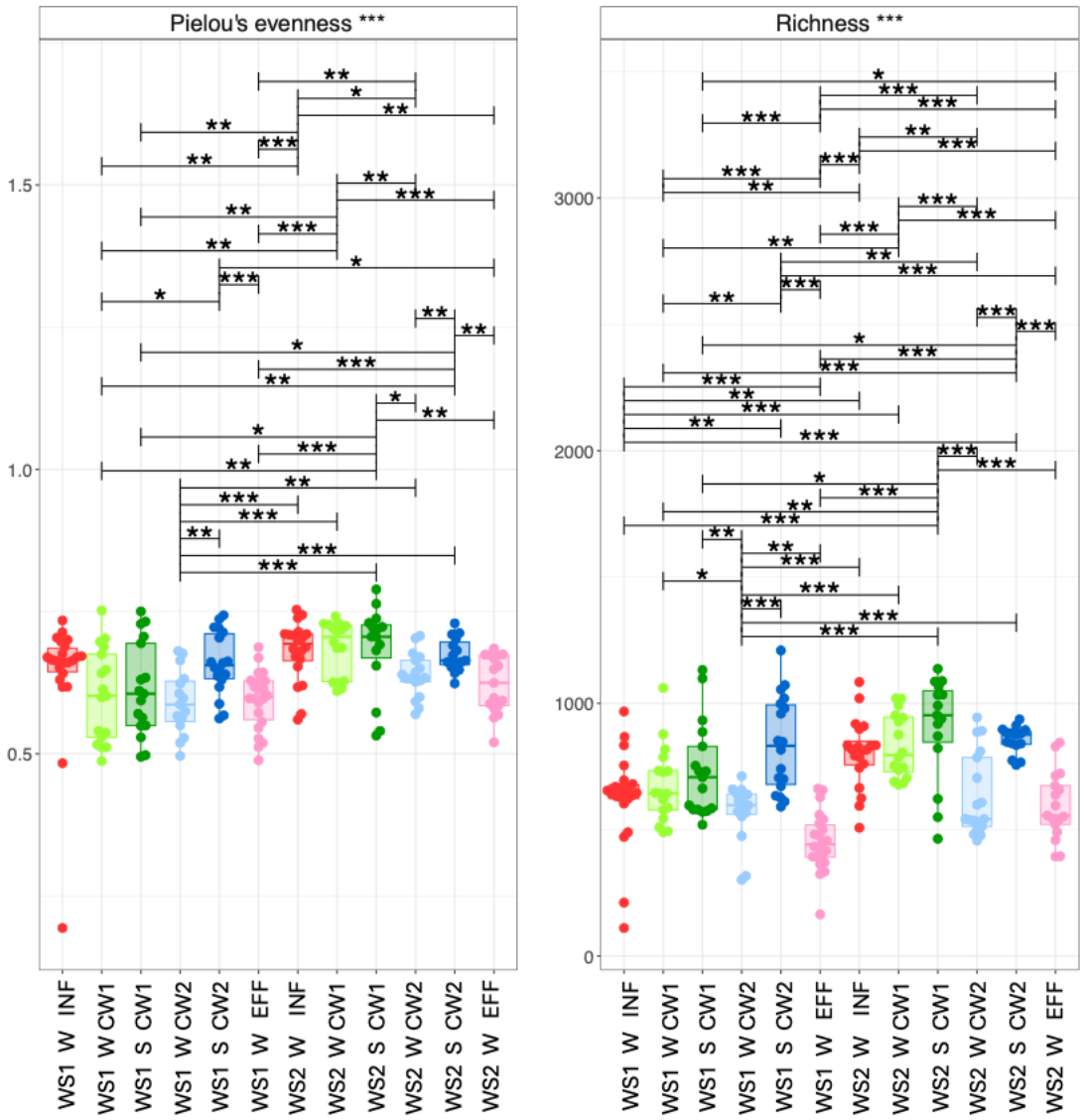

Figure 3-11 Pielou's evenness and richness value of the two WWTPs with samples grouped by system, location and sample type and analysed by ANOVA. Asterixis represent P value, where "*" p≤0.001, "**" 0.001 < p ≤ 0.01, "*" 0.01< p ≤ 0.05. WS1 and WS2 indicate the systems; W, water samples, S, sediment samples; INF, CW1, CW2 and EFF indicate sample location of anaerobic reactor (influent), horizontal constructed wetland (CW1), vertical constructed wetland (CW2) and holding tank (effluent), respectively (detailed in Figure 3-1). (n=211, where 111 from WS1 and 100 from WS2).**

Alpha diversity compares the diversity of microbial community within sample groups, where species richness focuses on the presence of different species and Pielous's evenness focuses on the distribution of the species. In this study, samples of each system from June 2020 to April 2021 were analysed, they were grouped and compared by locations (influent, CW1, CW2 and effluent) and sample types (water or sediment) (see Figure 3-11).

Species richness ranged from 110.87 ASVs to 1209.28 ASVs in WS1 and 393.13 ASVs to 1136.45 ASVs in WS2 (see Table 7-11 in appendix). Within the system, richness ranged from 110.87 ASVs to 1060.87 ASVs in water samples (influent, CW1, CW2 and effluent) and 519.47 ASVs to 1209.28 ASVs in sediment samples in WS1, in WS2 species richness ranged from 393.13 ASVs to 1084.26 ASVs in water samples (influent, CW1, CW2 and effluent) and 463.89 ASVs to 1136.45 ASVs in sediment samples. Despite this, WS2 had a higher species richness in both influents and effluents compared with WS1 (ANOVA: P=0.0012, influent; P=0.001, effluent). However, both systems showed the same trend of effluent having a lower species richness than the influent (ANOVA: $P=5.8X10^{-4}$, WS1; $P=4X10^{-5}$, WS2). Within the system, species richness decreased across the wetland - in the influent was the same as in the water in CW1, but reduced in the water in CW2 (ANOVA: P=0.034, WS1; $P=6.2X10^{-4}$, WS2), and further decreased in the effluent (ANOVA: P=0.0059, WS1). For the sediment samples, there was no significant difference between CW1 and CW2 in terms of species richness. When the species richness between water and sediment samples was compared within each wetland of both systems, there was no difference between CW1 of both systems, but they were different in CW2 (vertical wetland) of both system (ANOVA: $P=3.8X10^{-5}$, WS1; $P=3X10^{-5}$, WS2).

The evenness of all the sample groups showed a similar trend but there were less statistically significant differences than species richness. In WS1, the difference between water and sediment (ANOVA: P=0.0015) in CW2 was the only significant difference observed in terms of evenness. In WS2, influent differed from effluent (ANOVA: P=0.0018), meanwhile water in CW1 was different from water in CW2 (ANOVA: P=0.0036) and in CW2 water differed from sediment (ANOVA: P=0.0068) in terms of evenness. Furthermore, WS2 had a higher

evenness in water samples in CW2 than WS1 (ANOVA: P=0.0097), this difference was not seen in species richness.

Since those two identical systems worked well in treating household wastewater in the long term and both achieved stabilised removal at the same time, it was worth investigating the microbial community composition, that is likely driving/contributing to the carbon and nitrogen removal. In the long-term study, species richness showed the same trend of lower in the effluent and higher in influent. Also, when comparing the maximum species richness value, we observed that both the influent and effluent values were much higher in this long-term study compared to the data of first three months, which suggests the microbial community is getting more diverse with the maturating of the system. Our influent of the system was taken from the biodigester, increased species richness in this long-term study also indicates the biodigester was maturating over time. However, the species richness analysis is based on relative abundance, absolute cell count by *16S rRNA* qPCR would help to understand if there was an overall increase in cell numbers alongside the increase in species richness, this will be done in the future.

3.3.3 Microbial community composition changes overtime

Microbial community composition from both systems was compared by grouping samples of system (WS1 vs WS2), sample type (water (W) vs sediment (S)), sample time (month) and sample location (influent (INF) (sampled from AR), horizontal constructed wetland (CW1), vertical constructed wetland (CW2), effluent (EFF) (sampled from HT)). Figure 3-12 shown the Bray-Curtis and unweighted Unifrac of the bacterial communities within all samples collected from both systems, Table 7-12 was the PERMANOVA data of both systems. Meanwhile, significant differences were observed among all systems (P=0.001 for both Bray-Curtis and UniFrac), locations (P=0.001 for both Bray-Curtis and UniFrac), types (P=0.003, Bray-Curtis; P=0.001, UniFrac), and sample months (P=0.001 for both Bray-Curtis and UniFrac) in all groups. Furthermore, the microbial communities clustered by system, WS1 and WS2, in the overall phylogenetic abundance of unweighted UniFrac (Figure 3-12 b). While in the relative abundance of microbial community in Bray-Curtis dissimilarly clustered by location as opposed to system, with the influent and CW1 clustering

separately from CW2 and effluent. Since the microbial composition Figure 3-12 b) of two systems were different, the difference between sample location, sample month and sample type were analysed within each system.

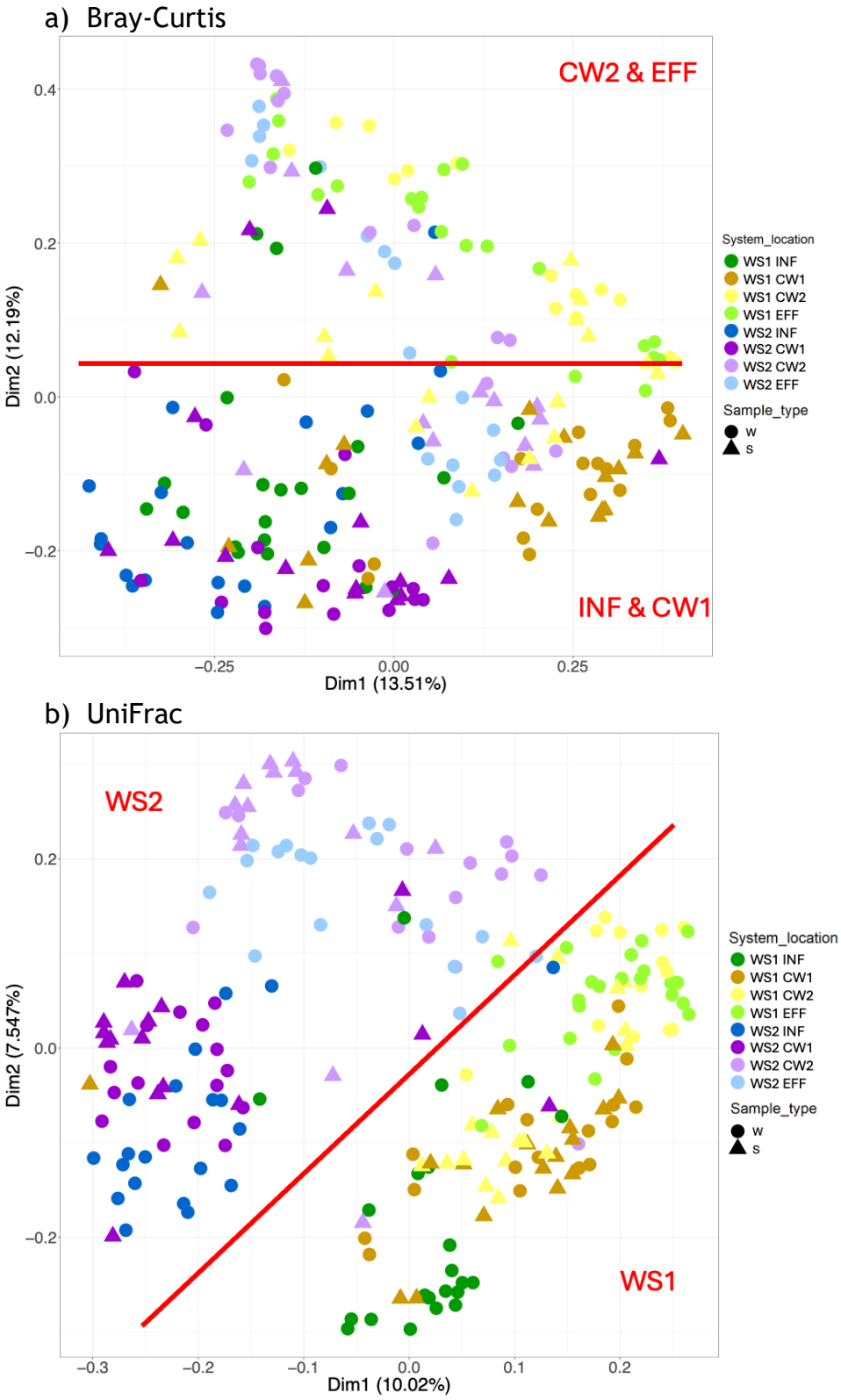

Figure 3-12 Beta diversity plotted in PCOA with a) Bray-Curtis dissimilarity and b) unweighted UniFrac distance with samples from both WS1 and WS2. Red line in both figures indicates the separation of samples. (n=211, where 111 from WS1 and 100 from WS2)

Samples from each system (include the first three months') were analysed with Bray-Curtis and unweighted Unifrac analysis of the beta diversity (Figure 3-13 and Figure 3-14). The microbial communities from each system clustered by location and month (P=0.001 for both Bray-Curtis and unweighted UniFrac). However, the differences between the microbial communities in the water (influent, CW1, CW2 and effluent) and sediments samples were different in each system. Microbial communities between water (influent, CW1, CW2 and effluent) and sediment in WS1 were different for both analysis (P=0.001 for both Bray-Curtis and UniFrac), while in WS2 the difference was only found in phylogenetic analysis of unweighted UniFrac (P= 0.088, Bray-Curtis; P=0.007, unweighted UniFrac). In both systems, microbial community from 2021 were more similar to each other compared to those from 2020 in both Bray-Curtis and unweighted UniFrac. Meanwhile for Bray-Curtis analysis of microbial communities in 2021, they formed four clusters by location (influent, CW1, CW2 and effluent) in WS1, while in WS2 microbial communities of influent overlapped with CW1 along with CW2 overlapped with effluent.

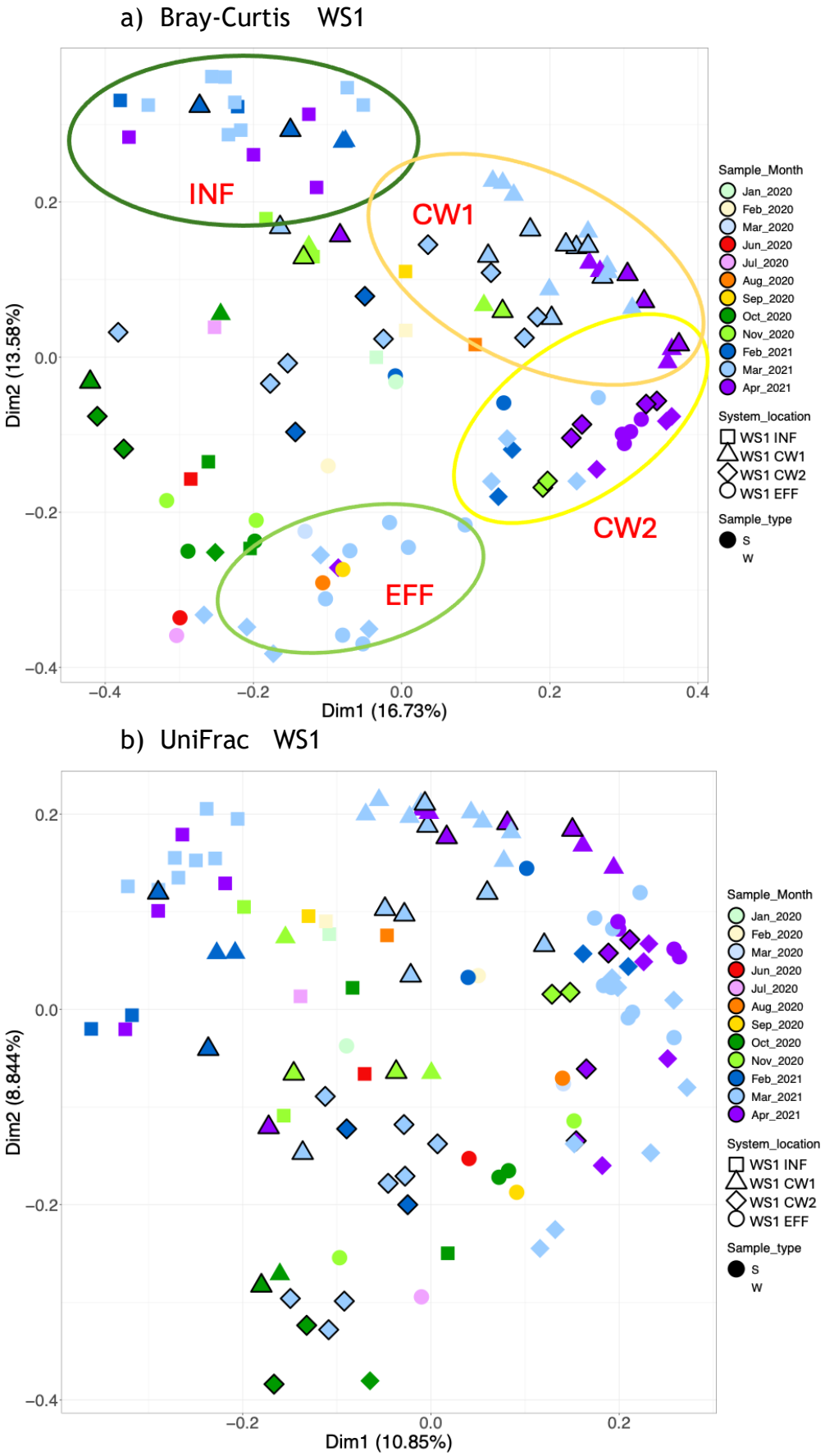

Figure 3-13 Bray-Curtis and unweighted UniFrac of all samples from WS1. a) Bray-Curtis dissimilarity. b) unweighted UniFrac distance. Ellipses in a) indicate the cluster of samples, where dark green indicates samples from influent, dark yellow indicates samples from CW1, light yellow indicates samples from CW2, light green indicates samples from effluent. (n=116, includes 5 samples from the first 3 months)

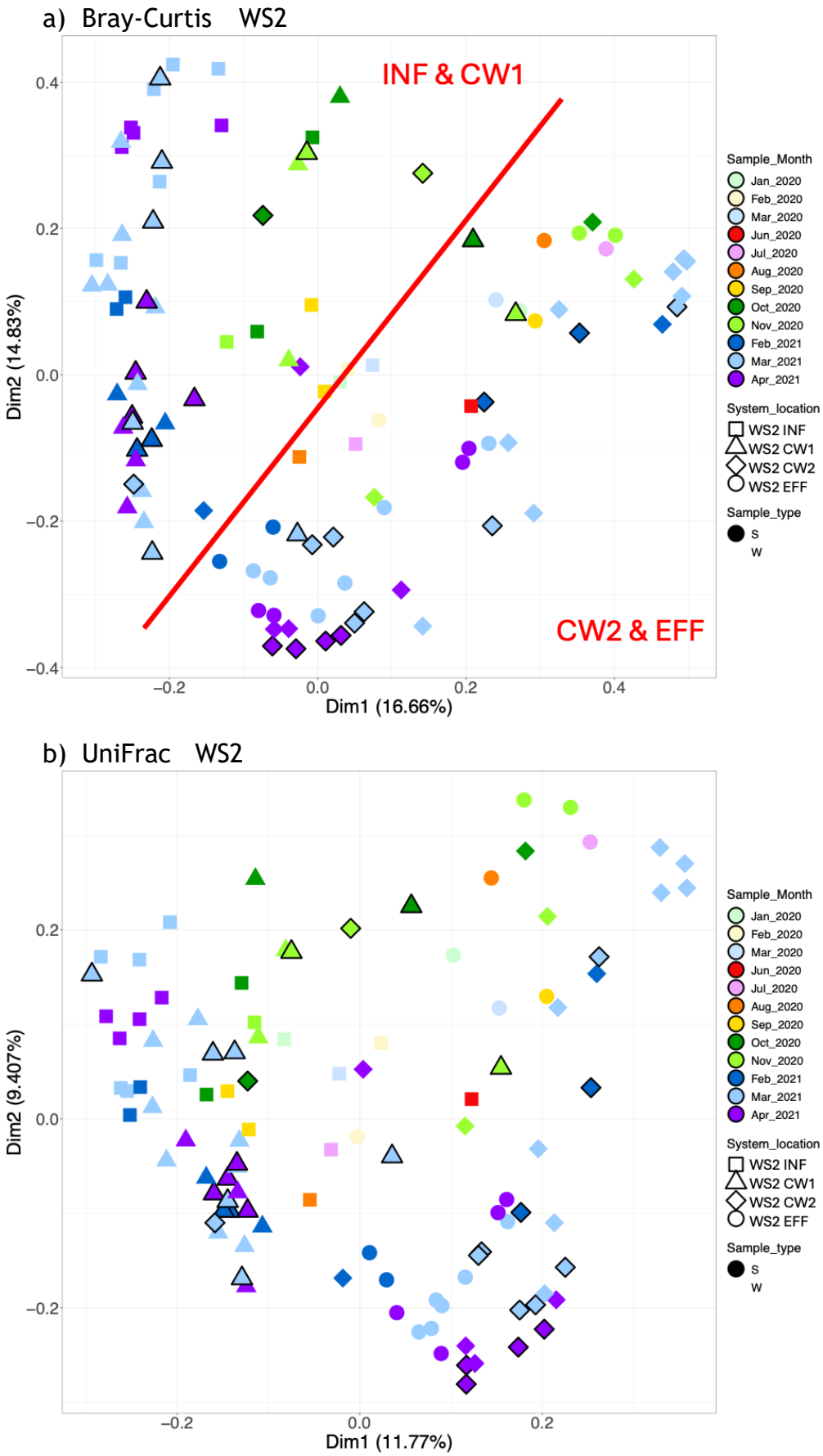

Figure 3-14 Bray-Curtis and unweighted UniFrac of all samples from WS2. a) Bray-Curtis dissimilarity. b) unweighted UniFrac distance. Red line in a) indicates the separation of samples. (n=106, includes 6 samples from the first 3 months)

Detailed comparison of the microbial communities from the wetland system were carried out by grouping samples in different ways. First overall changes in the microbial communities from influent to effluent across both wetlands was examined; 2) Changes in the microbial communities of the water as it passed through the wetland, from influent, through CW1 and CW2 to the final effluent; 3) a comparison of the microbial communities between the water and sediment within each constructed wetland (CW1 and CW2) was made (see Figure 3-15 to Figure 3-17 and Table 7-14).

The comparison of microbial community between influent and effluent showed statistically significant changes in the microbial communities from the influent compared to the effluent (P=0.001, WS1; P=0.001, WS2). Moreover, there was a temporal variation in the microbial communities of influent and effluent each month (P=0.001, WS1; P=0.001, WS2). In Figure 3-15, there was a clear separation between influent and effluent in both systems. The microbial communities of influent from 2021 in both systems were more similar to each other whereas the microbial communities from the samples collected in 2020 were more scattered.

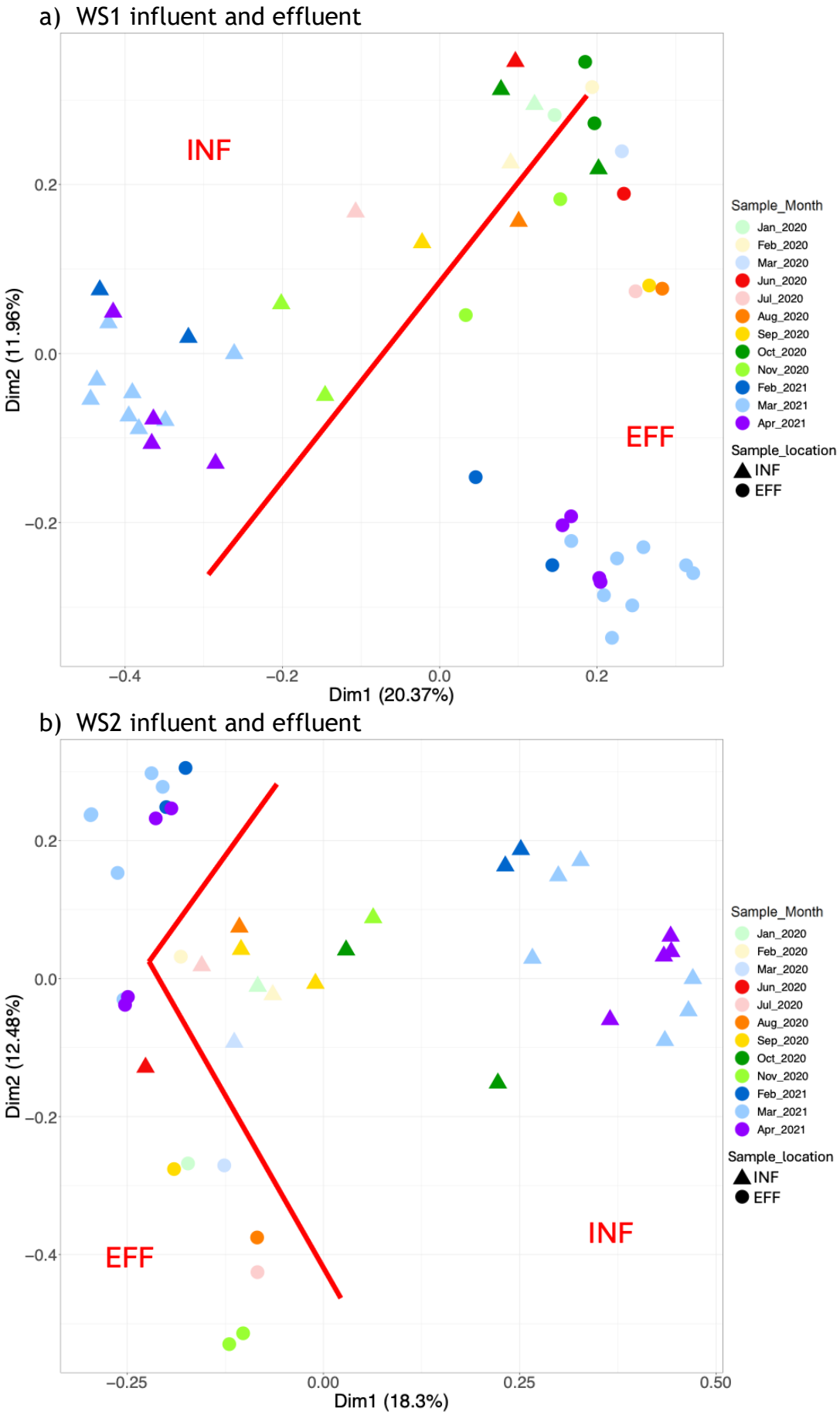

Figure 3-15 Bray-Curtis analysis of influent and effluent microbial communities within each system. a) WS1. b) WS2. Red line indicates the separation of microbial communities. (WS1, n=49, includes 5 samples from the first 3 months; WS2, n=43, includes 6 samples from the first 3 months)

Next, we examined changes in the microbial communities as the wastewater moved through each wetland system. Broadly, these changed significantly from the influent, through CW1 and CW2 to the effluent (P=0.001, WS1; P=0.001, WS2). There was also a significant effect of month (P=0.001, WS1; P=0.001, WS2). Again, as what was seen when only examining the influent and effluent communities, those collected in 2021 were more similar, while samples taken in in 2020, from both systems, were more scattered indicating little similarity between them. Microbial communities of influent and CW1 from 2021 were more similar in both systems, indicating little change in them as they moved through the first wetland, whilst the microbial communities of CW2 and effluent overlapped (see Figure 3-16) indicating that changes in the microbial communities occurred in CW2. For WS2, the influent and effluent microbial communities from 2020 were different, but this trend was not found in WS1.

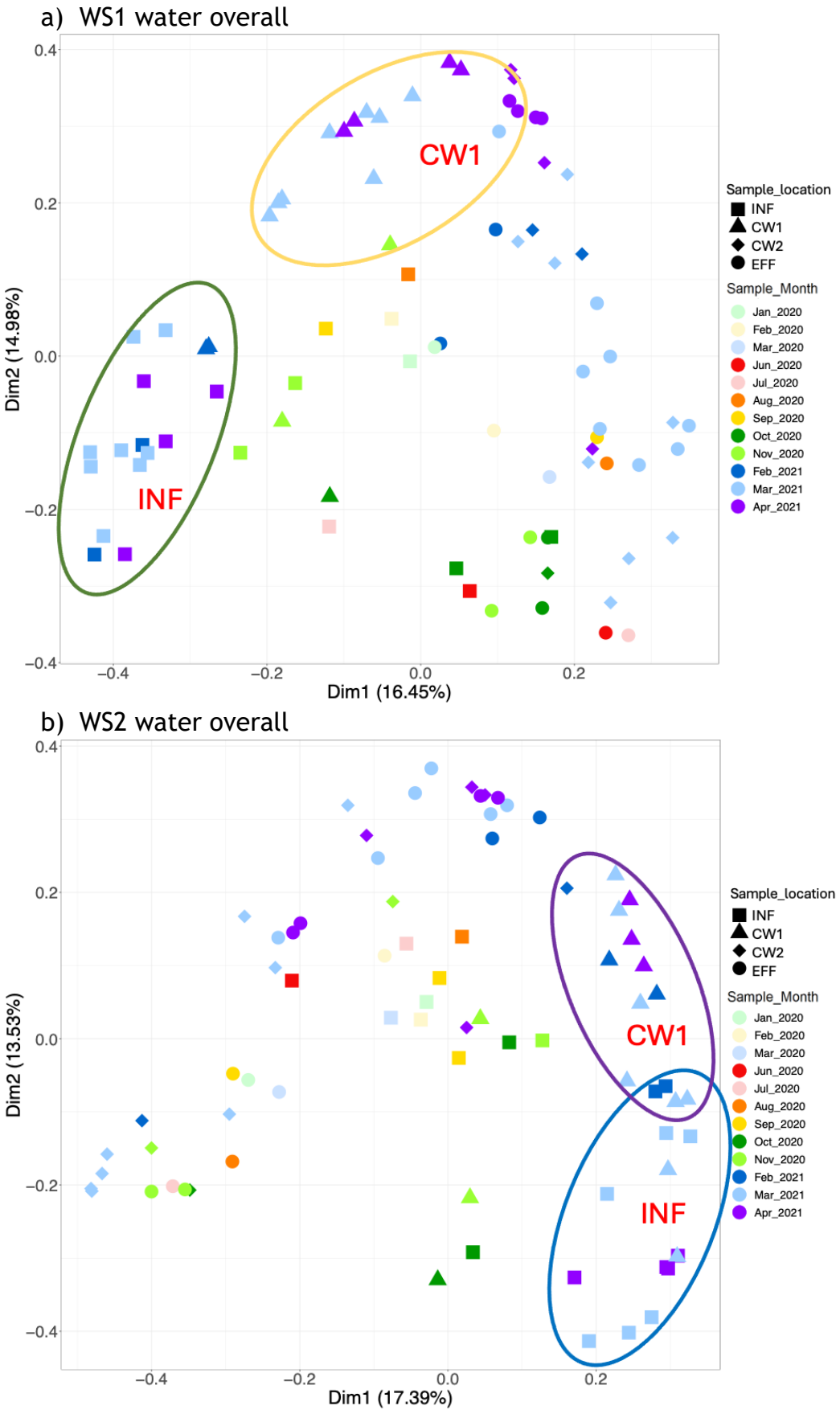

Figure 3-16 Bray-Curtis analysis of microbial communities in influent, CW1, CW2 and effluent within each system. a) WS1. b) WS2. Ellipses in both figures indicate the cluster of microbial communities. In a) dark green indicates influent of WS1, dark yellow indicates CW1 of WS1. In b) dark blue indicates influent of WS2, dark purple indicates CW1 of WS2. (WS1, n=81, includes 5 samples from the first 3 months; WS2, n=76, includes 6 samples from the first 3 months)

Next, we examined microbial community changes across the CW1 and CW2 within each system. Microbial communities were different in each system (P=0.001, WS1; P=0.001, WS2) overtime (P=0.001, WS1; P=0.001, WS2), however, the difference between wetland water and sediment was not observed in the Bray-Curtis analysis of WS2 (P=0.001, WS1; P=0.079, Bray-Curtis, P=0.005 unweighted UniFrac, WS2). In both systems, there was a separation of microbial community from CW1 to CW2 (see Figure 3-17), the separation in WS2 was clearer than WS1. Meanwhile, the microbial communities of the water samples from CW1 overlapped with their sediment samples, while in CW2 water samples clustered separately from the corresponding sediment samples.

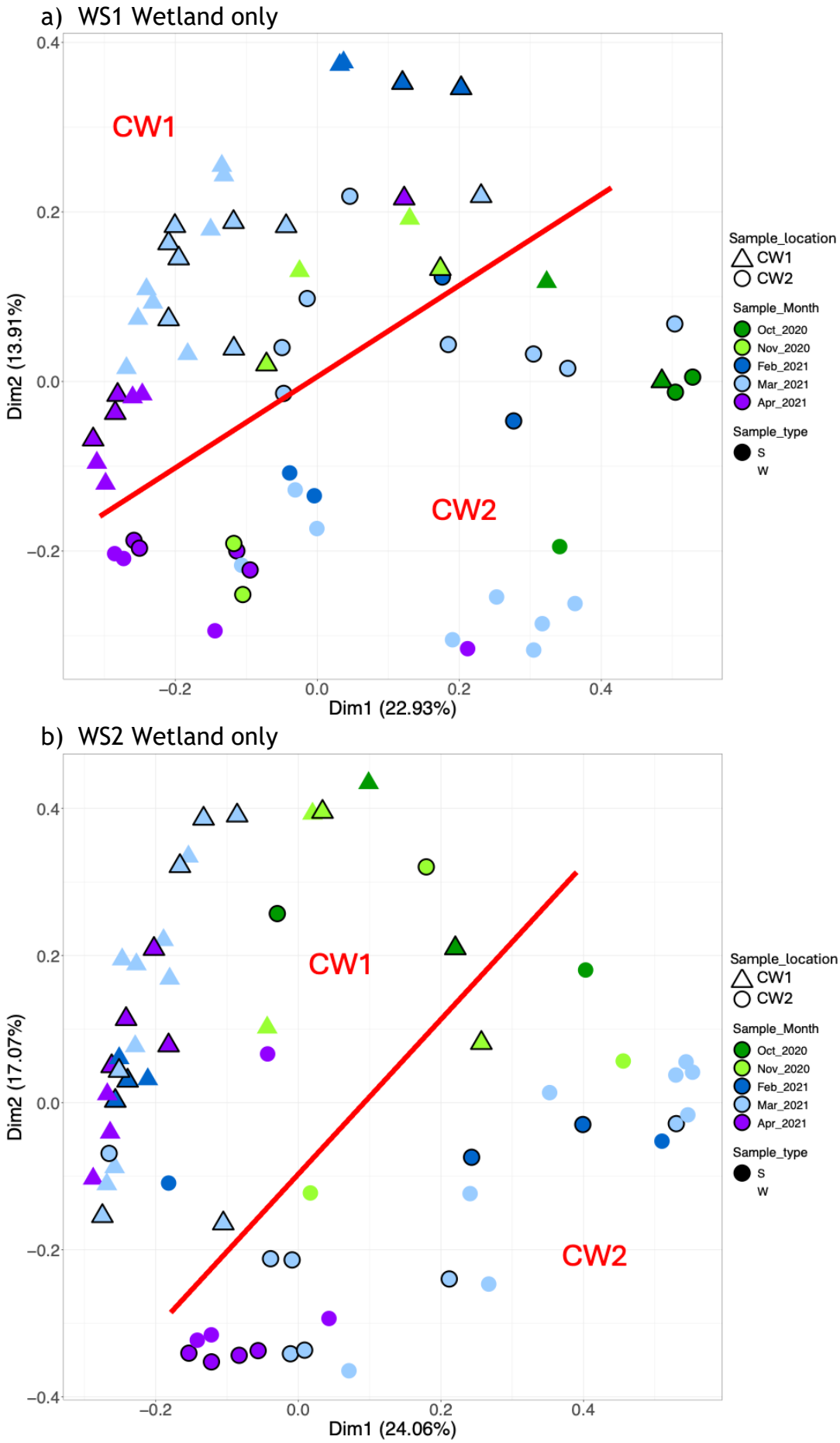

Figure 3-17 Bray-Curtis analysis of microbial communities of constructed wetlands within each system. a) WS1. B) WS2. Red line indicates the separation of microbial communities. (WS1, n=67; WS2, n=63)

Beta diversity reveals the similarity of microbial community composition of every sample, where in the short-term study, the microbial community composition in those two households converged in the third month despite differing influent communities. However, this was not observed over the long-term operation of the two wetlands with the microbial community composition in the two systems phylogenetically different. This result indicate that different influent microbial communities contribute to the difference of microbial communities in the whole system. Nevertheless, despite different microbial communities, the two systems have the same capacity to remove pollutants from wastewater. Interestingly, the removal rate of COD, NO_2^- and NO_3^- became stable from October 2020, whilst the microbial communities only became similar from February 2021. This indicates that system performance is not directly related to stable microbial communities, and microbial communities take longer to stabilise than the performance.

3.3.4 Variation of top 25 most abundant taxa overtime

Next, we examined changes in the microbial taxa, as indicated in beta diversity, the microbial communities were different in each system (WS1 verses WS2), and as such the comparison of the relative abundance of the top 25 most abundant taxa at ASVs level, was analysed across each system. In each system, the analysis was conducted as 25 most abundant taxa between water samples of influent, CW1, CW2 and effluent in each system (Figure 3-18 and Figure 3-19) and 25 most abundant taxa between water and sediment samples of two wetlands (CW1 and CW2) in each system (Figure 3-20 and Figure 3-21).

The overview of the taxa found out that there were six phyla shared by 46 ASVs across systems. 20 out of 46 ASVs belong to *Proteobacteria*, 13 ASVs belong to *Bacteroidota*, 7 ASVs belong to *Firmicutes*, and the rest were *Synergistota* (3), *Desulfobacterota* (2) and *Latescibacterota* (1).

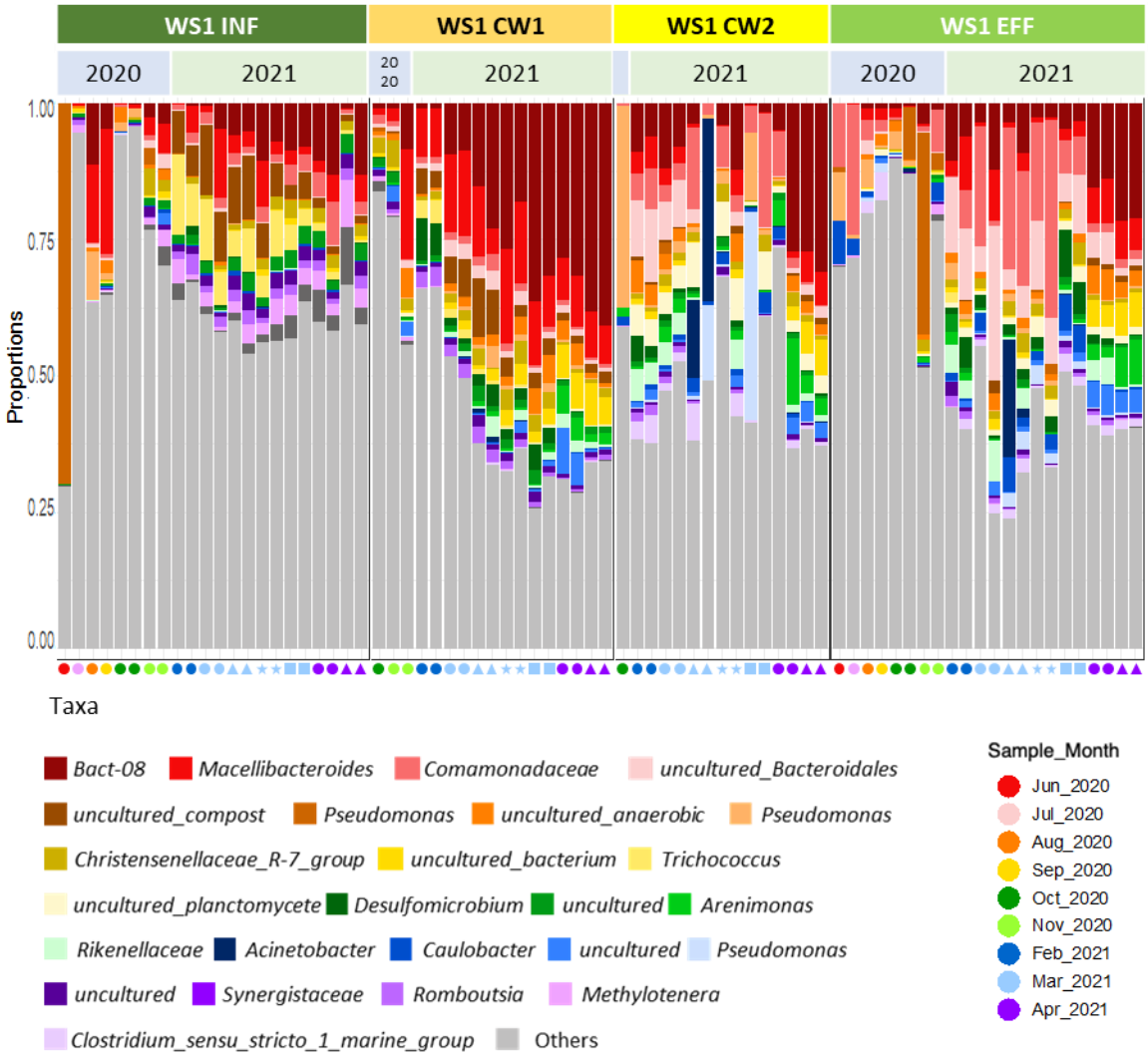

Figure 3-18 Top 25 most abundant taxa as ASV level of water samples from WS1. Taxa bars were grouped by influent, CW1, CW2 and effluent and the year of sampling is labelled separately. Colour of ●, ▲, ★ and ■ indicate the month of sampling. ●, ▲, ★ and ■ indicate the 1st, 2nd, 3rd and 4th sampling point of the month respectively. (n=76)

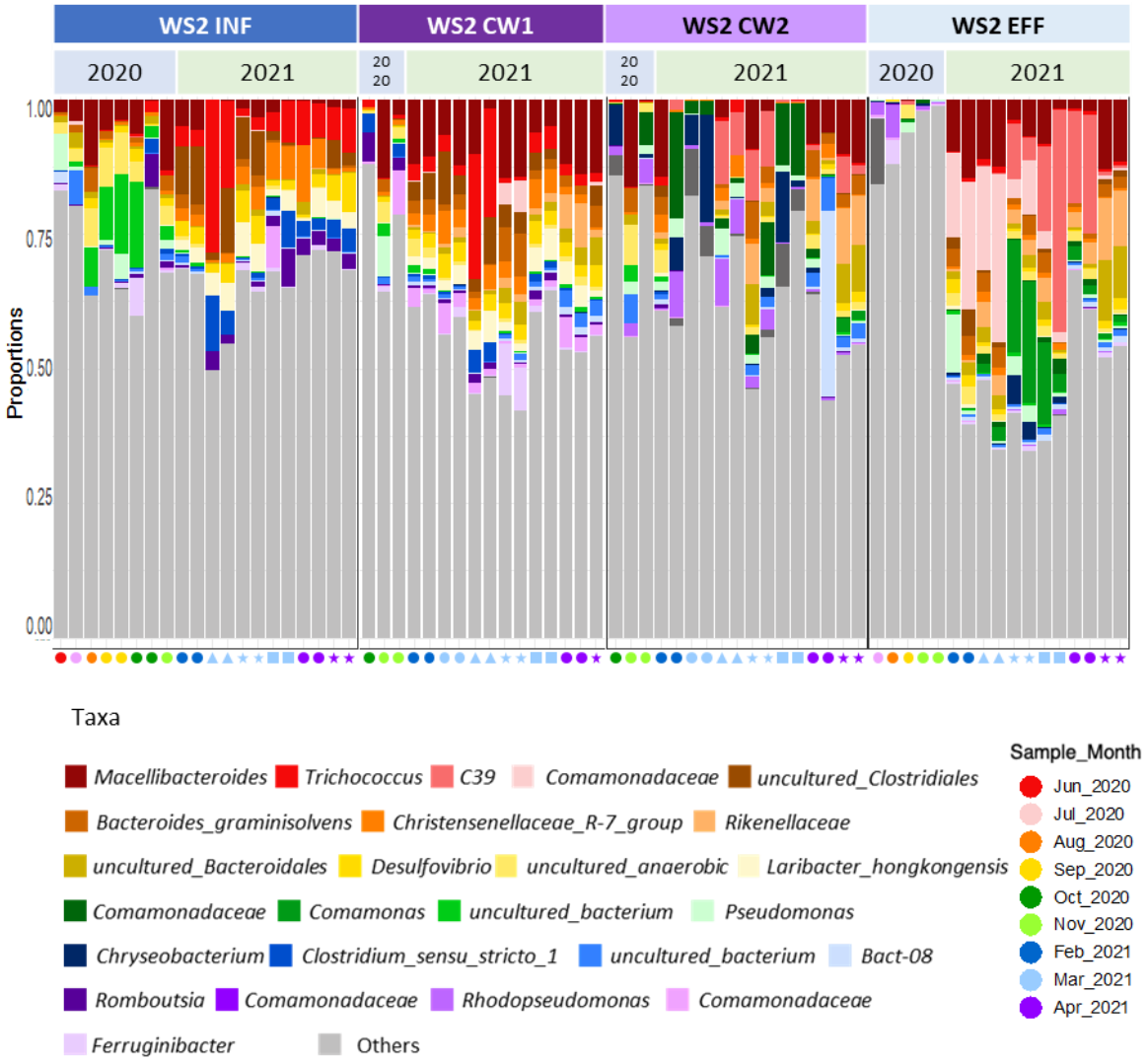

Figure 3-19 Top 25 most abundant taxa as ASV level of water samples from WS2. Taxa bars were grouped by influent, CW1, CW2 and effluent and the year of sampling was labelled separately. Colour of ●, ▲, ★ and ■ indicate the month of sampling. ●, ▲, ★ and ■ indicate the 1st, 2nd, 3rd and 4th sampling point of the month respectively. (n=70)

In WS1, the top 25 most abundant ASVs represented 4.27-76.18% of the microbial community of all water samples (Figure 3-18), and 0.15-80.81% of the microbial community from the two constructed wetlands (Figure 3-20). In WS2, the top 25 most abundant taxa covered 1.27% to 65.31% of the taxa in water samples (Figure 3-19), and 5.41% to 64% of the taxa in the two constructed wetlands (Figure 3-21). The coverage of top 25 most abundant taxa in WS1 was higher than in WS2, where more samples had 50% of the taxa coming from the top 25 most abundant taxa. This indicates that the microbial communities in WS2 were more diverse with less dominant ASVs than WS1. Whereas in the alpha diversity analysis of the microbial community, the value of evenness and species richness in WS2 were higher than WS1, which further supports that WS2 has a more diversity microbial community.

As seen in beta diversity plots that consider the total communities the influent and water from CW1 were closer to each other, where the water in CW2 and effluent were overlapped in both systems. In the top 25 most abundant taxa, the influent samples were very similar to each other and the same as the water samples from CW1. This trend was more obvious for the samples collected in 2021. In the water samples from CW2 and effluent samples, more variation in the AVSs was seen. Meanwhile, effluent samples collected in 2020 from both systems were very different compared with the samples in 2021. In 2020, the top 25 most abundant taxa only represented 10.0-48.41% of the taxa in WS1 effluent and 1.27-15.67% of the taxa in WS2, where the coverages of effluent in 2021 were much higher than 2020. In addition to this, in the beta diversity analysis of influent and effluent microbial communities, they were scattered in 2020 and became closer to each other in 2021 (see Figure 3-15), which further supports that there was a shift of microbial community in the effluent from 2020 to 2021 in both systems.

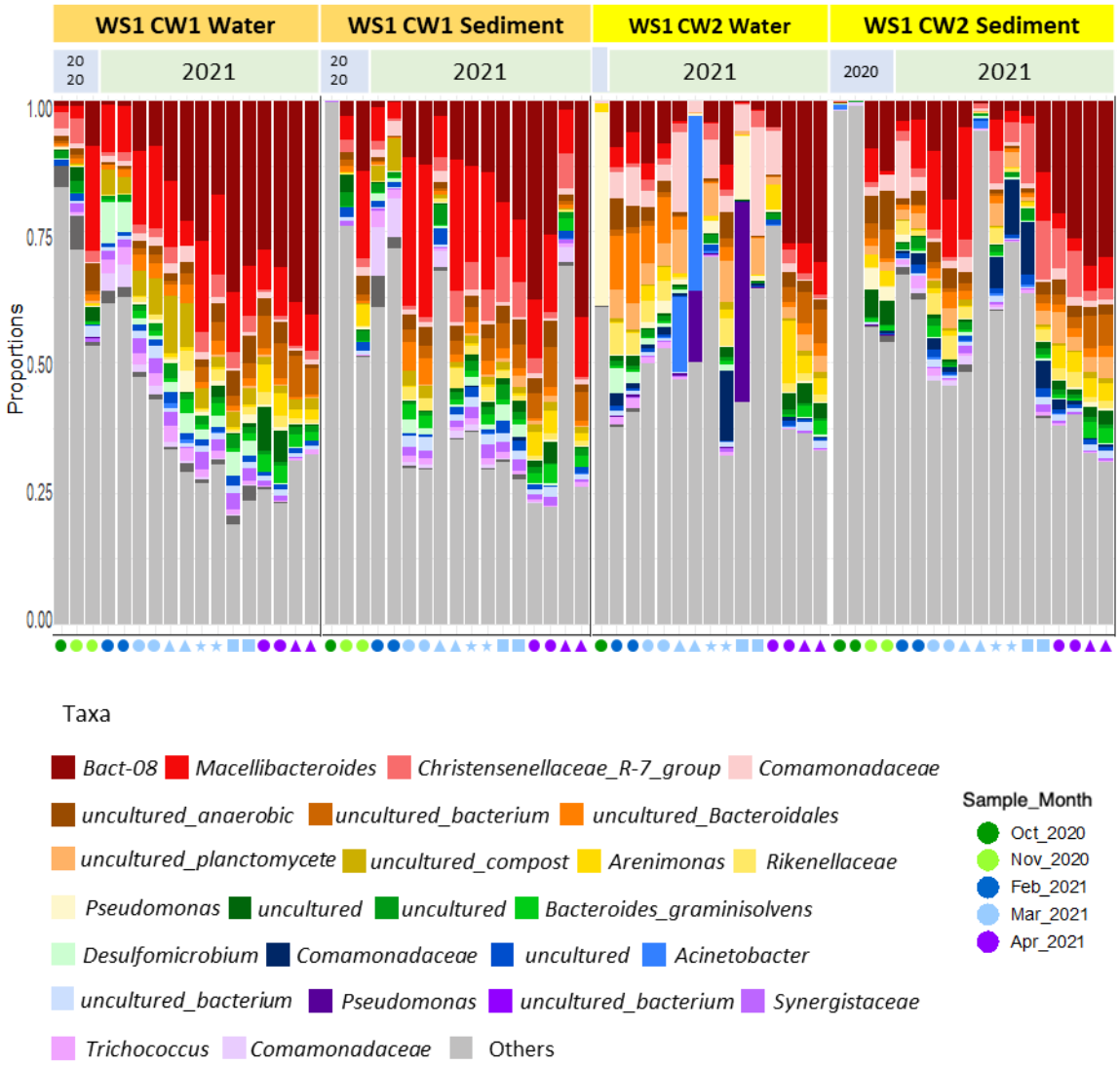

Figure 3-20 Top 25 most abundant taxa as ASV level of samples from constructed wetlands of WS1. Taxa bars were grouped by water samples from CW1, sediment samples from CW1, water samples from CW2 and sediment samples from CW2, and the year of sampling was labelled separately. Colour of ●, ▲, ★ and ■ indicate the month of sampling. ●, ▲, ★ and ■ indicate the 1st, 2nd, 3rd and 4th sampling point of the month respectively. (n=67)

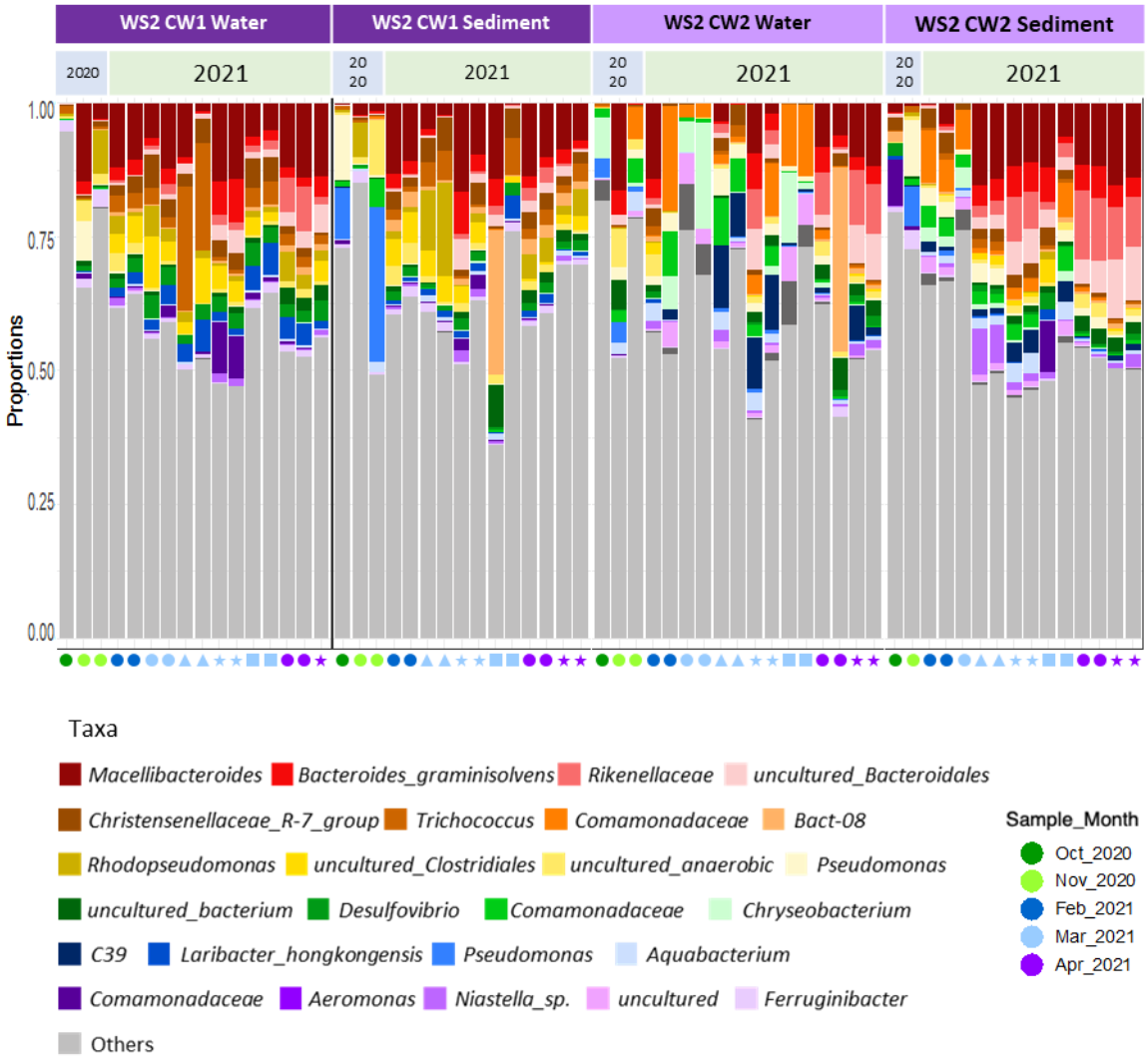

Figure 3-21 Top 25 most abundant taxa as ASV level of samples from constructed wetlands of WS2. Taxa bars were grouped by water samples from CW1, sediment samples from CW1, water samples from CW2 and sediment samples from CW2, and the year of sampling was labelled separately. Colour of ●, ▲, ★ and ■ indicate the month of sampling. ●, ▲, ★ and ■ indicate the 1st, 2nd, 3rd and 4th sampling point of the month respectively. (n=63)

The comparison between two constructed wetlands (CW1 and CW2) within each system showed that water and sediment samples were overlapped in CW1 and water and sediment samples were far away in CW2 were found in beta diversity (Figure 3-20 and Figure 3-21). Similar trends were observed in the top 25 most abundant taxa - water and sediment samples were similar in CW1 of both systems, while more variation was seen between water and sediment samples in CW2. Despite this trend being shared by both systems, *Bact-08* (ASV 1) from *Rikenellaceae* family was the most dominant ASV in CW1 of WS1 and *Macellibacteroides* from *Tannerellaceae* family was more dominant in CW1 of WS2. Even though water and sediment samples were different in CW2, the samples from the last two time points became similar to each other, where WS1 was dominated by *Bact-08* (ASV 1) from *Rikenellaceae* family and WS2 was

dominated by 4 ASVs, they were *Macellibacteroides* from *Tannerellaceae* family, *Bacteroides graminisolvens* from *Bacteroidaceae* family, and other two from *Rikenellaceae* family. The change of microbial community in CW2 suggests that the microbial community in vertical constructed wetland (CW2) takes a longer time to establish and mature.

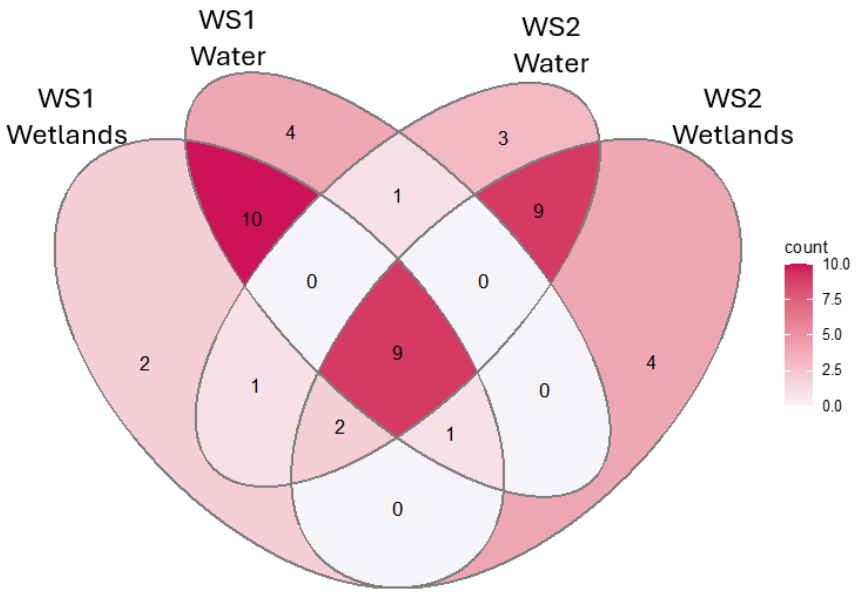

Figure 3-22 Venn diagram of top 25 most abundant ASVs shared between water and wetlands across systems in long-term study. ASVs used in this diagram were extracted from Figure 3-18 to Figure 3-21.

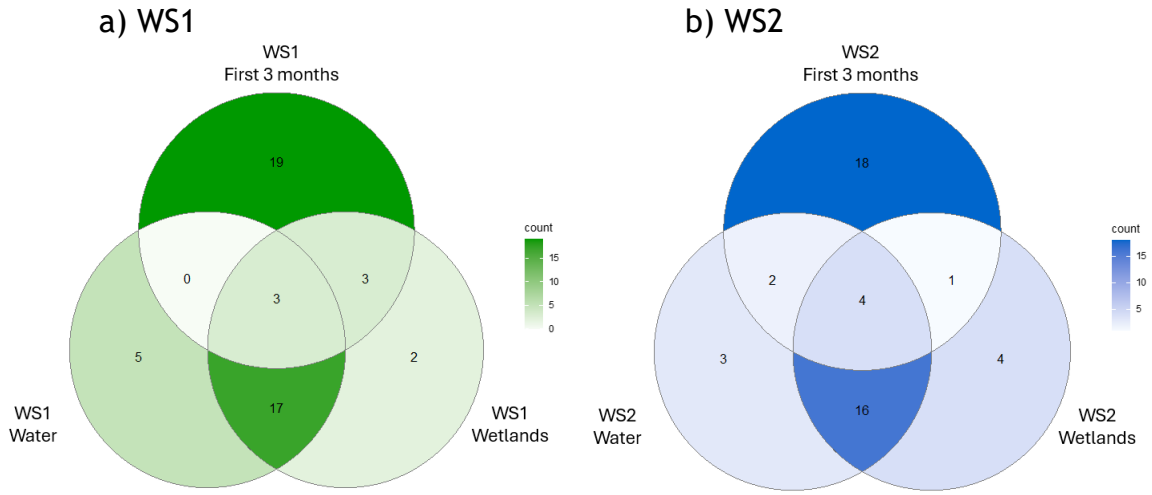

Figure 3-23 Venn diagram of top 25 most abundant ASVs shared within each system between first three-month and long-term study. a) shared ASVs in WS1 with data from Figure 3-18, Figure 3-20 and Figure 2-11; b) shared ASVs in WS2 with data from Figure 3-19, Figure 3-21 and Figure 3-18, Figure 3-20 and Figure 2-11.

Top 25 most abundant ASVs were analysed with different grouping of samples, there were 46 different ASVs shared among them (Figure 3-22). Only eight out of these 46 ASVs were shared with top 25 most abundant ASVs in the first three

months' study (Figure 3-23), this indicates that the dominant ASVs in the microbial community shifted during the long-term running. Meanwhile, only 10 ASVs were shared between water samples in those two systems, which was predicted by the unweighted UniFrac results, that showed that the microbial community composition of those two systems were different.

Within the top 25 most abundant ASVs in water samples of each system, the coverage of effluent of both systems in 2020 was much lower than others, whilst the coverage of influent fluctuated in 2020. Meanwhile, in WS1 the top 25 most abundant ASVs in 2020's effluent shared some similarity to the sample in 2021, while there was no similarity found in WS2 effluent, also the influents of both systems in 2020 were different from that in 2021. Combining the results of the microbial community composition analysis, where the samples in 2020 were scattered (less similar to each other) and samples in 2021 were clustered (more similar to each other), further indicating that the microbial community in 2020 was different from 2021 in terms of both the dominant ASVs and the composition. However, there were not enough samples in the two constructed wetlands to compare with influent and effluent at the same time. Therefore, it is hard to say if this change is caused by the microbial community in the influent or the maturation of the system.

3.4 Discussion

3.4.1 Wastewater treatment performance

COD, NO_2^-, NO_3^- and phosphate concentration were monitored for 10 months, where COD, NO_2^- and NO_3^- concentrations were much lower in the effluent than influent, whilst phosphate concentrations in the effluent were similar to influent. This indicates that our two small-scale constructed wetland systems were capable in removing COD, NO_2^- and NO_3^- from household wastewater in long-term. Despite large proportions of COD had been removed after treatment of the constructed wetland systems, there were still over 200mg/L of COD in average in the effluent of both systems. However, BOD_5 was not measured, it is unknown how much biodegradable carbon remained in the wastewater after treatment. It is also unable to evaluate if the treated wastewater has met the

Mexican standards for reuse treated wastewater (NOM-003-SEMARNAT-1997) (Gutiérrez, 2008) due to lack of BOD_5 data.

The initial short-term study found out that the concentrations of COD, NO_2^- and NO_3^- were high in our household wastewater, in this long-term study, high concentrations of COD, NO_2^- and NO_3^- in influent were observed throughout the monitoring period. Raw wastewater with over 1000mg/L of COD has been reported by De Anda et al. (2018), Verduzco Garibay et al. (2021) and Belmont et al. (2004) in decentralised wastewater treatment involved constructed wetlands in Mexico, however, in all three studies COD concentration has been reduced to less than 350mg/L before treating by constructed wetland units. Meanwhile, super low pollutant concentrations (COD < 53.6mg/L, total nitrogen < 18.5mg/L) are reported in some full-scale constructed wetland studies (Zhu et al., 2021, A et al., 2023), this suggests that constructed wetlands of various sizes are capable of treating wastewater with a wide range of pollutant level. However, there is no flowrate data available in this study, it is unable to evaluate the pollutant lading rate and treatment potential for our systems. Despite total nitrogen was not measured in our study, largely reduced NO_2^- and NO_3^- concentrations and relatively stable NH_4^+ concentration indicate that denitrification may be the main process of nitrogen removal in our systems. A et al. (2023) state that carbon sources are one of the important factors in nitrogen removal, higher C/N ratio promotes nitrogen removal as denitrification is the main pathway of nitrogen removal in subsurface constructed wetland. Although exact C/N ratio is unknown in our study, high COD concentration in wastewater may provide plenty carbon sources for denitrification, which should result a lower total nitrogen in the effluent.

The wastewater from both households were high strength with COD, NO_2^- and NO_3^- concentrations several times higher the centralised domestic wastewater treatment plants, even if so, they still achieved average removal rates of at least 73.42%. COD removal was particularly high in both of our systems, where average of 79.63± 7.87% of COD removed by WS1 and 84.08±7.40% of COD removed by WS2. Marín-Muñiz et al. (2023) summarised that 18 full-scale constructed wetland in Mexico removed 68.3-94.7% of COD from different resources of wastewater, the removal rates of our household systems fall into

this range. Average of 70.3±48.5% of BOD reduction in 21 single-house systems reported by Steer et al. (2002), average of 89.63% COD reduction in 20 systems reported by Alfiya et al. (2013) and 65.7-89.6% of COD reduction in nine systems reported by Obarska-Pempkowiak et al. (2015). COD removal rates of our systems were higher than most of the constructed wetlands in various sizes, the higher COD input in our systems could be the reason as higher organic load is associated with higher removal rate (A et al., 2023, Zhu et al., 2021, Sun and Saeed, 2009, Saeed and Sun, 2011). It is worth to mention that the wastewater composition in small-scale constructed wetland is more subject to change than full-scale constructed wetland due to its small operation volume. Our systems have an equalization tank before the biodigester (where our influents were taken), in which dramatical shift of influent in a short time should be minimised. Meanwhile, most of the small-scale constructed wetland studies like us only have one set of samples every month, those samples may not represent the true performance of a whole month.

It is interesting to see the removal rates of COD, NO_2^- and NO_3^- in both systems achieved a stable state 10 months after setting up. It is common to see in constructed wetland studies only describe that samples were taken after an initial operation period, which can vary from weeks (Hijosa-Valsero et al., 2012, Wu et al., 2018) to years (Wu et al., 2017, Yan et al., 2018), without knowing if the wetlands were stable at the time samples were taken. There are only a few long-term studies reports using constructed wetland to treat single household wastewater. 21 constructed systems were monitored over seven years by Steer et al. (2002), however, the result was reported as an average of the seven years. Same as 20 constructed systems over 1.5 years reported by Alfiya et al. (2013) and nine systems over 4 years reported by Obarska-Pempkowiak et al. (2015). 20 constructed wetland systems studied by Alfiya et al. (2013) started 10 months after setting up, which is around the time our systems achieved stable removal rate. In this case, our system with 79.63±7.87% of COD reduction in WS1 and 84.08±7.40% in WS2 are comparable to their 89.63% COD reduction, but our systems treated wastewater with 3-5 times higher COD concentrations than theirs. Although our study provides an insight into the time requires to achieve a stable pollutant removal, it is still unknown whether pollutant load or operation affected the time required to achieve a stable pollutant removal.

Phosphate accumulated in both systems, where the phosphate concentrations in the effluent increasing overtime. This result is expected as phosphate is removed from wastewater mostly through adsorption by the media in constructed wetland, desorption happens if the media is saturated (Vymazal, 2007). Microbes can only uptake a very low amount of phosphate and they tend to work better in less nutritious wetlands than the nutritious ones (Vymazal, 2007). Zhu et al. (2021) reported a constructed wetland system achieved an average 67.3% of phosphate removal over 10 years' time, however, the system received wastewater containing only 0.03-1.43mg/L phosphate, the filter media in this study is likely to take much longer time to saturate. In our study, phosphate accumulation may cause by the saturation of the filter media within constructed wetland, and WS2 had a poorer phosphate removal than WS1 may be explained by higher pollutant load in WS2 led to an earlier saturation and inhibition of biological phosphate removal. Although the water consumption is hard to monitor in single household system, which makes estimate the pollutant load harder. Monitor the change of phosphate concentration in small-scale constructed wetland may provide a guide for filter media replacement in further operation.

3.4.2 Microbial community analysis

Continuous monitoring of microbial communities in small-scale constructed wetland systems are barely see. Our study revealed that species richness in wastewater decreased throughout the treatment system, there was no difference in species richness in the sediment between both constructed wetlands in each system. Higher organic load and higher nutrients of nitrogen are associated with higher species richness in constructed wetland (Choi et al., 2022, Wu et al., 2023). This explains our observation of species richness reduced with lower COD and nitrogen concentrations in wastewater, and higher species richness in WS2 influent than WS1 influent due to higher COD and nitrogen concentrations in WS1 influent. However, Wang et al. (2022) summarised that high nitrogen concentration (>20mg/L) is associated with lower diversity and species richness in microbial community and Xiao et al. (2020) suggest that no pollutant removal related species are likely to be eliminated or suppressed under high pollutant concentrations (COD and nitrogen) may be the cause. But both of their high concentrations are just a fraction of the pollutant

concentrations in our systems, also, the species richness of microbial community is also affected by C/N ratio (Li et al., 2019), where in our study the C/N ratio was unknown. Compared with the microbial communities in the first three-month's study, species richness was much higher in the long-term study while the diversity remained the same. Tian et al. (2024) also observed increasing in species richness over 83 days in pilot-scale constructed wetlands, but the diversity of microbial communities were declining over time. Meanwhile, species richness and microbial community diversity were reduced after the maturation was observed by Wolff et al. (2024). The characteristics of wastewater such as carbon and nitrogen source or ratio are likely to contribute to this difference.

The function of plants within the constructed wetland is still debatable. Vymazal (2011b) claims that plants are essential for constructed wetland, and most studies suggest that vegetation is positively help in wastewater treatment. However, Nivala et al. (2019) and Wolff et al. (2024) found out that plants have minor or no effect on pollutant removal rate in the constructed wetland. Fu et al. (2019) observed that plants only changed the composition of potential functional bacteria in the soil bacterial communities but did not increase the overall diversity of the soil bacterial. As a result of the shift in bacterial community, the planted constructed wetland has a higher pollutant removal potential than the unplanted one. Similar change in microbial communities was also found by Zhao et al. (2012) and Tian et al. (2017), where the shift of functional microbial communities were seen in planted constructed wetlands. Our constructed wetlands have plants and plants were growing with the maturation of the constructed wetlands, however, there is no detailed record of plant growth and no unplanted constructed wetland to compare, it is unknown how the plants affected the performance and microbial community in our study.

Both systems, treatment performance achieved stable state after 10 months of operation, while microbial community only became similar after 14 months. Which indicate that the shift of microbial community composition does not affect the function of wastewater treatment. Hollstein et al. (2023) also found that a shift in microbial community structure is not necessary to affect the function of constructed wetland, which is the same as our systems. Indeed, previous studies with full scale WWTPs have also revealed that different

treatment has different microbial communities but they all can achieve similar effluent quality (Wu et al., 2019). Also, the microbial communities in the sediment were different between horizontal and vertical constructed wetlands in the same system in our study, the same was observed by Wu et al. (2016) from a full scale constructed wetland system consisting of three different constructed wetlands in sequence. The difference of sediment microbial community is likely to be caused by the change of COD and nitrogen concentrations in wastewater (Wu et al., 2019). The vast majority of studies of constructed wetlands only take samples at one time point or from matured constructed wetlands, whereas our study observed changes of microbial community overtime. Despite the change of microbial communities does not determine the performance of constructed wetland, it provides a better insight into microbial community than one-off sample.

Since most of the constructed wetland studies in the literature examining microbial community were analysed at phylum level, we looked the phyla of the 46 different ASVs shared in different analysis. *Proteobacteria* was the most abundant phylum, where 20 out of 46 ASVs belong to *Proteobacteria*. *Proteobacteria* is also one of the most reported phylum in both constructed wetland or full scale wastewater treatment plant, large numbers of species belong to *Proteobacteria* are positively associated with pollutant removal, includes various nitrogen removal processes, organic carbon removal, phosphate removal and so on (Wang et al., 2022, Wu et al., 2019).

Rikenellaceae and *Macellibacteroides* were the most dominant ASVs in WS1 and WS2 respectively, they both have been reported as anaerobic microorganisms in removal organic carbon in wastewater treatment (Graf, 2014, Nakasaki et al., 2020, Gallardo-Altamirano et al., 2021, Chen and Chang, 2017). This indicates that wastewater in both systems was likely to stay anaerobically throughout the treatment.

Comamonadaceae family was the most abundant family found in study, where 8 out of the 20 ASVs belong to *Proteobacteria* belonged to the *Comamonadaceae* family. *Comamonadaceae* family is reported to have positive association with improved organic carbon (Huang et al., 2021, Wu et al., 2019), whilst Wu et al. (2019) also reported the relative abundance of *Comamonadaceae* has strong

positive correlation with total nitrogen and phosphate removal. *Comamonadaceae* family is a phenotypic diverse family with more than 100 species, where both aerobic and anaerobic denitrifiers are reported as well as both heterotrophic and autotrophic bacteria (Willems, 2014, dos Santos et al., 2021, Petrilli et al., 2023). There was only one ASV in the most abundant 25 taxa belonging to the *Comamonadaceae* family in short-term study, but eight out of 46 most abundant ASVs shared between two systems belong to *Comamonadaceae* family in long-term study. This suggests that higher abundance of *Comamonadaceae* family appeared in our long-term study may positively associate with the higher performance of COD and nitrogen removal. *Rhodocyclaceae* family has also been positivity associated with COD removal rate (Wu et al., 2019) as well as nitrification and denitrification potential (Oren, 2014). In our study it was seen in the long-term study of WS2, suggesting that their increasing relative abundance could be associated with improved COD removal rate.

Pseudomonas appeared more in long-term study (6) than the short-term study (2) in the top 25 most abundant ASVs, whilst only one ASV was shared in both studies. Many strains in *Pseudomonas* genus are known to have denitrification abilities under anoxic condition (Lalucat et al., 2022, Fitzgerald et al., 2015), whilst this is more likely to happen as anaerobic *Rikenellaceae* and *Macellibacteroides* were the most dominants in WS1 and WS2. In additional to limited oxygen condition, aerobic denitrification by *Pseudomonas* has also been reported by Gao et al. (2023) and Huang et al. (2022). Meanwhile, many *Pseudomonas* strains have the ability of simultaneous nitrification and denitrification (Zhang et al., 2022, Li et al., 2015, Huang et al., 2022). Despite BOD was not measured in this study, super high COD concentration indicates high organic carbon resource for heterotrophic bacteria, where denitrifying bacteria from *Pseudomonas* genus from fall into this category (Rajta et al., 2020). Although total nitrogen was not measured in this study, combined with plenty of carbon resource provided by high COD concentration, the increased abundance of *Pseudomonas* in long-term study was likely to associate with improved denitrification in both systems.

3.4.3 Strengths and limits of this study

This long-term continuous study demonstrates that small-scale constructed wetland system is capable of treating high strength domestic wastewater from single household. Although a few studies have also observed the same outcome, none of them analysed microbial communities within the system. Similar sized constructed wetlands are commonly pilot scale tested with synthetic wastewater for a short time, although microbial communities are seen reported in those kinds of studies, it is unknown if the one-off sampling would represent the whole microbial communities. Our study observed the change of microbial communities over time and found out that the performance remained stable despite the shift of microbial communities.

Small-scale constructed wetland system study comes with its challenges. Unlike full-scale constructed wetland receiving large amount of wastewater all the time, the influent flowrate in small-scale system is variable and highly depended on the household activities. Our influent was sampled once from biodigester, although there is an equalization tank before the biodigester to minimise the fluctuation of wastewater characteristics, it would be better if the wastewater was sampled at several time point and homogenise for analysis. However, this practice is not commonly descripted in literature, Wolff et al. (2024) is one of the very few studies practised homogenised sampling. Meanwhile, the exact amount of wastewater flew into the system was unknown, water consumption from household water meter may help with this, it is unknown if rain has affected this open system or not. Lack of flowrate data also made it impossible to know the organic load and hydraulic retention time, which subsequently makes it hard to compare with other studies. Furthermore, this system is highly user-depended, it is unknown how irregular operation or non-operation for some time affects the performance.

The sampling campaign did not go as planned, although partial data monitored throughout the campaign still provided the performance evaluation. Biological replicates were not taken in the first six time points, if biological replicates were taken throughout the time, it would provide more robust support for the analysis. Organic carbon was only measured as COD not BOD_5, out data revealed both systems worked in removing COD as lower COD concentration in effluent

was seen, it would be better to have BOD_5 measured at some point to get the ratio between BOD_5 and COD which could be used in evaluating the actual organic carbon removal and also comparing with the wastewater reuse standard in Mexico (Gutiérrez, 2008). Nitrogen was only measured as NO_2^-, NO_3^- most of the time, where if total nitrogen and NH_4^+ were measured throughout the study they would provide much better view of nitrogen removal process within the system. Indeed, this system was designed to keep nitrogen in treated wastewater as fertilizer, knowing the nitrogen transforming within the system would provide better operational guidance for the users to keep nitrogen as they desired. Biomass was not measured in any form and DNA was only extracted from wet weight, future study should include biomass as it provides both physical and biological performance in the system. Samples within the constructed wetlands in the first seven time points (including three time points in chapter 2) were not measured in this study, those data would largely help tracking the change of microbial community and maturation of the constructed wetlands. Those missing data would largely support the evaluation of performance in this study, on the other side, those missing data provide a guide for planning further study.

3.5 Conclusion

Our two small scale operational constructed wetland systems worked well in removing COD, NO_2^- and NO_3^- from household wastewater in our year-long study, while the removal of NH_4^+ and phosphate were limited. The removal of COD, NO_2^- and NO_3^- stabilised after 10 months' operation. Microbial communities took longer (14 months) to stabilise than pollutant removal, and despite different microbial communities were found in these two systems they achieved comparable pollutant removal rates. The microbial communities in both systems shared a same trend, with the microbial communities in 2021 more similar to each other, and those in 2020 they were very different. The microbial communities in the CW1 were influenced by the influent while the microbial communities CW2 influenced the microbial communities of the effluent. Further, the microbial communities CW2 changed the most and there were less similarities between water and sediment of CW2.

Our small scale constructed wetland system received much higher loads of organic matter and achieved comparable pollutant removal rates as the full scale constructed wetlands. Given their relatively small footprint and their reliability in long-term operation, they offer a sustainable solution for off-grid water treatment, and it is recommended to use them as a solution to improve off-grid wastewater treatment in addition to septic tank. Meanwhile, the treated wastewater had a much lower NO_2^- and NO_3^- concentrations but retained NH_4^+ concentration, this relative high concentration of NH_4^+ suggests that the treated wastewater has the potential to be reused in irrigation, although the pathogens still need to be monitored.

4 Can optical density (OD) reflect the change of mixed microbial community response to a range of amoxicillin concentrations?

4.1 Introduction

Our small scale constructed wetlands removed pollutants from household wastewater. As they are designed to reuse treated wastewater, the potential of housing and spreading AMR within the system raise a key question about the risk of spread of AMR. In order to find a suitable method to monitoring the overall AMR in the system, we reviewed the common methods in AMR detection. Molecular methods such as HT-qPCR and metagenomics are always the first choice for environmental samples, as they are culture-independent therefore not affected by largely presented unculturable microbes in the environment. However, the high cost and long turnover time indicate they are not suitable for regular monitoring.

Observing limited inhibition of microbial growth after antibiotic exposure is a sign of AMR, and these kinds of observations started soon after the discovery of penicillin (Abraham and Chain, 1940). The isolation of bacterial cells and observation of inhibition of microbial growth when exposed to antibiotics is the standard method to test susceptibility of clinical pathogens (International Organization for Standardization, 2019, European Committee on Antimicrobial Susceptibility Testing, 2024). For environmental samples, most microbes are unculturable, but they can still be enriched as complex mixed microbial communities. Within the mixed microbial community, organisms may share ARGs among each other. Therefore, a measure of the change of whole microbial community growth when exposed to antibiotics may provide a view of how AMR affects microbial communities.

The growth of microbes is normally measured through the increase of microbes, the consumption of nutrients and the metabolic activity or product during microbial growth. Optical density (OD) is widely used to measure microbe concentration in liquid culture, as it is both time saving and high accuracy (Myers et al., 2013). OD measures the light absorbance and uses the Lambert-Beer linear relationship to reflect the change of microbes in liquid culture (Di

Caprio, 2020). However, OD is normally used in pure culture where the microbes share the same size and kinetic, it is unknown how accurate it would reflect the growth of a mixed community. Studies have shown that even in pure culture an optimised range where OD has a linear relationship with individual cell count through flow cytometer (FCM) still needs to be validated (Ogundero et al., 2022, Beal et al., 2020). Will there be an optimised range for mixed community?

4.1.1 Hypothesis and aims

The hypothesis for this experiment is that OD will reveal the change of microbial community growth rate response to a range of amoxicillin concentrations.

The aim of this chapter is to develop a rapid method (via OD) to measure complex mixed community growth rates in liquid culture subject to a range of amoxicillin concentrations and further exam the accuracy of microbial community growth measured by OD and cell counts through FCM.

4.1.2 Objectives

- Enrich an aerobic mixed microbial community from anaerobic sludge granules as ‘seed’ community for all the tests.

- Measure the growth rate of the ‘seed’ community under different amoxicillin concentrations using OD.

- Method validation of diluents, fixation and storage for cell count via FCM.

- Measure and compare the growth of mixed microbial communities under selected amoxicillin concentrations with OD and FCM.

4.2 Materials and methods

The experiment in this chapter was done in three stages - the preliminary experiment, method validation and secondary experiment.

4.2.1 Preliminary experiment

This experiment aims to develop a rapid method to measure antimicrobial resistance with potential application in measuring antimicrobial resistance in decentralised wastewater treatment. Since the septic tank (anaerobic digester) is the most common decentralised wastewater treatment, and the treated wastewater exposures to air once flows out of septic. Therefore, a 'seed' microbial community from anaerobic sludge was established to mimic the change from anaerobic to aerobic in wastewater treatment process. This mixed microbial culture was used to measure, via OD, the growth of the community, and summarise the change of growth when the 'seed' community exposed to a range of amoxicillin concentrations under aerobic condition.

4.2.1.1 'Seed' community preparation – glycerol stocks from anaerobic granular sludge

The anaerobic granular sludge used in this experiment was sampled on 6th July 2018 by Melissa Moore, PhD student at the University of Glasgow, from an expanded granular sludge bed bioreactor operated by the North British Distillery Company in Edinburgh. The granular sludge was stored in 30L jerry cans at room temperature in the fume hood in the Water and Environment research laboratory, University of Glasgow for 1.5 years.

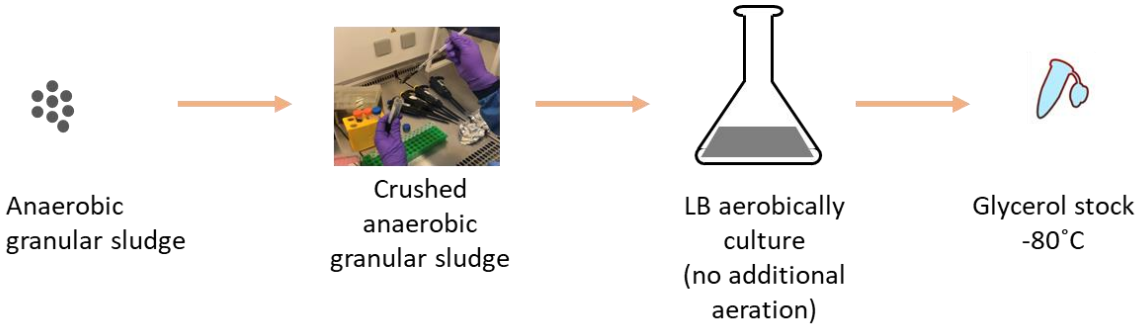

Figure 4-1 Preparation of glycerol stock from anaerobic granular sludge.

The preparation process of glycerol stocks from anaerobic granular sludge is shown in Figure 4-1. A sterile spoon was used to stir and sample the granular sludge from the jerry can. The granular sludge was centrifuged at 5000 rpm (Thermo Scientific Multifuge X1R) for 10 minutes in 50ml sterile falcon tube at room temperature. The supernatant was discarded and the solid sludge was transferred into a 15ml falcon tube and crushed with sterile glass bar manually.

Lysogeny broth (LB) medium was used in culturing the microbes throughout the experiment. LB medium used in this experiment was a sterile medium made of 1% (w/v) sodium chloride (Sigma-Aldrich, S3014), 1% (w/v) Tryptone (Formedium, TRP02) and 0.5% (w/v) Yeast extract (Formedium, YEA02) in weight and top up with 0.2μm filtered deionized water (DI). 0.25g of the crushed granular sludge was transferred into a 250ml sterile conical flask containing 100ml LB medium. The microbes in the conical flask were incubated at 37℃ shaking (120rpm) overnight. 1ml of the supernatant without uncrushed granular was incubated in 100ml LB for another 24h shaking (120rpm) at 37℃. The supernatant of the overnight culture was then mixed with equal volume of sterile 50% (v/v) glycerol (Fisher Chemical, G/0650/17) and stored in 2ml cryogenic tube at -80℃ until further use. All the overnight culture in the following tests were grown from this glycerol stock. The sterile LB medium in the flask was inoculated from the glycerol stock and incubated at 37℃ and shaking for 14-18 hours.

4.2.1.2 Preliminary experiment design – does OD reflect the growth of a mixed microbial community to a range of amoxicillin concentrations?

The mixed microbial community from glycerol stock (made in 4.2.1.1) grew under a range of amoxicillin concentrations and OD was monitored continuously by plate reader (see Figure 4-2).

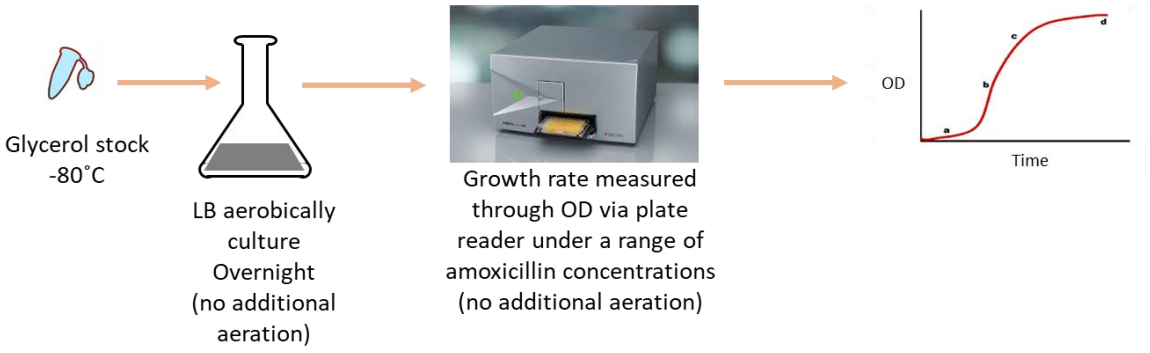

Figure 4-2 Experimental design of measuring antimicrobial resistance via the growth rate of aerobically cultured anaerobic granular sludge subject to a range of amoxicillin concentrations by optical density (OD). The overnight culture was cultured in incubator shaking (120rpm) at 37℃. Plate reader was set at 37℃ shaking orbital 6mm amplitude. No additional aeration for both overnight culture and plate reader.

4.2.1.3 Mixed community growth rate measurement

The mixed community used in this experiment was inoculated from glycerol stock grown overnight in LB shaking (120rpm) at 37 ℃ without additional aeration. 100µl of the overnight culture was reinoculated into 100ml of sterile LB. 1ml of the reinoculated culture or negative control was loaded into a well in 24-well clear plate, and 300µl of mineral oil (Thermo Scientific Acros, 415080025) was added into each well to minimise cross contamination. The plate was covered with clear lid shaking orbital 6mm amplitude at 37℃ without additional aeration and monitored by plate reader (Tecan, Nano Quant Infinite M200 Pro) every 30 minutes through OD at 600mm for 24 or 48h. Amoxicillin concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 10.0, 12.0, 16.0, 24.0 and 32.0 mg/L were used in this test, diluted from 1000mg/L amoxicillin (Sigma-Aldrich, A8523) stock. Positive control of this experiment was LB with microbe. Negative controls included LB, 2ml/L of amoxicillin (diluted with DI), LB containing 2ml/L of amoxicillin to ensure no contamination of LB, amoxicillin stock. All conditions were run triplicated. Experiments with amoxicillin concentrations 0-7.0mg/L were monitored for 24 hours, and experiments with amoxicillin concentrations of 8.0-32.0mg/L were monitored for 48 hours.

4.2.1.4 Growth rate and lag phase calculation

The growth rate and lag phase were calculated with the OD measured by plate reader over 24 or 48h with Microsoft Excel. The growth rate was calculated from the slope of ln (OD) against time from at least five consecutive points, where the highest coefficient of ln (OD) against time was the growth rate. The end of lag phase was considered as the last time point where OD value was below 0.1.

4.2.2 Method validation

Some changes of growth rate in mixed microbial community under a range of amoxicillin concentrations were captured in the preliminary experiment where the growth rate was measured with OD. It is not known how OD reflects the actual growth of the mixed community, a more robust method to measure the growth of mixed microbial communities with cell counts via FCM was validated. The method was then used to measure the growth of microbial communities with

selected amoxicillin concentrations simultaneous with OD and compared with OD measurement.

4.2.2.1 FCM method validation – which diluent to use and how long can a fixed sample be stored?

To keep the consistency of the experiment 'Seed' community stored in glycerol was used for all overnight culture in this test. Glycerol cells were inoculated into 100ml LB and grown at 37℃ shaking for 16 hours. Fresh sample of the overnight culture was initially diluted x1000 with LB and subsequently diluted x10 with phosphate Buffered saline (PBS) or DI then measured total cell count (TCC) and intact cell count (ICC) with FCM. A small portion of fresh overnight culture was diluted x1000 with LB and fixed with equal amount of 1% (v/v) glutaraldehyde, this fixed sample was then stored in dark at 4℃. This fixed sample is measured immediately and 24h, 48h, 72h after fixation, each time it further diluted with PBS or DI at the time of measuring and both TCC and ICC were measured with FCM (Figure 4-3).

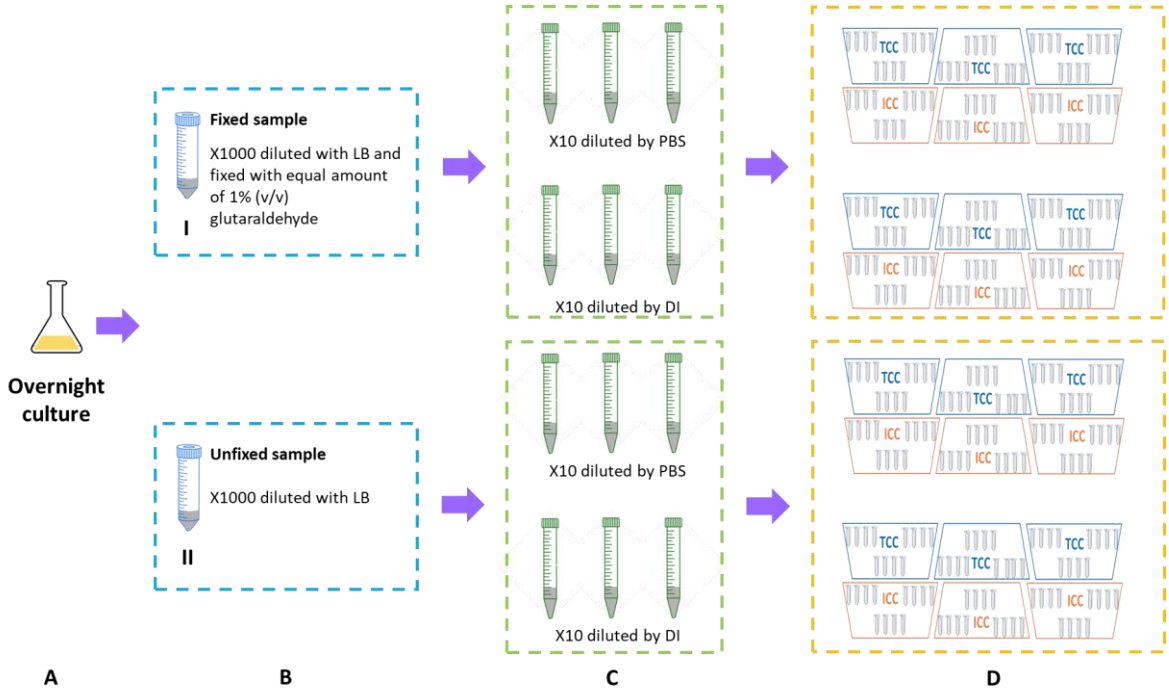

Figure 4-3 Experimental design of diluent (PBS or DI) and sample storage test for total (TCC) and intact (ICC) cell count via FCM. The fresh overnight culture was diluted x1000 with LB (B II) and further diluted with PBS or DI and then measured both TCC and ICC immediately. The fixed sample (B I) was diluted with PBS or DI and then measured TCC and ICC with both diluents immediately and 24h, 48h and 72h after fixation.

4.2.2.2 FCM method validation – Are fixation and high dilution factors necessary?

This experiment design was similar to the pervious one, where the overnight culture was prepared and measured TCC and ICC via FCM (see Figure 4-4). Two fixed samples diluted with LB at x100 and x1000 fixed with equal amount of 1% (v/v) glutaraldehyde and two unfixed samples diluted with LB at x100 and x1000 were prepared and stored in dark at 4℃ for 72h. They were measured immediately after preparation and 24h, 48h, 72h afterwards, each time they were further diluted x100 or x10 with PBS and both TCC and ICC were measured.

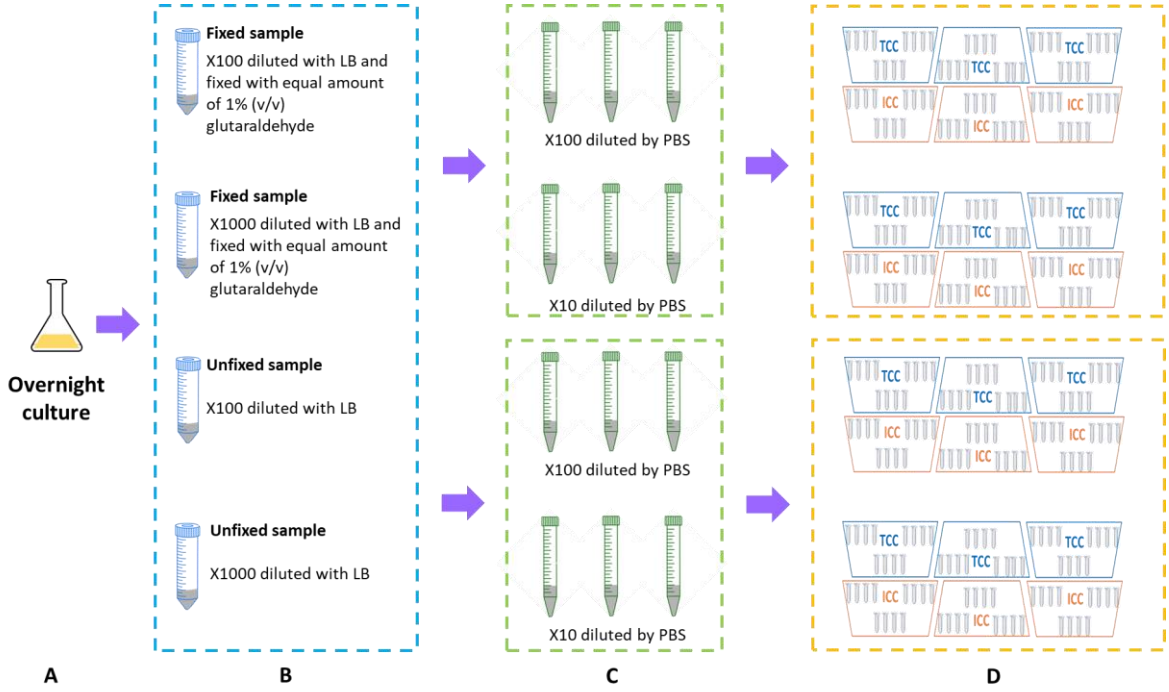

Figure 4-4 Experiment design of dilution factor and sample storage test for total (TCC) and intact (ICC) cell count via FCM. All samples in B were tested soon after prepared with steps in C and D, they were stored in dark at 4℃ and repeatedly tested 24h, 48h and 72h after preparation.

4.2.2.3 Total (TCC) and intact (ICC) cell count with flow cytometer (FCM)

Both experiments in 4.2.2.1 and 4.2.2.2 shared the same sample preparation and measuring processes which are listed below.

Fixative in both experiments was 1% (v/v) glutaraldehyde (Sigma, G5882) in DI water and filtered with 0.22μm sterilised filter. Sample was fixed with equal amount (1:1 in volume) of fixative under each condition and then measured immediately or stored up to 72 hours at 4℃ in dark. Diluent PBS used in this

experiment was prepared with one PBS tablet (Fisher Bioreagents, BP2944) in 200ml DI water and sterilised then filtered with 0.22µm sterilised filter, and diluent DI water was freshly filtered with every time with 0.22µm sterilised filter. During the dilution test, three samples were diluted with the same diluent under each condition. Each diluted sample was then prepared quadruplicate for measuring, where one sample was filtered with 0.22µm sterilised filter as background control, the rest three samples were for the measuring. One PBS sample, one DI water sample, one 1% (v/v) glutaraldehyde and one filtered store sample before diluting were also included in the following steps as background control.

The protocols for TCC and ICC were developed by Hammes et al. (2008) and Berney et al. (2007), in brief, 500µl of each sample stained with 10µl of respective stain separately. The TCC stain was made of 1% (v/v) SYBR Green (Sigma, S9430) in 0.22µm filtered tris-EDTA (Sigma, 93283) and the ICC stain was made of 1% (v/v) SYBR Green, 40% (v/v) propidium iodide (Sigma, P4864) and 60% (v/v) 0.22µm filtered tris-EDTA. Samples were stained and incubated in dark for 15 mins at 37℃ and measured by flow cytometer (BD Accuri C6 plus) immediately. The statistical analysis (T-test) was performed with Microsoft Excel.

4.2.3 Secondary experiment

The preliminary experiment showed that the effect of amoxicillin concentrations can be divided into three ranges based on the change of growth rate, where 0 - 1.5mg/L was the beneficial range, 1.5 – 4.5mg/L was the detrimental range and no further change range started from 4.5mg/L. In this study, amoxicillin concentrations of 1.5, 3.0, 4.5 and 8.0 mg/L were selected to cover all three ranges. The secondary experiment was run in two stages, where the stage 1 tested the microbial community growth without amoxicillin with the method developed in method validation and stage 2 tested microbial growth with selected amoxicillin concentrations with the same method in stage 1.

- **Stage 1 experiment – measure the growth of microbial communities without amoxicillin via OD and FCM**

Microbial communities were seeded from the glycerol stock used in the preliminary experiment and cultured the same way as detailed in 4.2.1.1. All the microbial communities were cultured in triplicate in 250ml flasks containing 100ml of LB medium and 100µl of overnight culture shaking at 37℃ (Figure 4-5). Abiotic control LB medium only was also included in the run. Samples were taken at 1.5 hours interval from the time of inoculation to 7.5 hours. All samples were measured with OD, cell counts of both ICC and TCC, a sample from overnight culture (beginning of the experiment) and a sample from 7.5 hours (end of experiment) were kept for DNA and RNA analysis in the next chapter.

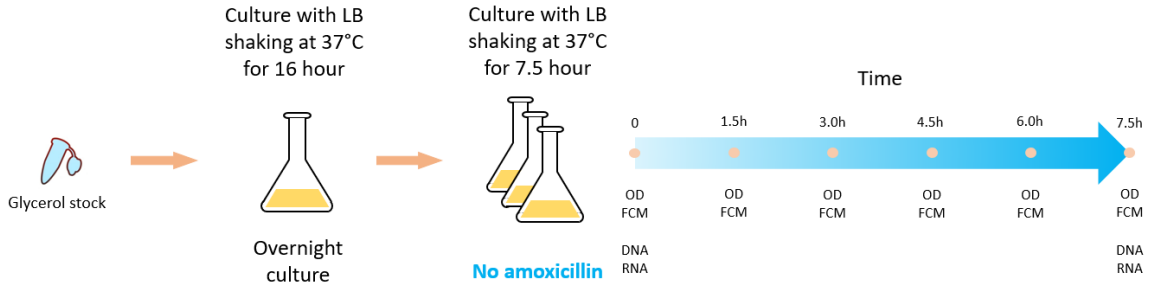

Figure 4-5 Stage 1 experiment design. Cell counts and OD were measured at six time points with 1.5h interval. Samples from the overnight culture and the last time point were kept for DNA and RNA analysis in next chapter.

- **Stage 2 experiment – measure the growth of microbial communities with amoxicillin via OD and FCM**

Each amoxicillin concentration was cultured in triplicate in 250ml flasks alongside triplicated no amoxicillin controls with 100ml of LB medium shaking at 37℃. Abiotic control LB medium only was also included in each run. Sampling intervals were chosen from the growth curve of the preliminary experiments with some adaptations, where detailed sampling time of each concentration listed in Table 4-1. The no amoxicillin microbial community controls in Stage 2 experiment were the same as Stage 1, so only OD was measured at each time point and cross checked with the results in Stage 1 to ensure the batch had a normal growth. OD and cell count via FCM (both ICC and TCC) were measured for all microbial communities cultured with amoxicillin at every time point. Meanwhile, samples at the first time point (beginning of the experiment) and

the last point (end of the experiment) were kept for DNA and RNA analysis in the next chapter, include both amoxicillin containing microbial communities and no amoxicillin controls (Figure 4-6).

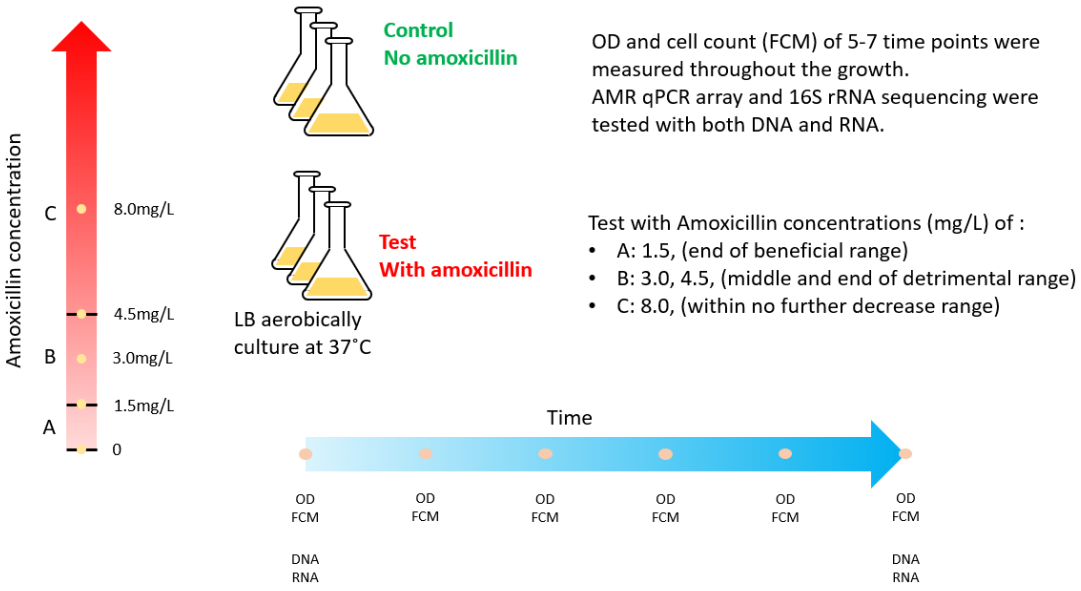

Figure 4-6 Stage 2 experiment design. Four amoxicillin concentrations from beneficial range, detrimental range and no further decrease range were tested with the same microbial community used in preliminary experiments, where samples were cultured in flasks with no amoxicillin controls. Cell counts and OD were measured at least five time points for all the microbial communities containing amoxicillin, whilst samples at the start and end were collected for DNA and RNA analysis in the next chapter.

Table 4-1 Sampling time of each amoxicillin concentration.

	Sampling time (h)								
Amoxicillin concentration	T0	T1	T2	T3	T4	T5	T6	T7	T8
0 mg/L	0	1.5	3.0	4.5	6.0	7.5			
1.5 mg/L	0	1.5	3.0	4.5	6.0	7.5	9.0	10.5	
3.0 mg/L	0	3.0	6.0	9.0	12.0	15.0			
4.5 mg/L	0	3.0	6.0	-	-	15.0	18.0	21.0	
8.0 mg/L	0	6.0	12.0	-	24.0	-	36.0	-	48.0

4.2.3.1 OD and cell count via FCM

OD was measured with a Hach portable spectrophotometer (DR 2800TM) at 600mm. TCC and ICC was performed using the method validated in 4.2.2.1 to 4.2.2.3, where all samples were fixed with 1% (v/v) glutaraldehyde and diluted x1000 times at the point of sampling, stored in dark at 4°C and measured within 24 hours and further diluted with PBS. R package 'Growthcurver' (Sprouffske and

Wagner, 2016) was used to generate growth curves and calculate the growth rates.

4.3 Results

4.3.1 Preliminary experiment

4.3.1.1 Mixed community growth rate under a range of amoxicillin concentrations

The ‘seed’ community was prepared and used in the test to measure growth rate. The change of OD overtime of the mixed microbial communities was captured and recorded by plate reader then analysed. The change of OD reflected the growth of mixed microbial community, where lag phase and exponential growth were clearly separated. Figure 4-7 showed an example of the change of OD over 24h with amoxicillin concentrations of 0, 0.5, 2.0, 8.0 and 32mg/L. The growth curves of other amoxicillin concentrations are shown in Figure 7-1 to Figure 7-5 of the appendix. The exponential growth appeared first in the samples without amoxicillin, whilst the exponential growth delayed with increasing amoxicillin concentrations. Meanwhile, the slope of the growth curve is flatter with higher amoxicillin concentration, which indicates a decline in growth rate as the growth rate is the slope of growth curve.

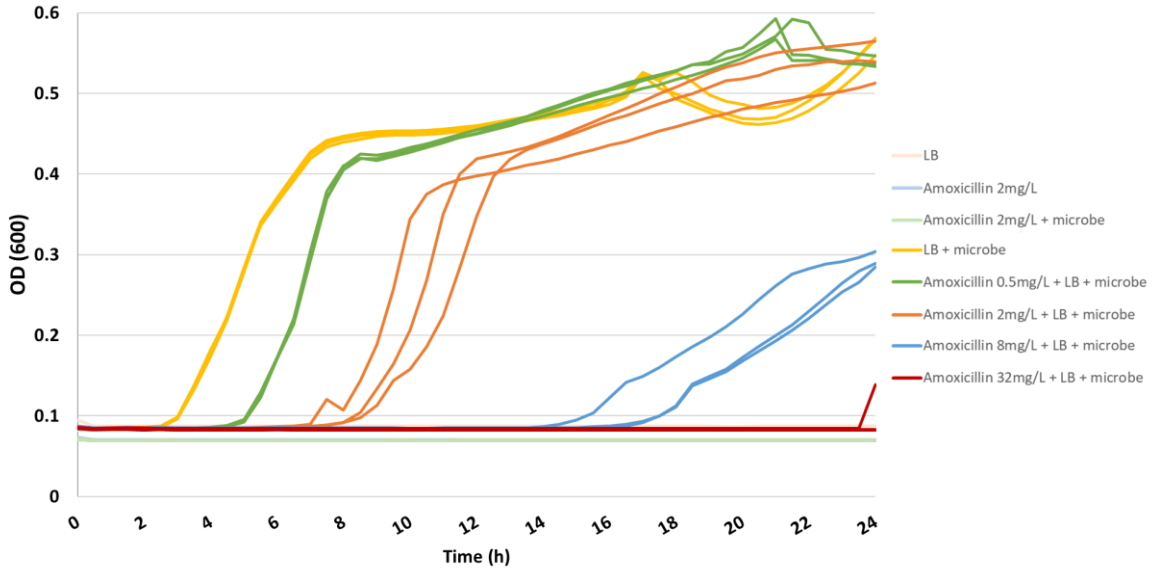

Figure 4-7 Community growth curve measured by OD via plate reader at amoxicillin concentration of 0, 0.5, 2.0, 8.0 and 32mg/L for 24h. Negative controls of culture media (LB), 2mg/L amoxicillin, microbe with 2mg/L amoxicillin were included. Each line represents the change of OD value from single well, all conditions were run triplicated.

Summary of the growth rate and the length of lag phase under all amoxicillin concentrations were calculated and shown in Figure 4-8 and Figure 4-9. The growth rate of this mixed community increased with increasing amoxicillin concentration at first, and then decreased with increasing amoxicillin concentration and no further decrease when amoxicillin concentration was higher than 4.5mg/L. Hence, those concentration ranges can be divided into beneficial range (0-1.5mg/L), detrimental range (1.5-4.5mg/L) and no further decrease range (greater than 4.5mg/L). However, there was no similar trend found in the length of lag phase, where it increased with increasing amoxicillin concentration.

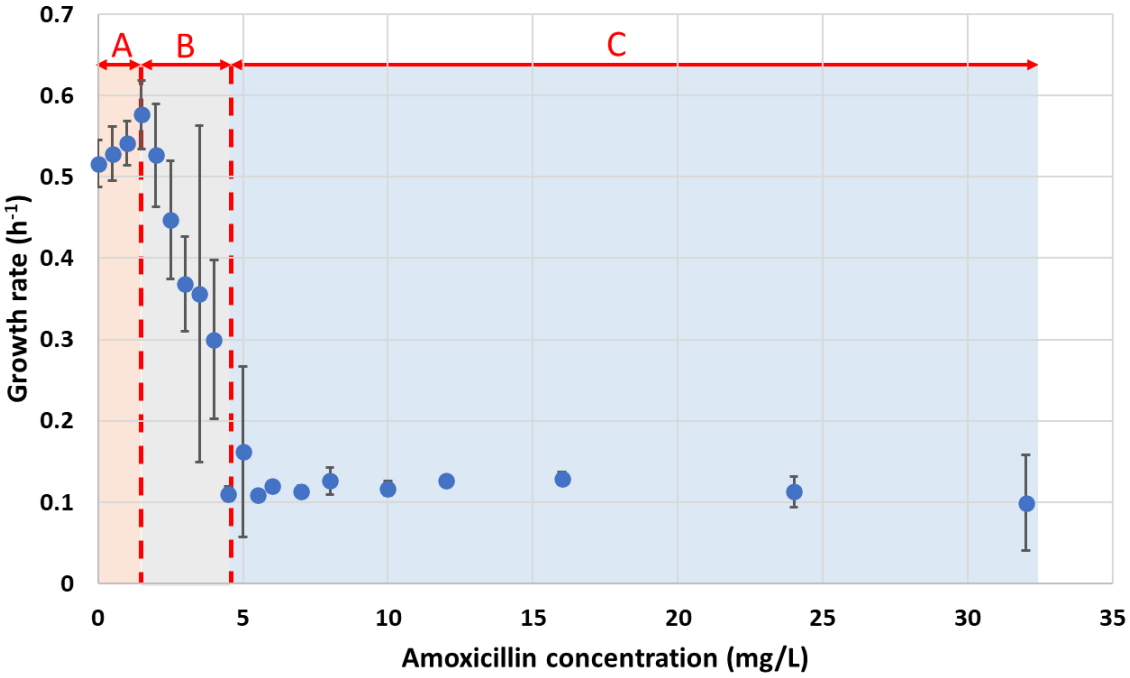

Figure 4-8 Calculated community growth rate measured by OD via plate reader at amoxicillin concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 10.0, 12.0, 16.0, 24.0 and 32.0 mg/L. Error bars represented the standard deviation of at least 3 measurements. A: beneficial range, B: detrimental range, C: no further decrease.

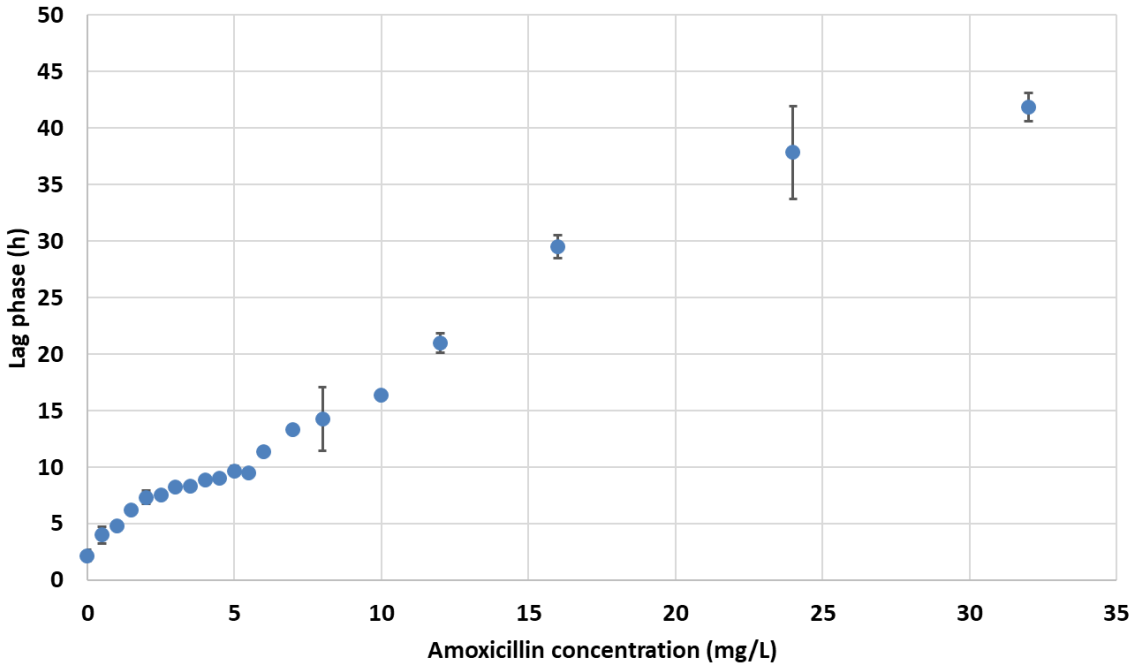

Figure 4-9 The length of community lag phase measured by OD via plate reader at amoxicillin concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 10.0, 12.0, 16.0, 24.0 and 32.0 mg/L. Error bars represent the standard deviation of at least 3 measurements.

It is not known if the mixed community would behave the same as pure isolates, and if the amoxicillin affected all members of the community in the same manner. It is also unknown how reliable the growth rate of a mixed community generated from OD is, since there may be different bacteria present in the community. Therefore, further study with all ranges of amoxicillin concentrations as well as characterisation of the mixed community are needed.

4.3.2 Method validation

4.3.2.1 Diluent and storage time determination with fixed sample

Since it is unknown how the growth of mixed community measured by OD reflects cell numbers, cell counts were conducted by FCM. However, it takes more effort to prepare and measure samples by FCM, and the preparation method may have a direct impact on the cell count results. Therefore, sample preparation methods were tested in two steps to find the best way with minimum disruption of the cell count. The first step of the method validation is to find the best diluent and storage time for fixed sample.

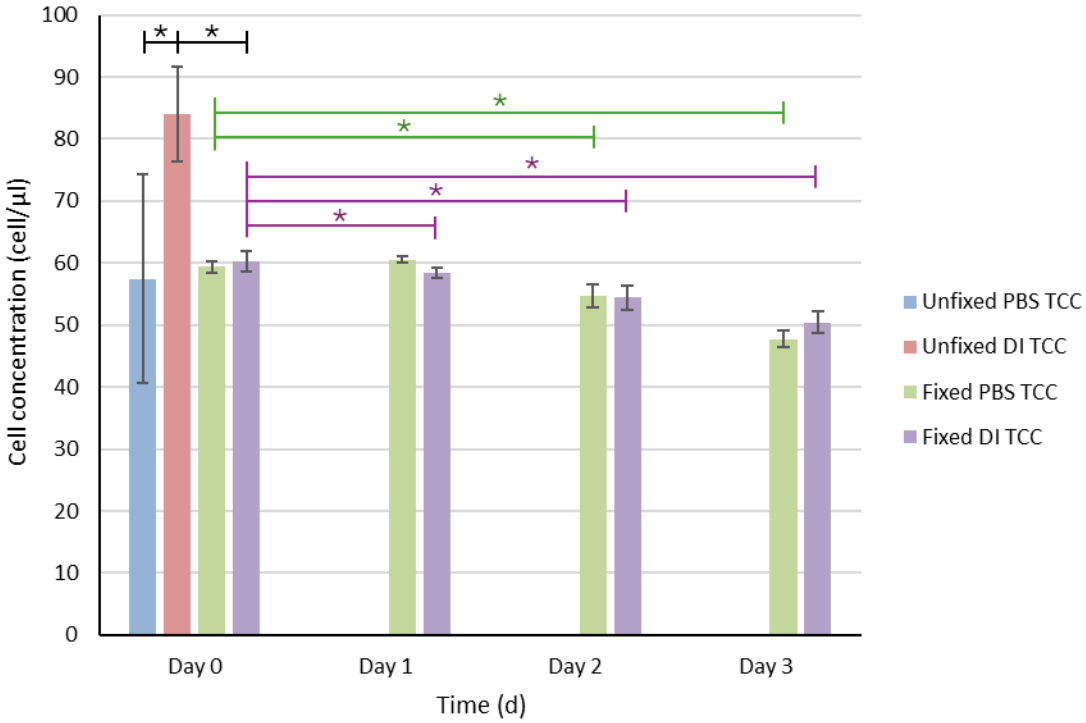

Figure 4-10 Total cell count (TCC) with fixed and unfixed sample diluted with PBS or DI water at the time of measuring. The unfixed samples were only measured on Day 0, and the fixed samples were measured every 24h from Day 0 to Day 3. Error bars stand for standard deviation from three biological replicates each measured triplicated. * Indicates statistically significant difference (P<0.05) in T-test.

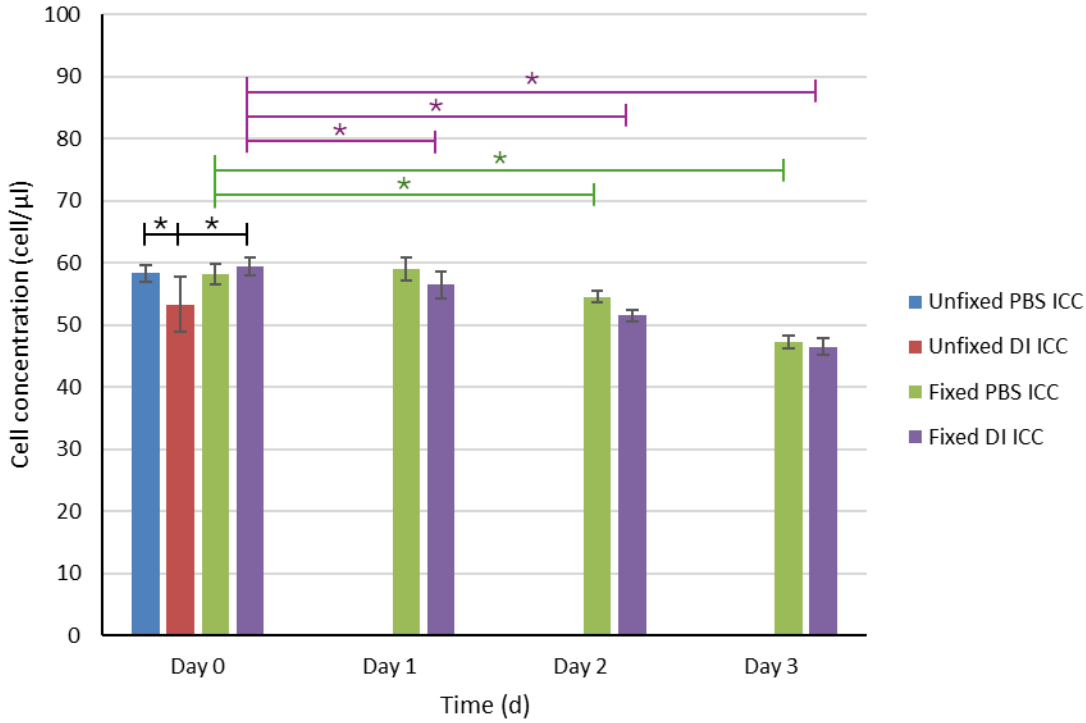

Figure 4-11 Intact cell count (ICC) with fixed and unfixed sample diluted with PBS or DI water at the time of measuring. The unfixed samples were only measured on Day 0, and the fixed samples were measured every 24h from Day 0 to Day 3. Error bars stand for standard deviation from three biological replicates each measured triplicated. * Indicates statistically significant difference (P<0.05) in T-test.

TCC and ICC were measured with samples diluted with PBS or DI water at the point of measuring, Figure 4-10 and Figure 4-11 were the results of TCC and ICC. The significant drop in cell concentration of all samples started from Day 2. On Day 1, there was no statistical difference found between TCC (P=0.077) and ICC (P=0.324) when fixed sample were diluted with PBS, while sample diluted with DI water returned statistically different in both TCC (P= 0.017) and ICC (P= 5.33 x 10^{-3}). There was no statistical difference between diluents on Day 0 in both TCC (P=0.294) and ICC (P=0.104) if samples were fixed with 1% (v/v) glutaraldehyde. On Day 0, no statistical differences between fixed and unfixed sample when PBS was used to dilute samples (ICC, P=0.772; TCC, P=0.803). However, there was a huge variation in TCC of unfixed samples, where the sample diluted with DI water was at least 39.5% higher than all the other samples on the same day, it was unknown if this contributed to the difference between fixed and unfixed sample in TCC when diluted with DI. Also, the measurement of LB as negative control returned much higher background noise than all the other negative controls, it is unknown if higher percentage of LB in unfixed sample contributed to the variation in TCC.

In summary, the results above recommend **that samples fixed with 1% (v/v) glutaraldehyde and further diluted with PBS and should be measured within 24h** to ensure the same result as the fresh sample.

4.3.2.2 Fixation and dilution factor determination

The last experiment made a conclusion of which diluent should be used at the point of measuring samples by FCM, however, samples were diluted x1000 at the time of storage, which is a large volume (50ml) and this also means potential risk of exposure to large amounts of glutaraldehyde especially when a large number of samples are generated in a short time. So, here it was tested if it was possible to reduce the total volume of samples stored and if it was necessary to fix the sample before storing?

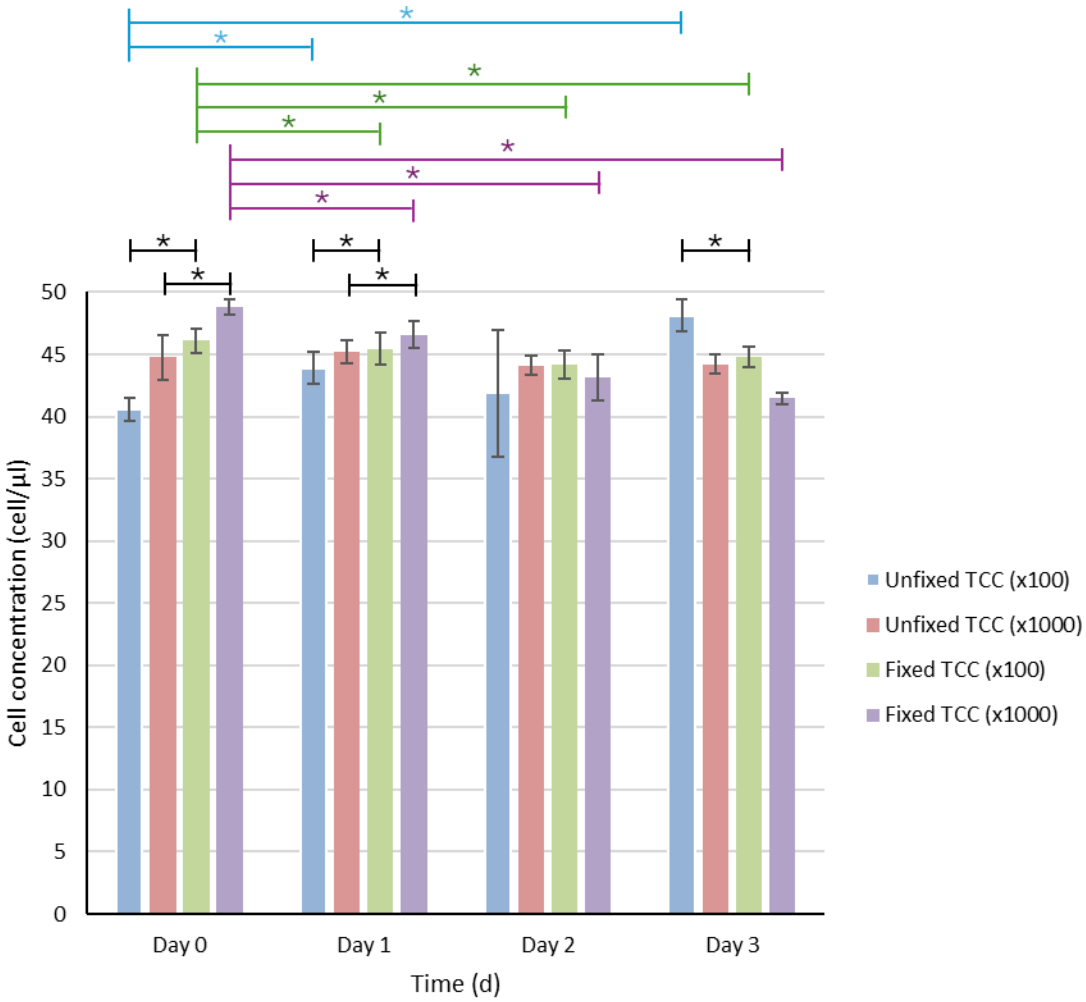

Figure 4-12 Total cell count (TCC) with fixed and unfixed samples diluted x100 or x1000 initially and stored up to 3 days. All samples were measured every 24h apart. Error bars stand for standard deviation from three biological replicates each measured triplicated. * Indicates statistically significant difference (P<0.05) in T-test.

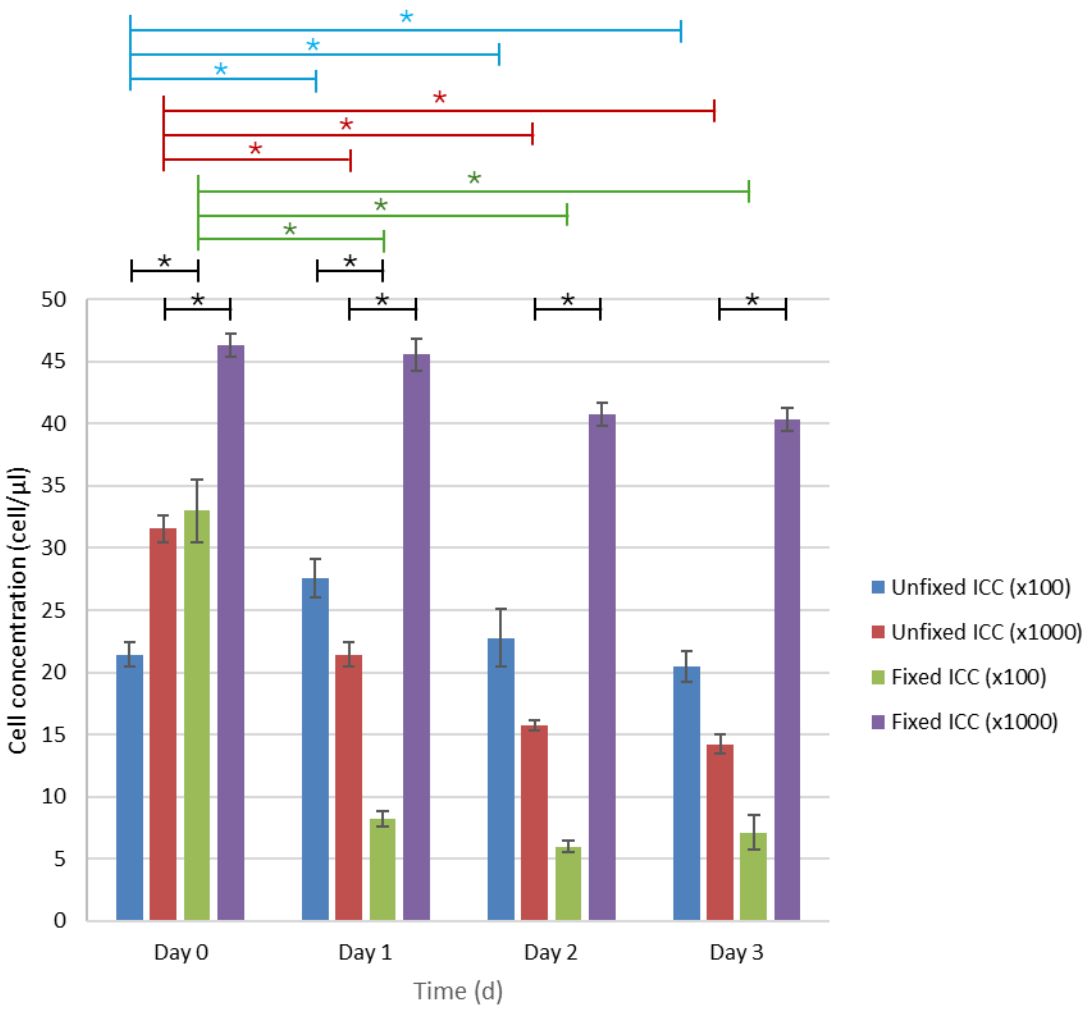

Figure 4-13 Intact cell count (ICC) with fixed and unfixed samples diluted x100 or x1000 initially and stored up to 3 days. All samples were measured every 24h apart. Error bars stand for standard deviation from three biological replicates each measured triplicated. * Indicates statistically significant difference (P<0.05) in T-test.

From the summary of previous experiment, diluting a sample with PBS is less likely to affect the cell count at the time of measuring. Both ICC and TCC were from x100 or x1000 initial dilution and fixed or unfixed sample were measured every 24 hours up to 72 hours (Day 3) as well as initial preparation (Figure 4-12 and Figure 4-13). The TCC results from all the conditions showed much less variation than the ICC, since both TCC and ICC of the same condition were measured from the same sample, this suggests that some changes happened during storage, but this was not captured by TCC. Also, fixed sample with initial dilution of x1000 was the only condition to return similar cell counts in both TCC and ICC on Day 0. A similar cell counts for both TCC and ICC from one initial dilution was expected as this was observed from the result of previous study in Figure 4-10 and Figure 4-11. Meanwhile, the condition of both fixed and unfixed sample with initial dilution x1000 were also measured in previous study, the ICC

of unfixed sample with x1000 initial dilution in this study was much lower than the previous one. Also, there was an interesting finding that TCC of unfixed x100 diluted sample returned a higher value in the following days, a similar result was found in ICC too, those suggest that under this condition the microbes were growing.

The statistical results suggest that unfixed samples with x1000 initial dilution returned no difference in TCC for three days following compared to Day 0 (Day 1, P=0.545; Day 2, P=0.349; Day 3, P=0.438). Although the TCC of fixed sample with x1000 initial dilution was different from Day 1 ($P= 1.11 \times 10^{-4}$), the absolute value dropped less than 5% (from 48.78 (Day 0) to 46.56 (Day 1) cell per µl). In terms of ICC, only fixed sample with initial dilution of x1000 on Day 1 returned no difference to Day 0 (P=0.181). This suggests that ICC measurements are only stable for up to 24 hours after fixation.

The purpose of this method validation experiment was to determine a way to store sample for the next step experiment with particular interest in ICC. For this, dilution of the sample x1000 and fixation with an equal volume of 1% glutaraldehyde is recommended and stored in dark at 4°C would likely to return the same cell count as fresh sample up to 24 hours after fixation.

4.3.3 Secondary experiment

4.3.3.1 Comparison of mixed microbial community growth rate measured by OD, TCC and ICC via FCM

OD and total and intact cell count via FCM were measured with samples from several time points throughout the growth of the mixed microbial community under selected amoxicillin concentrations. Figure 4-14 is the result of Stage 1 experiment where microbial community growth measured with OD, TCC and ICC over time without amoxicillin, results of microbial growth with amoxicillin are shown in Figure 7-6 to Figure 7-8 of appendix. The growth measured with OD was identical to the one in the preliminary experiment (Figure 4-8). Although both OD and TCC are supposed to measure all cells, the growth curve measured through TCC was slightly different from the one measured by OD, where the exponential growth and stationary growth both appeared later in TCC. The growth measured through ICC reached stationary stage earlier than others,

which was expected as ICC only measured the intact cells within the community, but other methods measured both live and dead cells.

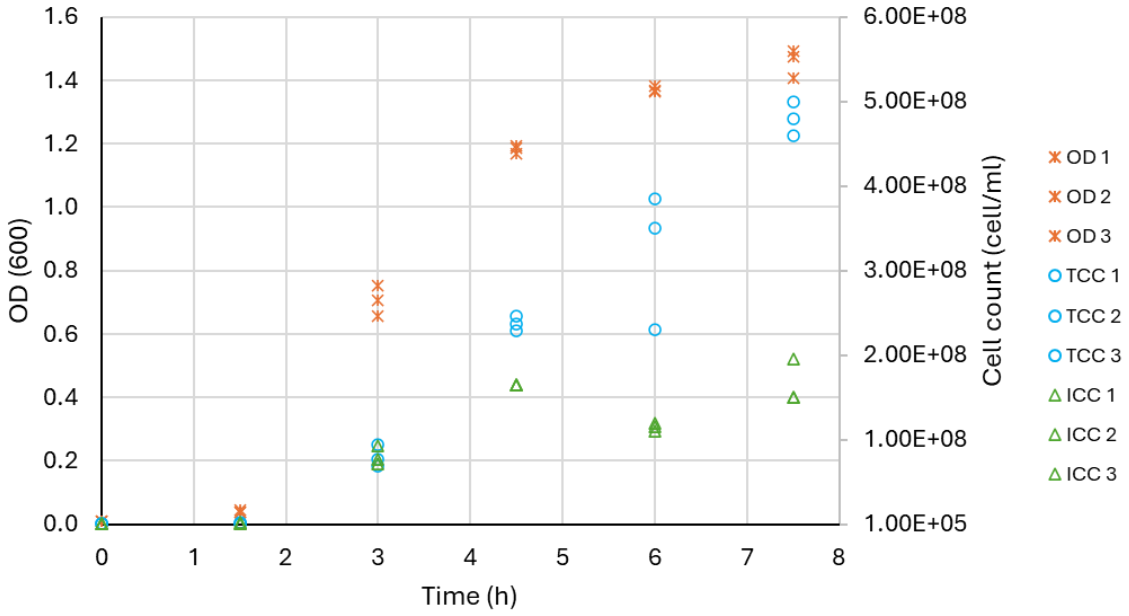

Figure 4-14 Comparison of microbial community growth measured by OD, TCC and ICC. OD, optical density; TCC, total cell count; ICC, intact cell count without amoxicillin. One measurement of OD, TCC and ICC from the same sample at the same time, result represent measurements from three biological samples (1, 2 and 3).

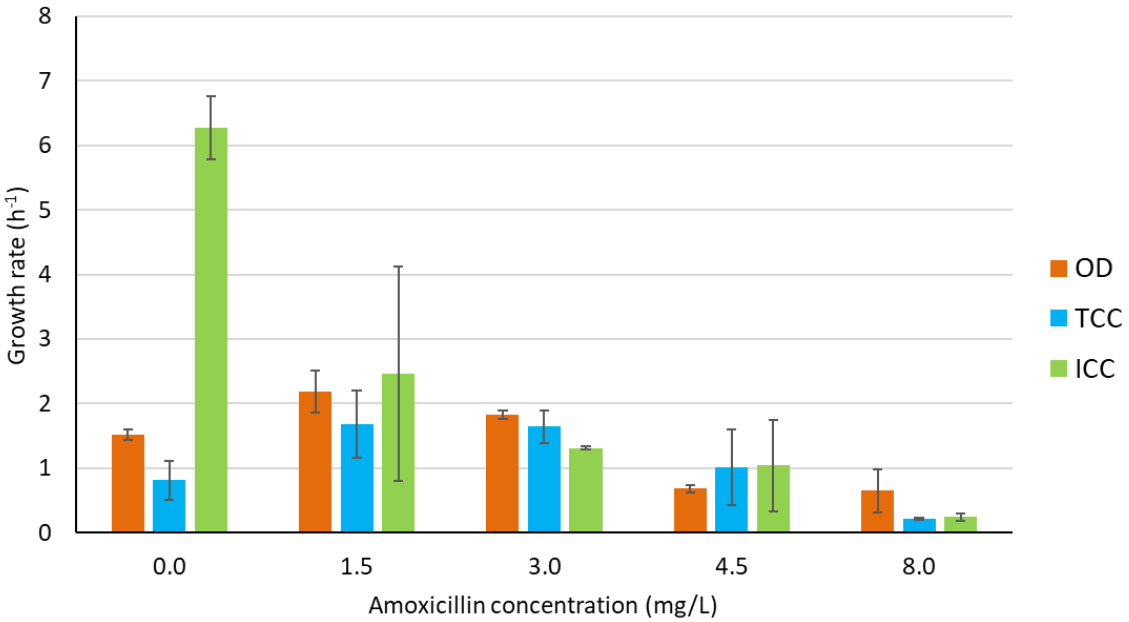

Figure 4-15 Summary of growth rate measured through OD, TCC and ICC with microbial community under amoxicillin concentrations of 0, 1.5, 3.0, 4.5 and 8.0mg/L. Error bars are standard deviation from three biological replicates. OD, optical density; TCC, total cell count; ICC, intact cell count.

Figure 4-15 is the summary of the growth rates of the microbial community in the presence of the selected amoxicillin concentrations through different

measurements. The trend of growth rate measured with OD was the same as the preliminary experiment, where the growth rate peaked at amoxicillin concentration of 1.5mg/L and decreased in higher concentration then no further decrease from 4.5mg/L amoxicillin. The trend of growth rate measured by TCC was not the same as OD, where it peaked at 1.5mg/L amoxicillin and decreased with increasing amoxicillin. The growth rate generated from ICC data was different from all the others, where it decreased with increasing amoxicillin concentration. However, the growth rates of the microbial community with amoxicillin may not reflect the true growth rates due to the limited available time points.

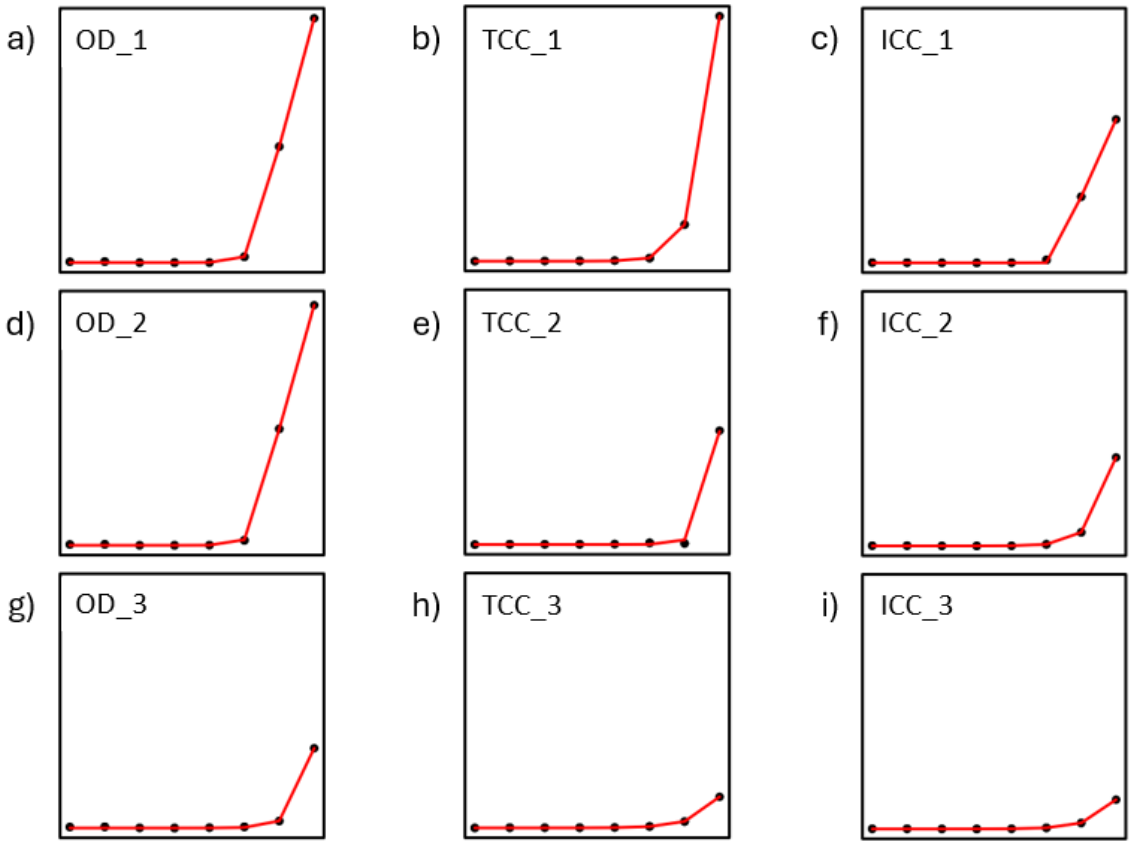

Figure 4-16 Growth curve of microbial communities with 1.5mg/L amoxicillin measured with OD, TCC and ICC. a), b), c); d), e), f); and g), h), i) were measured from replicate 1; 2; and 3, respectively. a), d) and g) are growth curve measured through OD; b), e) and h) are growth curve measured through TCC; c), f) and i) are growth curve measured through ICC. OD, optical density; TCC, total cell count; ICC, intact cell count.

Although all three ways of measuring microbial communities successfully generated growth rates through R package 'Growthcurver', the growth rate may not reflect the actual growth rate of the microbial communities. Figure 4-16 shows an example of growth curve measured with OD, TCC and ICC, where the

variations within the same measurement (OD, TCC or ICC) among replicates was seen. The microbial communities grew much slower in the third replicate compared with the other two biological replicates, and the exponential growth was unlikely to be captured in any of the measurements. Also, the time points and interval were selected based on the preliminary experiment where growth curves were measured through OD, the overestimation of the microbial communities growth by OD lead to finishing measurements too early. Therefore, although the growth of microbial communities was measured, the results may not be extensive enough to reflect the full growth of the microbial community.

4.4 Discussion

4.4.1 Preliminary experiment

The preliminary experiment was able to observe the change of growth rate of a mixed microbial community under a range of amoxicillin concentrations via OD. It is understandable that environmental antimicrobial resistance studies focus on DNA only since most environmental bacteria are not culturable. Whilst clinical antimicrobial resistance studies are mostly culture based as they focus on resistance developed by pathogenic species. Ottosson et al. (2012) tested the ciprofloxacin resistance of *Enterococci* sp. isolates from a hospital wastewater system. Interestingly, the ciprofloxacin resistant and intermediate isolates have a longer lag-phase than the sensitive isolates even without ciprofloxacin, while the generation times are the same for resistant, intermediate and sensitive isolates. In our study the lag-phase increased with amoxicillin concentration, this suggests the increased lag-phase in our study may cause by selecting of amoxicillin resistant bacteria. The growth rate of sensitive isolates decreased significantly with increasing ciprofloxacin concentrations, while the growth rate of intermediate and resistant isolates only decreased slightly with higher ciprofloxacin concentrations in the study conducted by Ottosson et al. (2012). Compared with this study, here we only observed a decrease of growth rate in the detrimental range (see Figure 4-8) concentrations, which suggests that amoxicillin resistance was developing or selecting in the detrimental range amoxicillin concentrations. Combining our results with the study by Ottosson et al. (2012) it shows that the microbial community developed resistance in the detrimental range (see Figure 4-8) of amoxicillin concentrations, but it is still

not enough to explain what has happened in lower and higher range of amoxicillin concentrations.

Single strain antimicrobial resistance study usually uses the MIC to determine the resistant. However, there is no single MIC for a mixed microbial community as one antibiotic does not work for all bacteria and even for the bacteria are sensitive to the antibiotic the MIC is different for every bacterium. It has been found that MSC can be much lower than MIC, where low concentration of antibiotic reduces the growth rate of susceptible strain but does not affect the resistant strain (Gullberg et al., 2011). We had seen an increase in growth rate and the length of lag phase in low amoxicillin concentrations (beneficial range in Figure 4-8), this may cause by the inhibited growth of amoxicillin susceptible strains which was competing with amoxicillin resistant strain when amoxicillin was not presented (Rajer and Sandegren, 2022). Meanwhile, the acquisition of resistance may also be happening at the same time, as low concentration of beta-lactam antibiotic have found to increase the rate of resistance mutations (Sandegren, 2014). Also, the fitness cost of developing antimicrobial resistance increased with antibiotic concentration (Andersson and Hughes, 2010), which may explain the length of lag phase increased with amoxicillin concentration (beneficial range in Figure 4-8) observed in our study. Meanwhile, one of the main amoxicillin (beta-lactam) resistance mechanisms is to break the beta-lactam ring through beta-lactamase, whilst under higher amoxicillin concentration more beta-lactamase is required to break the beta-lactam ring. It is possible that fewer species have the ability to produce enough beta-lactamase and survive, which consequently leads to a relative stable growth rate in higher amoxicillin concentrations (no further decrease range in Figure 4-8) in our study. Further test is required to find out the reason behind the change of growth rate and length of lag phase.

4.4.2 Method validation

Most studies employed flow cytometry only described the method used in their sample preparation, the comparison of sample preparation and storage methods are not commonly reported. Vignola et al. (2018) compared two fixation solutions, 1% glutaraldehyde and a solution of ethanol: PBS 50%, where 1% glutaraldehyde achieved better result in fixing biofilm in sand filter followed by

detachment. In this study, only 1% glutaraldehyde was tested to fix samples pre-diluted with different dilution factors, which resulted to 0.5% glutaraldehyde in the final stored samples. Glutaraldehyde as an additive fixation method which inactivates the cell and forms chemical bonds with protein to protect the cell structure. Our results saw a significant decline in TCC and ICC after fixation (x100) suggest that the volume of 1% glutaraldehyde used in the test was not enough to inactivate all the cells. Abay et al. (2019) tested the range of concentrations that reported in the literature and found out that glutaraldehyde concentration lower than 0.001% brings significant higher percentage of lysed red blood cells than higher glutaraldehyde concentration (>0.01%) or no glutaraldehyde. Although our concentration of 0.5% is much higher than 0.01%, the significant declined in ICC in later days suggests that cell lyse happened during storage and increase glutaraldehyde percentage or dilute cells are required. However, the exact amount of glutaraldehyde needed for a given number of cells has never been discussed, indeed fixation always happens before cell counting, therefore a better way to determine this is to test with actual samples.

There are some other parameters affect sample storage and test as well. Kamiya et al. (2007) suggested that fixation does not change the cell counts in seawater samples, but storage is a significant factor in total cell counts, particularly in the first three days of storage. In our study, significant drop in cell counts happened from Day 2 of storage. This difference may be because of differences in the physiology and taxonomy of cells in each sample (Kamiya et al., 2007). In addition to the characteristics of samples, Safford and Bischel (2019) also reported instrumental difference, where same sample measured through four different FCM returned different results. Although the difference in result may be minimised by adjusting the gating, it is unknown if the method reported in the literature will work for different sample without testing. Therefore, it is necessary to test sample with proposed fixation and storage method to find the best fit.

4.4.3 Secondary experiment

The community growth measured in three methods returned different results as expected. Although the OD value and TCC both measure the cells in the whole

community, TCC measures stained DNA from both intact and damaged cells, while OD measures everything in the liquid that changes light absorbance, where cell debris also count as cell by OD. In this study, OD values appeared in stationary earlier than TCC, which means OD reached maximum measurable absorbance earlier TCC and this may partially cause by overcounting debris as cells. Ogundero et al. (2022) also notice that OD value overestimates the cell counts at higher cell concentrations, it only shows a linear correlation with cell concentrations at lower concentrations. ICC only counts cell with intact membrane, which reflects the cell growth more accurately than the other two methods, but the presence of dead cell with intact membrane will still overestimate the cell number (Safford and Bischel, 2019). Although automatic measurement of both ICC and OD are available, ICC may be under estimated when cell concentration is too high where multiple cells may be counted as one cell due to aggregation (Safford and Bischel, 2019).

The growth rate measured by OD was slightly different from the preliminary experiment result, although they show the same trend with the change of amoxicillin concentrations. The difference of growth rate value may be caused by different measurement methods for OD, such as the cuvette and spectrophotometer (Sutton, 2011). Whilst a large-scale study conducted by Beal et al. (2020) compared *E. coli* cell counted by the same selected methods across over a hundred laboratories internationally and stated that OD is a robust method in estimating cell count. Nevertheless, the measurement of OD in the preliminary experiment is more frequent than this study, which largely affects the accuracy of the growth rate. It is hard to find a precise measuring interval for environmental sample since the doubling time and the length of exponential growth are unknown. A more frequent measurement provides better review of microbial growth. It was hoped that OD could reflect bacterial growth in an optimised range of time in which it could be used as a method to monitor the change in long-term, however, out data was not enough to make the conclusion that OD is enough to reflect the community growth rate.

Community growth rate measurement is challenge especially for environmental samples. Measuring substrate utilisation to reflect bacterial activity is more common, where essential resources for DNA and protein synthesis have been

measured by Fernández-Calviño and Bååth (2010) and carbon source utilisation via Biolog plate has been reported by Stefanowicz (2006). Domańska et al. (2019) used OD to measure bacterial contamination in water and wastewater, although a growth curve was reported, the detection of bacteria growth is more important than growth rate in this study. Another way for community growth rate measurements to be taken is to measure the growth rate of pure culture and predict the growth rate of the community using computational approaches (Ram et al., 2019). Despite methods varied, lab culture introduces the bias by the selection of growth media and monitoring time, it is likely that only fast-growing bacteria capable of growth in the selected media would be selected.

4.5 Conclusion

This chapter set out to establish methods to rapidly monitor mixed microbial community growth rate in the presence of amoxicillin using OD and to establish robust methods to validate the growth rate with cell counts via FCM.

The method of sample dilution, fixation and storage for the measurement of intact and total cell count via FCM was validated, which suggests that sample pretreatment of diluting fresh sample x1000 with LB and adding equal amount of 1% glutaraldehyde then stored in dark at 4℃ for up to 24 hours is less likely to change the cell count results than if the sample is diluted with PBS at the time of measuring.

The change of growth rate of mixed microbial communities under the impact of amoxicillin was captured by the change of OD. Microbial communities' growth of selected amoxicillin concentrations was measured with OD, TCC and ICC simultaneously, however, the results are not enough to conclude that OD is sufficient to reflect the 'true' growth of microbial communities. Further work is needed to determine if the change in growth rates within mixed microbial communities is caused by a shift of microbial communities or the development of antimicrobial resistance genes responding to the presence of amoxicillin or indeed a combination of these two factors.

5 Effects of increasing exposure to amoxicillin on microbial community growth rates, community composition and AMR genes and expression.

5.1 Introduction

Antibiotics work by inhibiting the growth of bacteria using a variety of different mechanisms. Each antibiotic class shares the same target and action mechanisms and therefore has similar impacts on susceptible bacteria (Peach et al., 2013). Amoxicillin belongs to beta-lactam antibiotic class, which disrupts cell wall synthesis by targeting the penicillin-binding proteins. In other words, the growth of bacteria without penicillin-binding proteins will not be affected by amoxicillin or other beta lactam antibiotics. The microbial community composition is expected to shift after antibiotic exposure as the abundance of antibiotic susceptible bacteria is likely to reduce. The shift of gut microbial community composition after antibiotic treatment has been reviewed by Fishbein et al. (2023), where the change of microbial community composition varied when different antibiotics were used.

WWTPs are considered 'hot spots' for AMR. As they are not designed to remove antibiotic or AMR, both antibiotics and ARGs are found in the effluent of WWTP. Antibiotics concentrations is reported to be lower in the effluent than influent. For example, 79%-88% total antibiotics have been reported to be removed by Sabri et al. (2020b) in three full-scale WWTPs in the Netherlands, while 42.2% of tetracycline and 83% of sulfonamide removal in a full-scale WWTP was reported by Gao et al. (2012). Furthermore, ARG diversity in the effluent was lower than the influent in full-scale WWTPs (Yang et al., 2014, Ju et al., 2019), whilst the relative abundance of ARGs in the effluent was seen to increase by Pärnänen et al. (2019) and Ju et al. (2019) even though the biomass was more reduced in effluent than influent. While the microbial community composition of full-scale WWTPs has been reported from all over the world, they largely provide just a snapshot of the microbial communities since most of the studies only include one sample point (Sun et al., 2023, Ju et al., 2019) or analyse the pooled sample from different time points (Yang et al., 2014). Although the microbial communities in full-scale WWTPs are relatively stable, it is unknown how the presence of antibiotics act on the establishment of microbial communities.

Small-scale WWTPs have a much lower capacity compared with full-scale WWTPs, where the influent is generally more variable than full-scale WWTPs due to their small capacity. Antibiotics and ARGs have been studied in small-scale WWTPs, but the changes of microbial communities after antibiotic exposure are not commonly investigated. In a series of mesocosm-scale CWs treating domestic wastewater with various substrates, hydraulic loads, flow configuration and plant species used was studied by Chen et al. (2016a). They reported lower antibiotic concentrations and abundance of ARGs in the effluent compared to influent. Similar results of reduced antibiotic concentrations and abundance of ARGs in the effluent were observed by Gentile et al. (2024). However, if and how these affected changes in the microbial communities within these systems was not studied. The shift of microbial communities after expose to sulfadiazine and trimethoprim for 60 days has been studied by Kruglova et al. (2019) in lab-scale bioreactors treating synthetic wastewater, showing lower nitrifier activities after antibiotic exposure, but changes in the underpinning ARGs was not measured. It has been reported that *E. coli* resistance development is correlated with the relative concentration of antibiotics in wastewater (Sutradhar et al., 2023). But it is unknown how the development of overall resistance in the environment after antibiotic exposure and if the change of microbial community composition is happening at the same time of AMR development.

Our batch experiment mimicked the change of small-scale WWTP where wastewater flows from anaerobic to aerobic condition with exposure to antibiotics. The change of growth rate has been observed, but it is unknown how the microbial community responded to amoxicillin, and what ARGs underpinned these changes during their growth. Furthermore, as it was a short batch experiment, the length of the experiment may not have been enough to capture microbial activity via DNA and growth, whereas RNA provides a better view of instantaneous changes in gene expression. Therefore, DNA alongside mRNA were both considered for the study on the effects of a mixed microbial community exposure to increasing concentrations of antibiotics, coupled with a high-throughput qPCR array to monitor changes in AMR genes and transcripts.

5.1.1 Hypothesis and aim

The change of microbial community growth rate after amoxicillin exposure was observed in Chapter 4, we hypothesise that the change of microbial community growth rate is associated with the shift of microbial community composition and development of antimicrobial resistance.

The aim of this study is to determine mixed microbial community response to an amoxicillin concentration gradient.

5.1.2 Objectives

- To analyse the change of microbial community composition under different amoxicillin concentrations.

- To test if there are changes in ARG expression within the mixed microbial community across the different amoxicillin concentrations via HT-qPCR.

5.2 Materials and methods

DNA or RNA used in this chapter were collected from the secondary experiment in 4.2.3, Table 5-1 details samples numbers used in this chapter.

Table 5-1 Summary of sample numbers collected from the experiment described in 4.2.3 and used in this chapter. T0 was sampled from overnight culture, which was subsequently reinoculated for 'T end control' and 'T end amoxicillin' to ensure all those Tends were biological replicates. T end (control) was sampled 7.5 hours after reinoculation, T end (amoxicillin) was sampled from 10.5 to 48 hours after reinoculation. (see Table 4-1 for detail)

Sample numbers	T0		T end (Control)		T end (Amoxicillin)	
Amoxicillin concentration (mg/L)	DNA	cDNA	DNA	cDNA	DNA	cDNA
0	1	1	3	3		
1.5	1	1	3	3	3	3
3.0	1	1	3	3	3	3
4.5	1	1	3	3	3	3
8.0	1	1	3	3	3	3

5.2.1 *16S rRNA* annealing temperature optimisation

DNA was extracted from 2ml of overnight culture with FastDNA™ SPIN Kit for Soil (MP Biomedicals, 116560200) and was used in this optimisation. PCR reaction condition and primers (515F (barcoded) & 926R) were detailed in 2.2.4, where only annealing temperature was tested with the DNA here. The annealing temperature of 56℃, 58℃, 60℃, 62℃, 64℃ and 66℃ were applied in the test. Figure 5-1 shown the gel image of the annealing temperature optimisation, where the 62℃ was the highest temperature with the brightest bands. Therefore, 62℃ was the annealing temperature used for all PCR reactions with DNA or cDNA samples from this experiment.

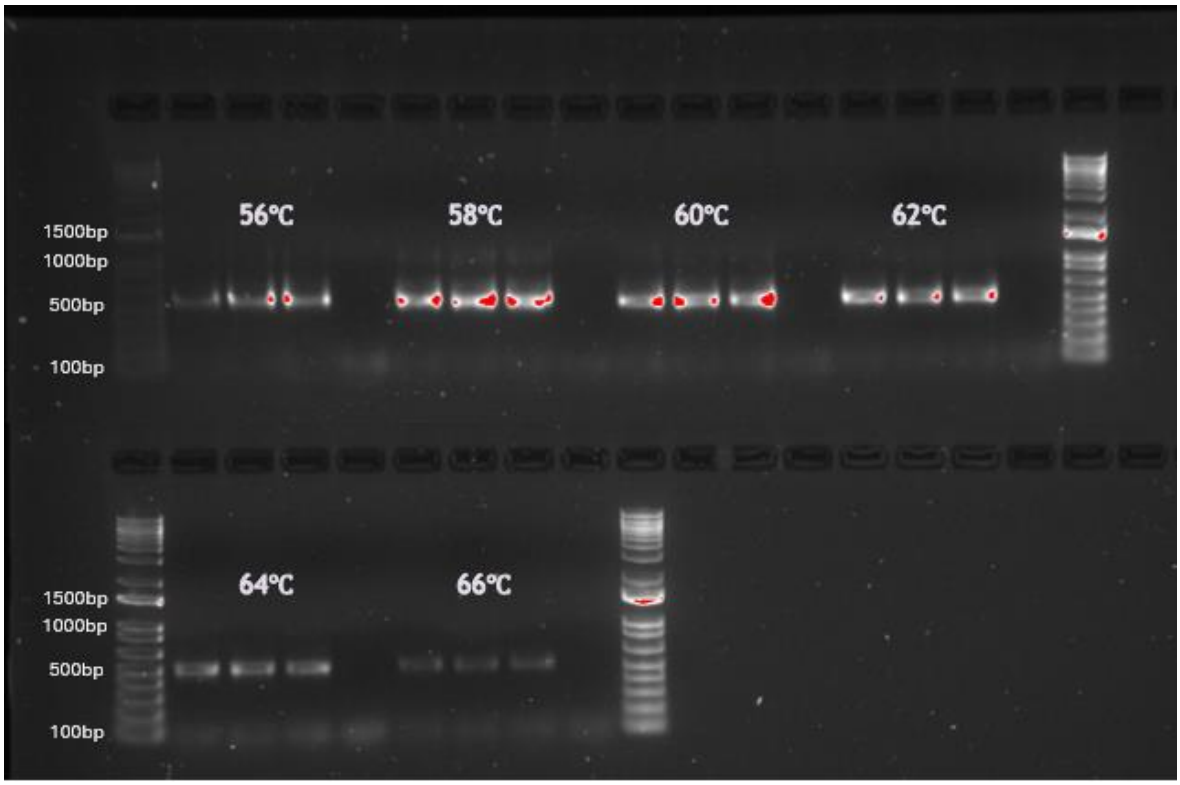

Figure 5-1 Gel image of *16S rRNA* PCR annealing temperature optimisation with temperature ranged from 56℃ to 66℃. The PCR products of 15µl each was mixed with 1.5µl loading dye and loaded onto the gel against the 1kb plus ladder respectively. 62℃ appeared to be the brightest one with the highest annealing temperature. (515F (barcoded) & 926R were used in the test, see detail at 2.2.4)

5.2.2 DNA & RNA co-extraction and quality check

4ml of liquid sample from the beginning of the experiment (overnight culture) and the end of the experiment in the secondary experiment in chapter 4 was kept for use in this chapter. The 4ml from each flask was spun down at x13000g 4℃ for 5 mins, the supernatant was removed and pellet snap frozen at -80℃

until extraction. DNA and RNA were co-extracted with RNeasy PowerMicrobiome Kit (Qiagen, 26000-50) following the manufacture's manual. 80% of each sample was used for RNA extraction and eluted with 70µl of DNase/RNase-Free water and 20% of each sample was used for DNA extraction and eluted with 50µl of DNase/RNase-Free water. Both DNA and RNA were then quantified with Qubit BR Assay for DNA (Invitrogen™ Q33266) and RNA (Invitrogen™ Q10210) respectively and visualised on 1% agarose gel. DNA was then stored at -80℃ until further use. The workflow of DNA & RNA co-extraction and quality check are showed in Figure 5-2.

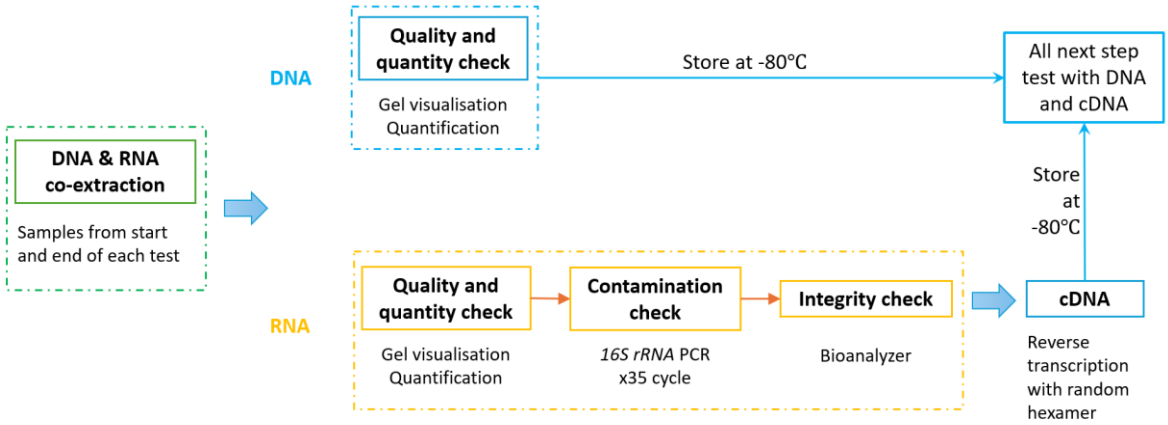

Figure 5-2 DNA & RNA extraction and quality check processes.

The RNA quality check started with a *16S rRNA* PCR to check for contaminating DNA, primers and conditions were detailed in 2.2.4, except that it was run with 35 cycles and with the annealing temperature of 62℃ optimised in 5.2.1. PCR products were visualised on 1% (w/v) agarose gel (in TAE buffer) to check if there was DNA presence. Figure 5-3 shows an example of DNA free RNA samples, where no band was found with RNA samples or NTC but very bright bands of positive controls. The quality of DNA free RNA samples was checked with RNA 6000 Nano kit on 2100 Bioanalyzer platform (Agilent Technologies) followed the manufacture's manual. After all the quality check, the cDNA was generated by reverse transcription with random hexamers via Invitrogen™ SuperScript™ IV (Invitrogen™, 18091050). 4µl of RNA template mixed with 1µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix and 7µl of DNase/RNase-Free water and incubated at 65℃ for 5 mins then cooled on ice for at least 1 min. Then, 4µl of 5x SSIV Buffer, 1µl of 100 mM DTT, 1µl of Ribonuclease inhibitor and 1µl of reverse transcriptase were added into the previous mix. The mixed then

incubated at 23℃ for 10mins and 55℃ for 10mins, followed by 80℃ for 10mins. The cDNA then quantified with Qubit DNA HS kit and stored at -80℃ until further use.

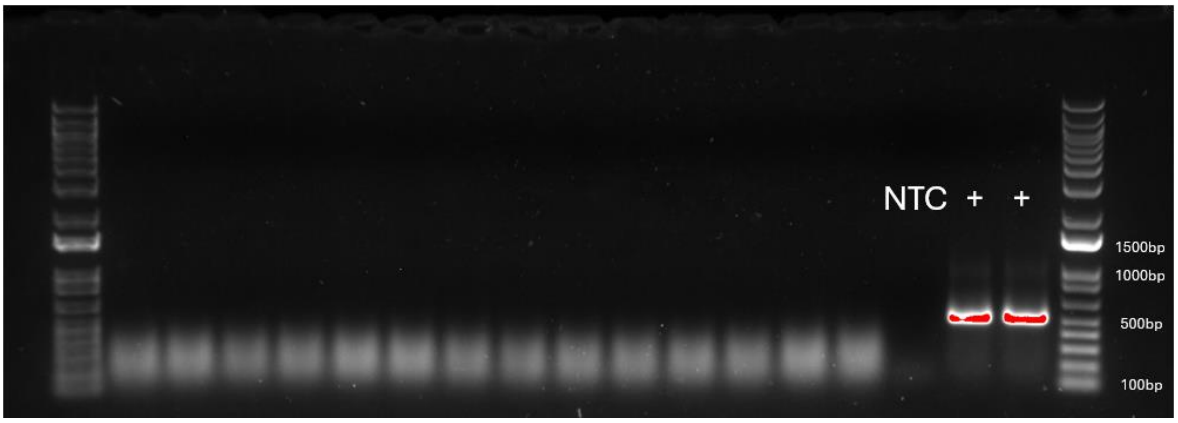

Figure 5-3 Gel image of 35 cycles of *16S rRNA* with RNA, negative control and positive control against 1kb plus ladder. Two positive controls showed very bright bands, while no band was found in negative control or RNA samples.

5.2.3 *16S rRNA* sequencing and statistics

To identify the microbial community present and how they changed over the experimental conditions, *16S rRNA* amplicon sequencing was undertaken using one step PCR with barcoded primers (515F, 926R) descripted in 2.2.4. The PCR conditions were the same as 2.2.4, only the annealing temperature was optimised with DNA sample extracted from this experiment. In addition, *16S rRNA* gene expression was also analysed by sequencing of amplified cDNA.

All samples of DNA and cDNA were used for library preparation. The sequencing library was prepared with the same way detailed in Chapter 2 and sequenced by the same platform by Earlham Institute (Norwich, UK). The sequencing data was processed the same as described in 2.2.5, except a read length of 280bp was used. 41 ASVs generated from sequencing data were manually searched against NCBI (Sayers et al., 2022) database. Analysis and visualisation of alpha and beta diversity, taxa were described in 2.2.6.

5.2.4 High-throughput quantitative polymerase chain reaction (HT-qPCR) for antimicrobial resistance gene (ARG) and mobile genetic element (MGE) screening and testing

Antimicrobial resistance gene (ARG) and mobile genetic element (MGE) were detected via a High-throughput quantitative polymerase chain reaction (HT-

qPCR) using a SmartChip Real-time PCR system (TaKaRa Bio) by Resistomap (Helsinki, Finland). Briefly, each SmartChip has 5184 reaction wells with a volume of 100nl. Each reaction well included 1x SmartChip TB Green Gene Expression Master Mix (TaKaRa Bio), nuclease-free PCR-grade water, 300nM of each primer and a DNA template of 2mg/µl (Majlander et al., 2021). Real-time PCR reaction conditions were 10 mins at 95℃ followed by 40 cycles of 30 sec at 95℃ and 30 sec at 60℃ and a final melting curve analysis performed automatically by TaKaRa Bio software (Wang et al., 2014). The 5184 reaction wells on the SmartChip allows up to 1728 genes to be amplified against one sample in triplicate simultaneously in one run. The primer sets used in this test were selected from ARG qPCR array 2.1 provided by Resistomap (Helsinki, Finland).

The tests were conducted in two rounds, where the first round screened the gene targets of ARG and MGE with pooled samples and the second round tested samples with the selected gene targets based on the results of the first round's test. The first round tested 216 primer sets against 6 pooled samples and 2 non-template controls (NTCs) with one SmartChip, the second round tested 60 samples against 54 primer sets in two SmartChip with 2 NTCs on each SmartChip. The 216 gene targets in the first test were selected from 384 gene targets in ARG 2.1 provided by Resistomap. Details of the primer sets used for the gene targets are listed in Table 7-15 and Table 7-16 of appendix, summary of gene targets are shown in Table 5-3. The whole available set of ARG from antibiotic resistance group of beta lactam, multidrug resistance (MDR) and tetracycline, in addition to integrons and mobile generic element (MGE) were selected for the array. In addition to a selection of ARGs that included genes for Aminoglycoside, macrolides, lincosamides and streptogramin B (MLSB), phenicol, sulfonamide, trimethoprim, vancomycin and quinolone resistance. Pooled samples used in the first round's tests were listed in Table 5-2, where the samples were diluted with DNase/RNase-Free water to around 10ng/µl measured by Nanodrop 1000 spectrophotometer (Thermo Scientific) as requested and pooled equimolarly with a final volume of more than 100µl. Samples used in the second round's test were listed in Table 5-1 with the exception of 2 'T-end' control DNA and 2 'T-end' control cDNA samples from 0 amoxicillin. Samples were diluted to 10ng/µl in a volume of at least 100µl as described in first round's samples. All the

samples (include NTCs) were amplified in triplicated against each primer set. The results received from Risistomap were raw data in excel and the analysis included R and manual check with Microsoft 365.

Table 5-2 Pooled sample (T1-T6) for the first round's AMR array test to identify gene targets.

Sample numbers	T0		T end (Control)		T end (Amoxicillin)	
Amoxicillin concentration (mg/L)	DNA	cDNA	DNA	cDNA	DNA	cDNA
0	1	1	3	3	-	-
1.5	1	1	3	3	3	3
3.0	1	1	3	3	3	3
4.5	1	1	3	3	3	3
8.0	1	1	3	3	3	3
Pooled sample	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**

Table 5-3 Gene targets for the high throughput qPCR test. *16S rRNA*, mobile genetic elements of integrons and MGE and all the classes of AMR.

	Gene target (antibiotic class)	Number of genes tested	
		1st test	2nd test
1	*16S rRNA*	1	1
2	Beta lactam	54	11
3	Integrons	4	0
4	MDR*	39	10
5	MGE**	48	11
6	Tetracycline	26	8
7	Aminoglycoside	5	0
8	MLSB***	19	5
9	Phenicol	3	1
10	Sulfonamide	5	3
11	Trimethoprim	5	2
12	Vancomycin	5	2
13	Quinolone	2	0
	Total	216	54

***MDR, multidrug resistance; **MGE, mobile generic element; ***MLSB, macrolide, lincosamide, streptogramin B resistance.**

5.3 Results

5.3.1 Effect of increasing amoxicillin exposure on alpha diversity of microbial communities

The alpha diversity of *16S rRNA* sequencing results was first analysed examining species richness and evenness. The microbial community compositions were compared between the beginning of the experiment (T0), the end of the experiment without amoxicillin (T-end control) and with amoxicillin (T-end amoxicillin), as well as between DNA and cDNA.

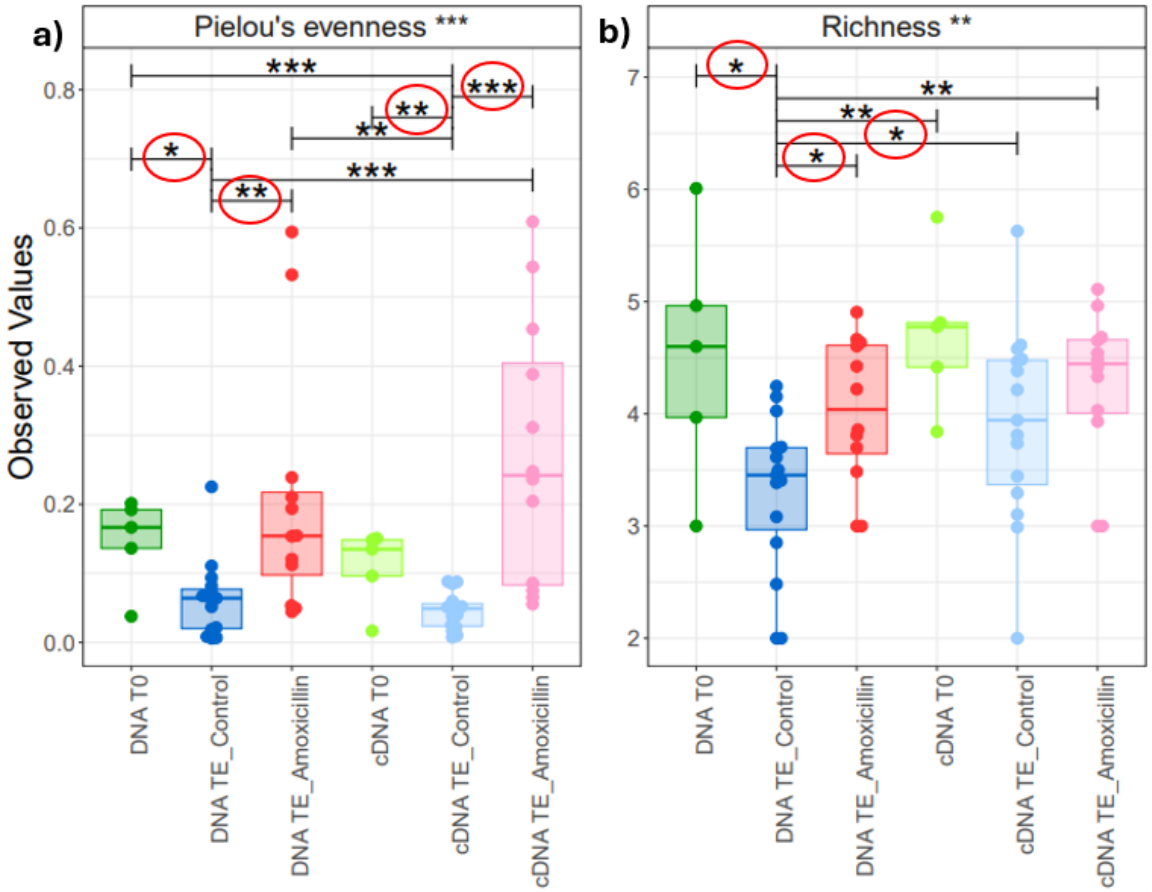

Figure 5-4 Alpha diversity of microbial communities analysed through a) Pielou's evenness and b) species richness. n=64. Asterisk represents P value analysed by ANOVA, where "*" p≤0.001, "**" 0.001 < p ≤ 0.01, "*" 0.01< p ≤ 0.05.**

The overall species richness was very low and ranged from two to six per sample (Figure 5-4 b)) given only 41 ASVs were identified, therefore the values of species richness were expected to be low. The change of species richness in the DNA showed the same trend as it in RNA (cDNA). The microbial communities in T0 had the highest species richness (3.00 to 6.01 ASVs), followed by T-end amoxicillin (3.00 to 5.11 ASVs). The communities from T-end control had the

lowest species richness, while species richness increased with increasing amoxicillin in T-end amoxicillin (see Table 5-4). However, the significant differences were only observed in DNA, where they were found in microbial communities T0 and the T-end control (ANOVA, $P<0.05$), and also the between T-end control and T-end amoxicillin ones (ANOVA, $P<0.05$). Meanwhile, the species richness in the microbial communities of the RNA from experiment-end control was significantly higher than their DNA microbial communities (ANOVA, $P<0.05$).

Table 5-4 Species richness in the microbial community of T0, T-end control and T-end amoxicillin.

	T0	T-end control	T-end amoxicillin			
			1.5 mg/L	3.0 mg/L	4.5 mg/L	8.0 mg/L
DNA	4.51 ±1.00 SD	3.31 ±0.68 SD	3.39 ±0.29 SD	4.17 ±0.23 SD	3.90 ±0.78 SD	4.63 ±0.02 SD
cDNA	4.72 ±0.62 SD	3.91 ±0.84 SD	4.55 ±0.16 SD	4.53 ±0.48 SD	3.84 ±0.62 SD	4.13 ±0.83 SD

The evenness of microbial communities ranged from almost 0 to above 0.6, indicating they are dominated by a single to a small number of organisms, supporting the species richness observations. The highest values were found in the microbial communities of both DNA and RNA at T-end amoxicillin, where the evenness increased with increased amoxicillin concentration in DNA and RNA except 4.5mg/L amoxicillin (see Table 5-5). The trend of evenness was the same in microbial communities of both DNA and RNA, where the microbial communities of T-end control was the lowest in terms of the first quartile and median, followed by T0 (ANOVA, $P<0.05$, DNA; $P<0.01$, RNA), then T-end amoxicillin. The evenness of microbial communities at T-end amoxicillin was significantly higher than T-end control (ANOVA, $P<0.01$, DNA; $P<0.001$, RNA).

Table 5-5 Pielou's evenness in the microbial community of T0, T-end control and T-end amoxicillin.

	T0	T-end control	T-end amoxicillin			
			1.5 mg/L	3.0 mg/L	4.5 mg/L	8.0 mg/L
DNA	0.15 ±0.06 SD	0.06 ±0.05 SD	0.11 ±0.05 SD	0.07 ±0.03 SD	0.43 ±0.19 SD	0.21 ±0.02 SD
cDNA	0.11 ±0.05 SD	0.05 ±0.03 SD	0.11 ±0.07 SD	0.13 ±0.08 SD	0.48 ±0.09 SD	0.36 ±0.13 SD

It is interesting to see that the microbial communities from T0 had higher species richness and evenness than T-end control. Since the microbial communities at T-end control was reinoculated from T0 and cultured in the same medium and at the same temperature, they were expected to be similar. However, the microbial communities at T0 were cultured for 16 hours, where T-end control were only cultured for 7.5 hours. Those time difference may contribute to the differences in microbial communities. Also, microbial communities in T0 had a high species richness while low evenness, this suggests that the microbial communities in T0 were dominated by fewer species. In contrast, the microbial communities of T-end amoxicillin had a medium species richness but high evenness, which suggests that the species in those microbial communities were more equally distributed and the presence of amoxicillin may play a role in maintaining diversity.

5.3.2 Bray-Curtis beta diversity

The diversity within each sample was compared to each other using a Bray-Curtis beta diversity of microbial community composition analysed microbial communities grouped as T0, T-end control, T-end amoxicillin as well as DNA and RNA.

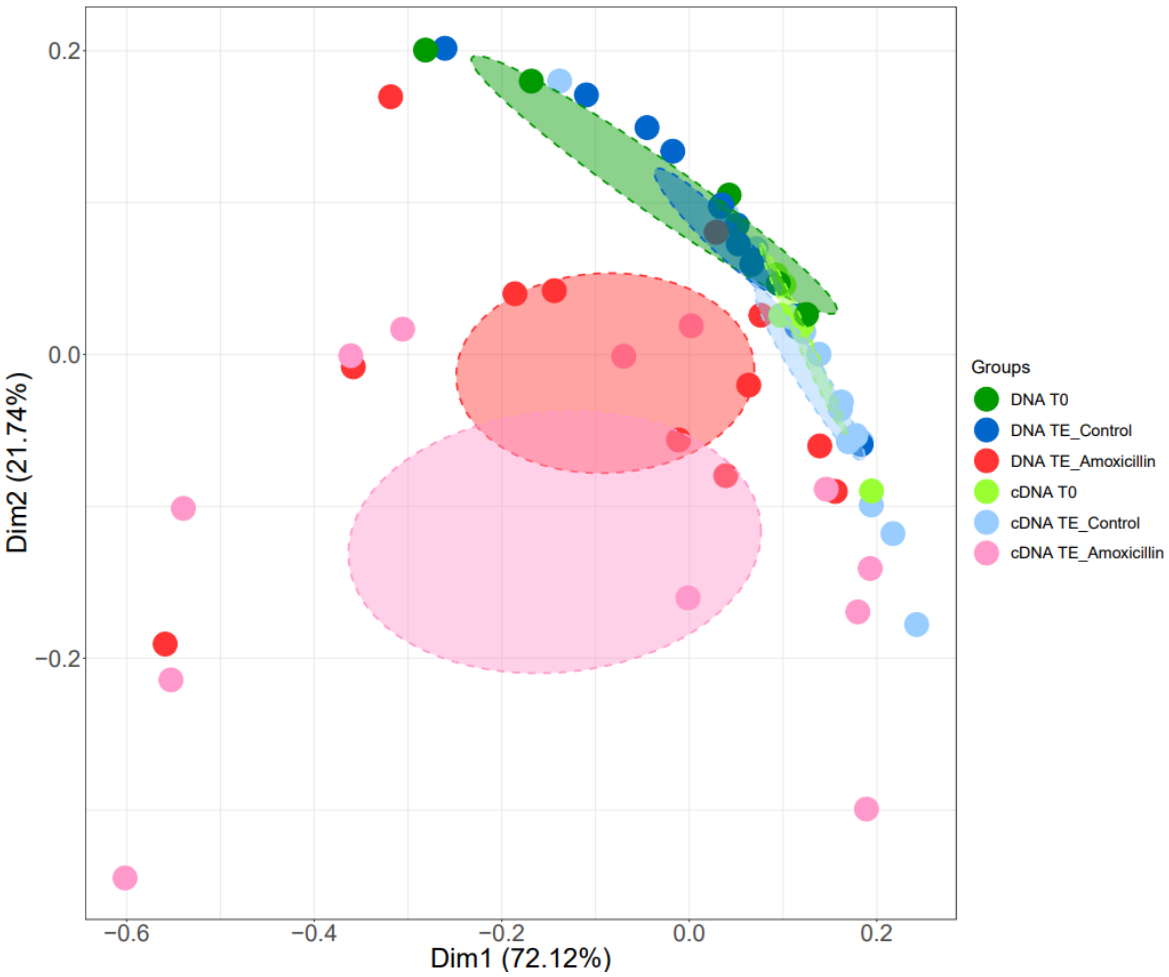

Figure 5-5 Bray-Curtis Beta diversity. n=64, ellipses stand for standard errors.

The microbial communities of T0, T-end control and T-end amoxicillin were different from each other (PERMANOVA, P=0.001), despite DNA microbial communities of T0 and T-end control overlapped in Figure 5-5 and Table 7-17. Microbial communities of T-end amoxicillin were scattered and separated from T0 and T-end control, where T0 and T-end control were much closer to each other in both DNA and RNA. The microbial communities of DNA were different from RNA (PERMANOVA, P=0.012), but the DNA microbial communities were not very different from their corresponding RNA microbial communities. The microbial communities of T-end amoxicillin were also analysed against amoxicillin concentrations, where the community compositions were different under different amoxicillin concentrations (PERMANOVA, P=0.004). This suggests that the presence of amoxicillin contributes to the change of microbial community composition, meanwhile, the T-end amoxicillin groups had much longer culturing time than the no amoxicillin one - this may also contribute to the change of microbial community composition. Since the culture time of T-end

amoxicillin was much longer than any of the no amoxicillin once, so it is not enough to conclude that amoxicillin is the only cause of increased diversity in that group.

5.3.3 Taxa in the microbial communities

The Bray-Curtis beta diversity analysis has shown that the microbial community composition of amoxicillin presented group was different from the ones without amoxicillin. Next, the taxa of each sample were examined (see Figure 5-6). The diversity of microbial communities was expected to be low as indicated in the alpha diversity. Low diversity in the community composition was expected as the anaerobic microbes were cultured aerobically in LB. Likely culture conditions resulted in a small portion of the microbes, capable of aerobic and/or facultative metabolisms, to be selected.

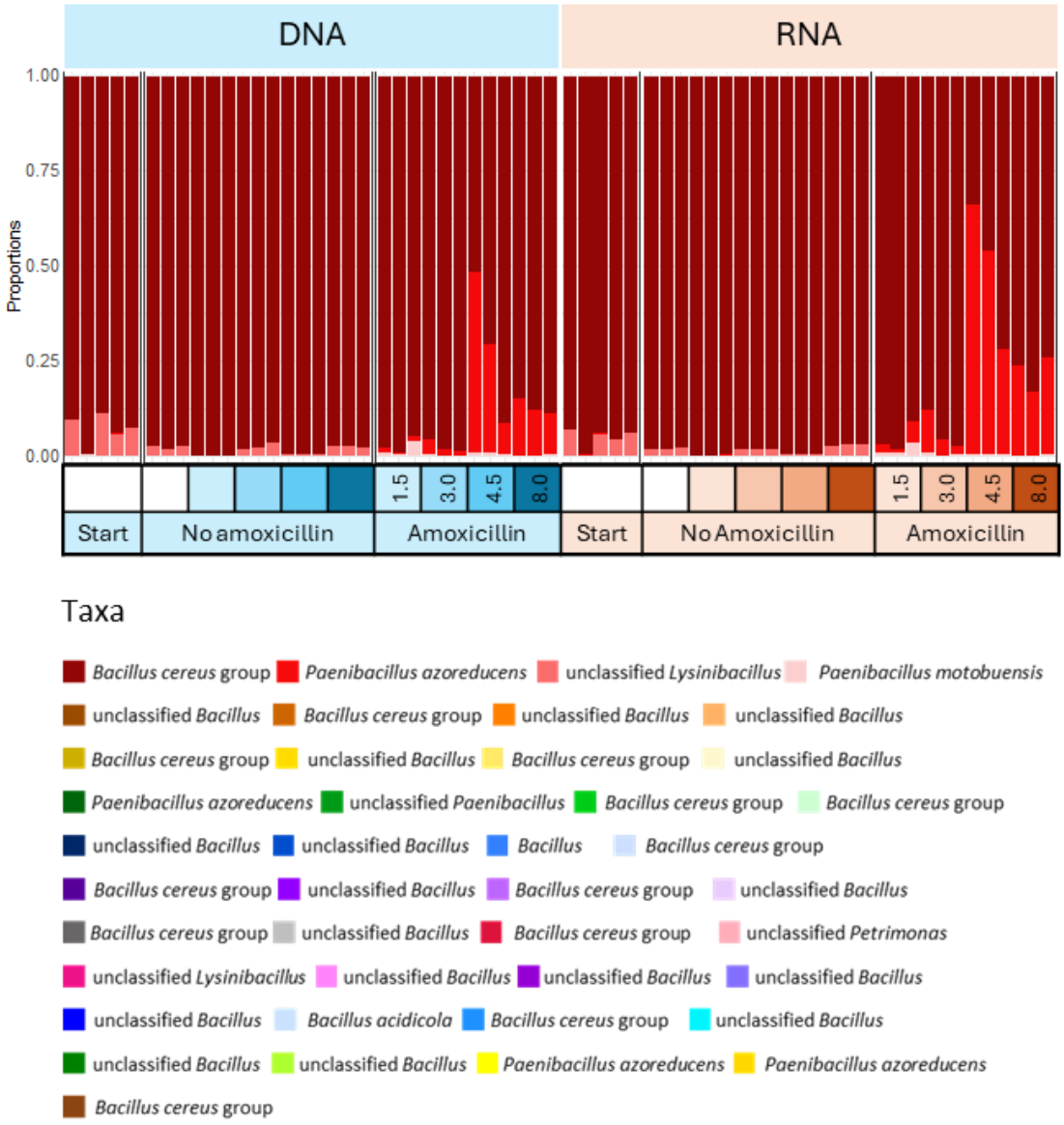

Figure 5-6 Taxa bars at ASV level of both DNA and RNA in microbial communities. n=64.

Taxa were dominated by *Bacillus cereus* group, *Paenibacillus azoreducens*, unclassified *Lysinibacillus* and *Paenibacillus motobuensis*. As all the dominated ASVs are from *Bacillales* capable of facultative anaerobic respiration and endospore formations. The community composition at the start and end of no amoxicillin controls were dominated by *Bacillus cereus* group and unclassified *Lysinibacillus*, whilst they were also the most active species at the time of sampling revealed by 16S rRNA transcripts. Differences in the microbial community from DNA and RNA shown in the beta diversity plot (Figure 5-5), suggests that low abundance species are more transcriptionally active.

The culturing time may also play a role in the shift of dominant ASVs, where the culturing time of each amoxicillin concentration was selected based on the preliminary experiment. It is known that all the communities reached stationary

stage at the time of sampling (cell count 1.3-5.30 x10^8 cell/mL measured via FCM in 4.2.3, data not show), but how long they have stayed at stationary stage is unknown as the length of stationary stage can last for hours but cell count remains almost the same. Also, as indicated in the preliminary experiment result (Figure 4-9), the length of lag phase increased with increased amoxicillin concentration, which makes it difficult to predict the exactly time when stationary stage was reached. In this study, the only similar culturing time was the starting microbial communities (seeded from overnight culture, 16h) and communities with 3.0 mg/L of amoxicillin (15h). The abundance of unclassified *Lysinibacillus* decreased, while *Paenibacillus azoreducens*, *Paenibacillus motobuensis* and unclassified *Bacillus* increased in both DNA and RNA under amoxicillin 3.0mg/L, which suggests that the shift of microbial composition is associated with the presence of amoxicillin.

Meanwhile, the addition of amoxicillin impacted the community, and the communities exposed to the highest concentration of amoxicillin showed more difference. Specifically, there was an increase (up to 264-fold) in the relative abundance of *Paenibacillus azoreducens* in both DNA and RNA in the community exposed to 4.5mg/L of amoxicillin. Furthermore, there was a higher relative abundance of *Paenibacillus azoreducens* in RNA (1.24-65.72%) than DNA (0.47-47.59%) at T-end communities. The highest relative abundance of *Paenibacillus azoreducens* was higher in RNA of 4.5mg/L amoxicillin (65.72%) followed by RNA in 8.0mg/L amoxicillin (25.37%) a slight (up to 11.18%) increase was also seen in the communities with 1.5 and 3.0mg/L amoxicillin. The abundance of unclassified *Lysinibacillus* was not seen in the communities exposed to amoxicillin, but it was found in the communities without amoxicillin (up to 11.11%).

5.3.4 Quality control and primer selection of first round's HT-qPCR results

The SmartChip HT-qPCR was used to evaluate ARG and ARG expression in our experiment. In the first round we were selecting gene targets available on the appropriate for our samples. Further we had to develop an approach to analyse the data from the chip to ensure we has a rigorous data set from which to derive meaning from our experiments.

The 1st round of ARM array tested 216 AMR genes with six pooled samples and two negative controls (NTCs) of water on one chip. Results returned the cycle threshold (Ct) value, primer melting temperature (Tm), efficiency and note (Flag) of the melting curve analysis of all the assays. Flag includes 'low efficiency', 'high efficiency', 'multiple melt peaks', 'Ct is large', 'Ct is small', 'curve fit failed', 'no amplification', 'high baseline', 'saturation baseline ratio is low' and 'bad R^2' (see HT-qPCR results). No individual melt curve or any other information was provided. The 1st round's test was set to screen the best AMR primer set with the potential to capture the change of AMR in different conditions, so the analysis of the results focused on the detectability and quality of primer sets.

First stage of analysis was to retain gene targets that had at least 1 log fold (3.32 Ct) difference between the pooled sample and non-template control (NTC) (Smith et al., 2006), ensuring real amplification of the gene target above the negative control in our samples. Firstly, assays with no amplification in any of the three technical replicates were removed. Secondly, amplification found in only one of the three technical replicates was considered as unreliable amplification, and these samples were also removed. Whilst for NTCs, any amplification in one of the replicates was kept and considered as true amplification. Thirdly, for the remaining assays average Ct value of the samples was compared to the average Ct value of the NTCs. All the samples were compared with technical replicates from one of the NTCs at a time, where 38 primer sets were removed by NTC 1 and 38 primer sets were removed by NTC 2. Lastly, the removed primer sets in the last steps were cross checked. 32 of the removed primer sets were shared between both of the NTCs, 12 primer sets were only removed by one of the NTCs. The analysis in this stage was to find if those gene targets were presented in the samples or not, so the 12 primer sets only removed by one of the NTCs were kept and the shared 32 primer sets were removed.

The second stage of the analysis focused on the quality of the primer sets by looking into the melting curve analysis results provided in the 'Flag' section of the raw data. We paid extra attention to 'multiple melt peaks' in the 'Flag' section as it may indicate non-specific amplification. Any primer sets with more

than 20% of all the tests had 'multiple melt peaks' in the 'Flag' section were excluded, therefore a further 130 primer sets were removed in this step.

After those two stages' analysis, 54 ARG primer sets were kept for the second round's test. The summary of the class of the resistance genes were listed in Table 5-3, details of each primer set were listed in Table 7-16 of appendix. As differences was observed in the NTCs, which brings question if this is an occasional or constant issue. Therefore, in the second round's test, two NTCs were included in each SmartChip.

5.3.5 Quality control of second round's HT-qPCR results

The 2nd round's results received as the same format of integration for real amplification as the 1st round (see HT-qPCR results) (see analysis steps in Figure 5-7). The analysis of the results started by comparing the average Ct value between samples and NTCs, again, any results of less than 3.32 Ct difference were removed. The comparison of the Ct value difference was conducted with one of the NTC at a time, and the remaining assays were cross checked between these two different normalisations. There were 5 primer sets only appeared when normalising with one of the NTCs, those primer sets were removed as this round's test was set to quantify the change of the gene targets.

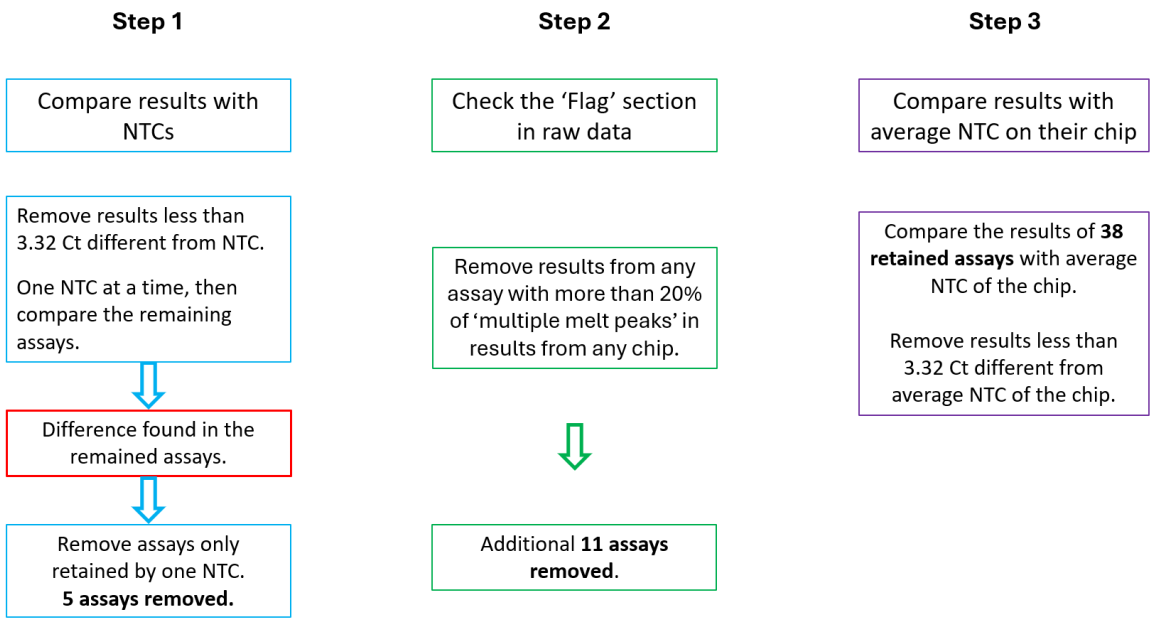

Figure 5-7 Steps of analysis second round's HT-qPCR results.

As the results of normalising with different NTCs were different again, which brings doubt to the reliability of this qPCR array. In order to check the quality of the result, we then looked into all the Ct values of the *16S rRNA* first (Table 5-6), as it would be used to normalise the change of all the other genes. All the samples had a relative consistent Ct value in all three SmartChip (both 1st and 2nd tests), but the average Ct value of NTCs were much lower in the 1st round's (25.97) test compared with the 2nd round (28.32 and 28.25). Although the Ct values of NTCs in the second test were close, they were very close (1.73Ct with *yidY mdtL*; 0.38 Ct with *bla L1*; 2.32Ct with *ampC/blaDHA*) to most of the positive samples in the rest of tested primer sets. Next, we checked the 'multiple melt peaks' in the 'Flag' section of all the results (Table 5-7). In the 1st round's test, only 12.5% of the test had a 'multiple melt peaks' in the 'Flag', hence the *16S rRNA* gene was kept for the 2nd round's test. However, in the 2nd round's test, the result from one SmartChip reported 32.29% 'multiple melt peaks' and the other one reported 15.63%.

Table 5-6 Summary of *16S rRNA* Ct values in two round's qPCR array test.

16S rRNA	1st round	2nd round	
		SmartChip 1	SmartChip 2
Average Ct value of samples	12.64	12.67	12.45
Average Ct value of NTCs	25.97	28.32	28.25

Table 5-7 Summary of the numbers and percentage of 'multiple melt peaks' of *16S rRNA* in the 'Flag' of the qPCR array raw data.

16S rRNA	1st round	2nd round	
		SmartChip 1	SmartChip 2
Number of tests	24	96	96
Number of 'multiple melt peaks'	3	31	15
Percentage of 'multiple melt peaks'	12.50%	32.29%	15.63%

In our data analysis, we deleted primer sets with high percentage of 'multiple melt peaks' from the automatic melt curve analysis. It was supposed to have been low in the second round, however, the high percentage of 'multiple melt peaks' in *16S rRNA* was very different from the first round's test. High percentage of 'multiple melt peaks' indicates the potential of nonspecific amplification, if the gene copy from *16S rRNA* is not reliable, normalising all the

other gene copies with *16S rRNA* gene will not provide useful information in the abundance of each gene. Therefore, we decided to **only keep gene expression data by comparing Ct values of the samples with their NTCs.**

Since huge differences in the percentage of 'multiple melt peaks' appeared in two rounds' test of *16S rRNA* drew our attention to check if this also happened in other gene targets. 11 out of 49 remaining gene targets were found to have at least 20% of 'multiple melt peaks' in at least one of the SmartChip's result (Table 5-8). All assays with those 11 gene targets were removed from further analysis. As a result of those analysis, only 38 out of 54 gene targeting AMR and MGE were retained for next step analysis.

Table 5-8 Summary of gene targets with higher than 20% of 'multiple melt peaks' in melting curve analysis of the second round's test.

	Gene target	Antibiotic class	Percentage of 'multiple melt peaks'		
			SmartChip 1	SmartChip 2	Average
1	*16S rRNA*		32.29%	15.63%	23.96%
2	*blaCARB*	Beta Lactam	38.54%	35.42%	36.98%
3	*blaKPC*	Beta Lactam	44.79%	38.54%	41.67%
4	*blaSHV11*	Beta Lactam	38.54%	48.96%	43.75%
5	*acrB 1*	MDR	30.21%	17.71%	23.96%
6	*acrR 1*	MDR	42.71%	25.00%	33.85%
7	*IncI1 repI1*	MGE	31.25%	26.04%	28.65%
8	*IS613*	MGE	23.96%	23.96%	23.96%
9	*erm35*	MLSB	27.08%	41.67%	34.38%
10	*yidY mdtL*	Phenicol	23.96%	26.04%	25.00%
11	*sul4*	Sulfonamide	27.08%	31.25%	29.17%

5.3.6 Comparison of the ARG and MGE gene expression between treatments

Since the shift of microbial community composition associated with amoxicillin concentration was observed, we next asked if the expression of ARG and MGE genes changes under different amoxicillin concentrations. Total ARGs and MGEs detected in DNA and mRNA of each sample are shown in Figure 5-8. More ARGs were detected in DNA than expressed in the RNA from all samples. On average

across all samples there were 23.34 (± 5.01 SD) ARG and MGE presented, but only 7.13 (± 2.62 SD) were expressed.

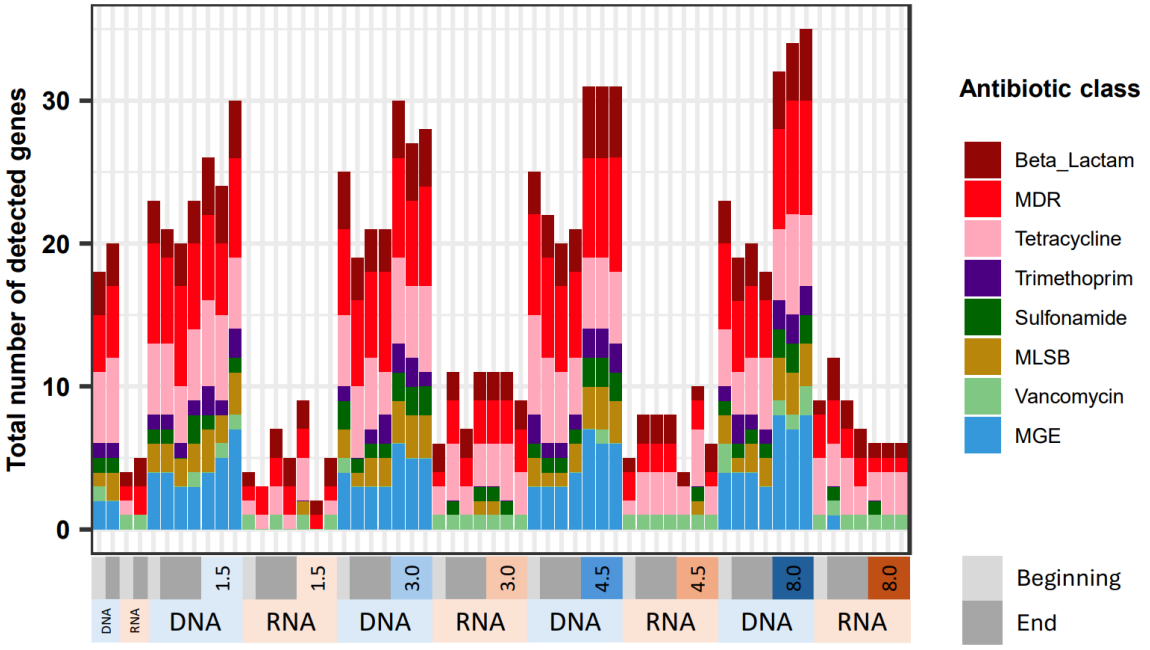

Figure 5-8 Total number of ARG and MGE genes presented and expressed (by antibiotic class) in each sample. Numbers label the amoxicillin concentration, samples without number are 0 amoxicillin. n=60, each bar represents detected gene from a single sample. MDR: multidrug resistance; MLSB: macrolide, lincosamide, streptogramin B resistance; MGE: mobile genetic element.

For each set of amoxicillin concentration, there were three samples with amoxicillin and four samples without amoxicillin, the no amoxicillin samples included one from overnight culture (T0, incubated for 16h) which acted as the seed for the test, and three were from the no amoxicillin control (T-end control) sampled 7.5h after seeding. In all four different amoxicillin concentrations, DNA samples from T0 had highest number of genes detected than the three T-end controls (T-test, P=0.012). TCC of T0 (5.50 $x10^8$ cell/ml) was similar to T-end control samples (4.80 (±0.16 SD) $x10^8$ cell/ml), which suggests that higher number of gene presented may be affected by longer culturing time. Whilst this trend was not observed in RNA, despite the ICC of T0 (5.10 $x10^8$ cell/ml) was around three times higher than T-end controls (1.65 (±0.21 SD) $x10^8$ cell/ml) (see Figure 4-14), there was no statistical difference between average total number of genes expressed in T0 and T-end controls (T-test, P=0.086). Those suggests that neither culturing time nor cell concentration affected the number of total number of gene expressed.

The detection results of ARG and MGE presented in the samples with amoxicillin (T-end amoxicillin) were significantly higher than their no amoxicillin controls (T-end controls) (T-test, P=0.026, 1.5mg/L; P=0.035, 3.0mg/L; P=0.003, 4.5mg/L; P=0.006, 8.0mg/L), the differences became wider with increasing amoxicillin concentration. Meanwhile, samples with higher amoxicillin concentration were cultured longer compared to lower amoxicillin concentration or no amoxicillin controls (see Table 4-1). Higher total ARG and MGE detection value was seen in the T-end control with longer culturing time, we then looked into the total detection between amoxicillin concentration of 3.0mg/L and overnight culture as they share similar culturing time 15h vs 16h. Total detection of ARG and MGE were 28.33 (±1.25 SD) (amoxicillin 3.0mg/L) and 25 (overnight culture) in DNA, while 10.33 (± 0.94 SD) gene expressed in amoxicillin (3.0mg/L) sample and 6.0 in overnight culture. Although total detection of ARG and MGE was higher in amoxicillin 3.0mg/L than overnight culture, there was not enough data to conclude that amoxicillin contributes more than culturing time in ARG and MGE in DNA. Despite gene expression was higher with 3.0mg/L amoxicillin compared with overnight culture, no significant difference (T-test, P=0.086) in total gene expressed between samples with amoxicillin and their controls, this suggests that there is no direct link between amoxicillin exposure and gene expression.

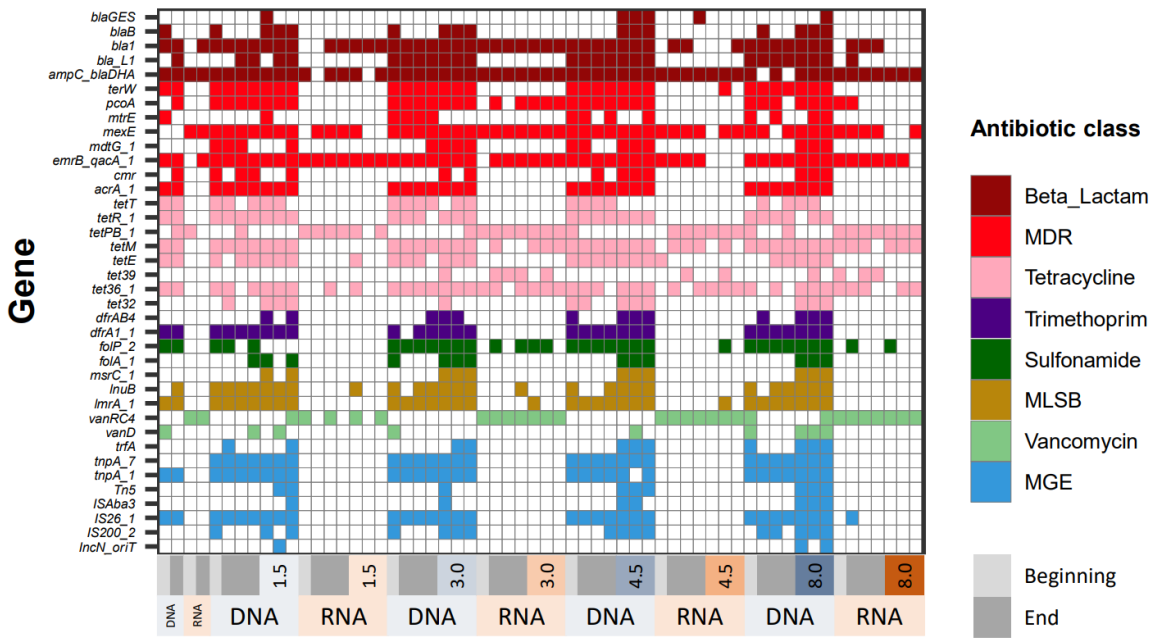

Figure 5-9 ARG and MGE detected within each sample. Numbers label the amoxicillin concentration, samples without number are 0 amoxicillin. n=60, each column represents detected gene from a single sample. MDR: multidrug resistance; MLSB: macrolide, lincosamide, streptogramin B resistance; MGE: mobile genetic element.

Since changes in total gene expression were linked with amoxicillin concentration, we examined which gene were affected by amoxicillin exposure (Figure 5-9). As amoxicillin belongs to beta lactam antibiotic class, it was therefore expected to see more beta lactam resistance genes expressed. After a quality check only five out of the 11 selected beta lactam resistant gene were kept, these all showed different trends. The most frequently detected and expressed beta lactam resistance genes were *ampC/blaDHA* and *bla1*, where *ampC/blaDHA* was detected in almost all DNA and RNA samples, and *bla1* were less likely to be found in RNA with higher amoxicillin concentrations (4.5mg/L and 8.0mg/L). This suggests that the presence and expression of *ampC/blaDHA* was independent of amoxicillin exposure and *bla1* expression may even be inhibited by increasing amoxicillin exposure. *blaL1* and *blaB* were mostly detected in DNA, where *blaL1* was detected in the majority of DNA samples, while *blaB* was found in all amoxicillin containing samples but only four no amoxicillin samples. This suggests that the presence of *blaL1* is not directly linked to amoxicillin exposure, while *blaB* is more likely to be induced by amoxicillin exposure.

MGEs are not an ARG but have the potential to carry a number of different AMR genes and act as a vector to facilitate horizontal gene transfer of ARGs. In all eight MGEs tested, only *IS26 1* was seen expressed in one sample with the highest concentration of amoxicillin (8.0mg/L), the rest were only present in DNA samples. Three MGEs frequently detected MGEs were *IS26 1*, *tnpA 1* and *tnpA 7*, where *IS26 1* was detected in every DNA sample, *tnpA 1* and *tnpA 7* were detected in almost every DNA sample. *Tn5*, *ISAba3*, *IncN oriT* were only detected in DNA samples with amoxicillin, where higher amoxicillin concentration was associated with more detections. *trfA* and *IS200 2* were detected in both amoxicillin containing samples and no amoxicillin samples, in which more detections were seen in amoxicillin containing samples than no amoxicillin samples.

The majority of ARGs detected in DNA, but three ARGs (*vanRC4*, *tetPB 1* and *tet39*) were expressed more in RNA than DNA. *vanRC4* which encodes vancomycin resistance was only detected in three DNA samples but gene expression was detected in almost every RNA sample. The detection of *tetPB 1*

and *tet39* in tetracycline was similar to *vanRC4,* was also more commonly detected in RNA rather than DNA. This detection pattern indicates these three genes are low abundant genes but highly expressed. ARGs present in DNA but not expressed as mRNA, were widely seen across every antibiotic class, such as *mtrE*, *mdtG 1*, *cmr* and *acrA 1* encode MDR; *tetT*, *tetR 1* and *tet32* encode tetracycline resistance; *dfrAB4* and *dfrA1 1* encode trimethoprim resistance; *folA 1* encode sulfonamide resistance; *msrC 1* encodes MLSB resistance; and *vanD* encodes vancomycin resistance. Some ARGs were only occasionally expressed in RNA, but widely presented in DNA, such as *terW* encodes MDR; *tetE* encodes tetracycline resistance; and *lnuB* and *lmrA 1* encode MLSB. In all those genes mentioned above, *mdtG 1* and *cmr* encode MDR; *tet32* encodes tetracycline resistance; *dfrAB4* encodes trimethoprim resistance; and *msrC 1* encodes MLSB resistance were showing strong association with amoxicillin, where majority of those resistance genes were detected in DNA samples with amoxicillin. This suggests that those low abundance ARGs were likely to be enriched by amoxicillin exposure. In contrast, *terW*, *pcoA* and *acrA 1* encode MDR; *tetR 1* encodes tetracycline resistance; *dfrA1 1* encodes trimethoprim resistance; and *lnuB* and *lmrA 1* encode MLSB were detected in almost all DNA samples, which indicates the presence of those AMRs has no association with amoxicillin.

5.4 Discussion

5.4.1 Effect of increasing amoxicillin exposure microbial communities

It was expected to have lower species richness than the environmental samples since the culture condition and culture medium have changed, but the species richness in this experiment was much lower than expected. The same microbial community were sequenced by Bruns-Moore (2022), but were cultured anaerobically and fed with glucose, resulting in a diverse microbial community of 1491 ASVs verses the significantly reduced community of 41 ASVs in this study. Culture media and condition affect the diversity of microbial communities, with the majority of microbes in a viable but non culturable state, and electron donors and acceptors in the culture media strongly selecting for culturable groups. For example, Pédron et al. (2020) compared the number of OTUs in an

environmental water samples and then cultured the same water in the laboratory on three different media, resulting in only a small portion of the OTUs from environmental water samples shared in the cultured samples. Similar Fang et al. (2023) also observed a shift of microbial community composition after running lab-based reactor with lake water feeding with glucose. Our experiment aimed to mimic the transition of wastewater flows from anaerobic reactor to constructed wetland in aerobic condition, this change of condition would inevitably result in a reduced diversity in microbial community. In this study, the microbial community seeded from anaerobic reactor and cultured in aerobic condition and the microbes have been stored in our lab for 1.5 years before starting this experiment, this may also significantly contribute to the reduction of species richness. Nevertheless, the significantly declined microbial community diversity highlights the challenges of lab mimicking experiment and suggests this should be taken into consideration in future planning.

Shift of microbial community after acute antibiotic exposure is expected, although our microbial community is less diverse than expected the change within the *Bacillota* phylum was observed. The dominant *Bacillus cereus* group was well-studied due to their pathogenic potential (Ehling-Schulz et al., 2019), and it is known that *Bacillus cereus* group have low genetic diversity, yet noticeably different pathogenic behaviours (Pfrunder et al., 2016). Some *Bacillus* species especially *Bacillus cereus* group have the ability to produce beta lactamases, which is responsible for beta lactam antibiotic resistance (Harirchi et al., 2022). In contrast, *Paenibacillus azoreducens* is a less known species with only a few reports, it first isolated from textile wastewater by Meehan et al. (2001), then isolated from soil (Lee et al., 2018), wastewater (Jałowiecki et al., 2018) and rotten saccharified rice (Krusong et al., 2022). A *Paenibacillus azoreducens* isolated from a wastewater treatment plant was shown to be resistant to 35 antibiotics from eight antibiotic classes by Jałowiecki et al. (2018). Since almost all species found in our study are known to have beta lactam resistance, the increased abundance of *Paenibacillus azoreducens* after amoxicillin exposure suggests that *Paenibacillus azoreducens* are more resistance than *Bacillus cereus* group. Also, some species in *Paenibacillus* genus and *Lysinibacillus* genus are known to have antimicrobial properties (Grady et al., 2016, Hashmi et al., 2020, Ahmad et al., 2014), it is unknown if the

increased abundance of *Paenibacillus azoreducens* brought in antimicrobial compounds that inhibits the growth of other species. However, there may be multiple *16S rRNA* transcripts per DNA, the higher abundance of transcript indicates the species were more active, also the 16S *rRNA* turnover time is longer than other transcripts, *16S rRNA* sequencing result may not be the best indicator for whole community activities (Wang et al., 2023b).

5.4.2 Comparisons of ARG and MGE gene expression between treatments

Data analysis and quality control of the results from HT-qPCR is a challenge as the huge data generated from one test and lack of information for individual assay. Four HT-qPCR platforms employed in ARG profiling are reported in literatures, where Takara Bio SmartChip real-time PCR system (WaferGen™ previously) first introduced by Wang et al. (2014) and later became the most mentioned system (Waseem et al., 2019). Relative abundance of ARGs and MGEs against *16S rRNA* gene has been reported with the comparative Ct (threshold cycle) method (Equation 2) suggested by Schmittgen and Livak (2008) and relative gene copy number calculated by Equation 3 (Looft et al., 2012). Since the Ct value of each gene target was measured directly, the selection of Ct cutoff value would largely affect the gene copy number in the analysis.

$$\Delta C_t = C_{t\,(ARG)} - C_{t\,(16S)},\ \Delta\Delta C_t = \Delta C_{t\,(Target)} - \Delta C_{t\,(Reference)} \qquad \text{Equation 2}$$

$$gene\ copy\ number = 10^{(C_t\ cutoff - C_t)/(10/3)} \qquad \text{Equation 3}$$

Waseem et al. (2019) summarised that Ct cutoff value of 31 is the most reported value in published analysis by October 2018, we looked into the development of HT-qPCR primer sets and Ct values. The first primer sets for HT-qPCR were designed by Looft et al. (2012) for OpenArray™ platform, where 271 primer sets targeting 174 ARGs were validated and 26 was used as Ct cutoff value in analysis. Zhu et al. (2013) designed additional 89 primer sets target new ARGs with the same method as Looft et al. (2012) and Ct 27 was used as detection limit for no amplification. Then Wang et al. (2014) introduced WaferGen™ platform (Takara Bio SmartChip real-time PCR system nowadays) and used 295 validated primer sets with a Ct cutoff value of 31. However, there is no clear

statement if all 295 primer sets were newly designed by Wang et al. (2014) or not. Later on, Primer set 2.0 was designed and validated by Stedtfeld et al. (2018) based on the latest sequences deposited to several online database, 209 new and 175 retained primer sets made up the primer set 2.0. Stedtfeld et al. (2018) reported that by average Ct cutoff value of 31 overestimates gene copy number by 10-fold compared to 28, meanwhile, higher Ct cutoff value (31) also has a higher false positive rate compared to 28. Although Ct cutoff value of 28 was suggested by Stedtfeld et al. (2018), Ct cutoff value of 27 appeared the same true positive rate and lower false positive rate compared to 28 in the result of Stedtfeld et al. (2018). No other report has ever discussed the relationship between Ct cutoff value and the detection. And there is no more major new primer set design for HT-qPCR report after Stedtfeld et al. (2018).

Ct cutoff value has been described in HT-qPCR analysis in almost all published literatures, but quality controls of other criteria are barely mentioned. Our inconsistence *16S rRNA* results brought our interest to investigate if there are any similar data analysis has been done in all published literature employed HT-qPCR. More than one NTC in HT-qPCR has been reported by Tyrrell et al. (2023) (two NTCs) and Sacristán-Soriano et al. (2024) (three NTCs), but how they affect the results are not mentioned in their report. Deleting assays with multiple peaks based on the melt curve analysis has been done by several studies (Zhou et al., 2023, Santosaningsih et al., 2023, Kasuga et al., 2022), but there is detailed description of the criteria. Indeed, HT-qPCR is good for screening ARGs in environmental samples, the reliability of our results suggests extra caution may need in data analysis.

It is expected to see high beta lactam resistance genes detected across all samples as *Bacillus cereus* group was the dominant organism in our microbial community. Penicillin resistance as well as resistance to other beta lactam antibiotics in strains belonging to *Bacillus cereus* group have been isolated from all kinds of samples all over the world (Chen et al., 2022, Adamski et al., 2023, Mills et al., 2022, Fiedler et al., 2019, Mohammadi et al., 2023). All five beta lactam resistance genes are chromosomal encoded genes and they share the same resistant mechanism of drug inactivation (Stedtfeld et al., 2018, Alcock et al., 2023), where bacteria with those genes are able to produce beta lactamase

which hydrolyses the beta lactam ring in amoxicillin. However, it is unknown if those five beta lactam resistance genes are able to represent the change of all beta lactam resistance genes. Also, due to lack of abundance data, it is unknown if amoxicillin exposure has increased the abundance of those beta lactam resistance genes.

Mohammadi et al. (2023) reported that 95% of *Bacillus cereus* isolates are susceptible to tetracycline and vancomycin, Fiedler et al. (2019) noticed that isolates with *tet45* gene were resistant to tetracycline. Given *vanRC4*, *tetPB 1* and tet39 were highly expressed, our microbial communities are likely to show resistance to both tetracycline and vancomycin. Many *Bacillus cereus* isolates are resistant to trimethoprim and sulfonamide (Chen et al., 2022, Mohammadi et al., 2023). In our results, most of the trimethoprim and sulfonamide resistance genes were detected in DNA and some were associated with amoxicillin exposure, which suggests that our microbial communities have higher potential to resistant to trimethoprim and sulfonamide antibiotic. Meanwhile, MDR was defined as resistant to antibiotics from more than one antibiotic classes (Magiorakos et al., 2012). As resistance from more than one antibiotic class was detected widely across all the samples, it was expected to see high detection of MDR.

ARG studies antimicrobial resistance potential, which is not always the same as phenotypic resistance. Test on *Bacillus cereus* group find out that genotypic resistance is different from phenotypic resistance, where more ARGs encoding resistance from more antibiotic classes were detected than antimicrobial susceptibility test (Farina et al., 2024). There is positive association between antibiotic residue in wastewater and abundance of ARGs encoding the resistance of the antibiotic at country level (Hendriksen et al., 2019). In hospital wastewater the abundance of vancomycin and carbapenem resistance genes are positively associated with their usage (Perry et al., 2021). However, same as our study ARGs are not quantified by Farina et al. (2024), it is unknown whether ARG abundance is correlated with phenotypic resistance or not. The aim of this experiment was to design a rapid tool to detect antimicrobial resistance in decentralised wastewater treatment based on the phenotypic resistance. ARG

abundance along with ARG expression on top of phenotypic resistance would provide better overall evaluation of the antimicrobial resistance detection tool.

5.5 Conclusion

The aim of this chapter was to explore and compare microbial community and ARGs from microbial communities under different amoxicillin concentrations. The microbial community composition was analysed through *16S rRNA* sequencing of both DNA and RNA. Although the microbial communities were less diverse than expected, the shift of microbial community composition was associated with the presence of amoxicillin. ARGs confer different antibiotic classes were screened and tested through HT-qPCR, total ARG expression increased with increasing amoxicillin concentrations. However, samples from higher amoxicillin concentrations were cultured for a longer time which may also have impacted ARG expression, this cannot be ruled out due to insufficient data. ARG expression reveals that despite only beta lactam antibiotics used in this study, ARGs belong to other antibiotic class were also expressed. Some ARG expressions were irrespective of amoxicillin, however, it is unknown that how the abundance of those ARGs was changed due to poor data quality. HT-qPCR is not recommended for total ARG study as the reliability was lower than expected.

6 Conclusion and future work

This thesis focuses on improving decentralised wastewater treatment by small-scale constructed wetland system. The aims of this thesis were:

1) Monitor the performance and the changes of the microbial communities in operational small-scale constructed wetlands treating domestic wastewater from start-up (first 3 months) and long term (10 months) in Mexico City.

2) Develop a rapid approach to detect the antimicrobial resistance in mixed microbial community from wastewater.

3) Analysis the change in microbial communities and ARGs in response to a range of amoxicillin concentrations.

6.1 Conclusion

6.1.1 Small-scale constructed wetland system is a solution for improving decentralised wastewater treatment

Septic tanks are one of the most commonly used sanitation solutions in rural areas globally. Their performance varies and often the effluent quality barely meets wastewater discharge standards. Our small-scale constructed wetland systems aimed to employ a horizontal constructed wetland and a vertical constructed wetland to remove pollutants from septic tank effluent. Three systems, two household and one school system, were built in Mexico City and monitored from start-up, with all of them successful in removing pollutants (COD, NO_2^- and NO_3^-) from domestic wastewater from the beginning of operation. The school system (WS3) was monitored for three months before it shut down due to the Covid pandemic. Although it received a lower strength wastewater the pollutant removal was comparable to the other two household systems. Two household systems (WS1 and WS2) were monitored for 16 months, pollutants removal fluctuated at the beginning and stabilised after nine months' operation. The removals of COD, NO_2^- and NO_3^- in our long-term monitoring were comparable to full-scale constructed wetland. Meanwhile, the removal of NH_4^+ and phosphate were limited in our long-term monitoring. All those performance

monitoring suggests that our small-scale constructed wetland system achieved our aim of reducing pollutants from the effluent of septic tank via small-scale constructed wetland system.

One of the challenges in small-scale constructed wetland system for single household is the nutrients in the influent are unpredictable and it changes overtime with the daily activities. In contrast, full-scale WWTPs are more resilient to this change as they have larger treatment volume and wastewater is normally homogenised better than small-scale WWTPs. Our systems have equalization tank and a biodigester before the constructed wetlands to minimise the fluctuation, however from our monitoring results the constructed wetlands still received wastewater with various nutrients level, double or even tripled COD concentrations were observed. Even though, the effluent quality remained stable, which suggests that our systems are capable of dealing with nutrient fluctuations. Meanwhile, the average COD concentration received by our WS1 and WS2 was very high compared to the guideline of maximum 400mg/L of COD for constructed wetland influent (Zurita et al., 2012). They still worked with comparable COD removal of the full-scale constructed wetlands receiving much lower strength wastewater. This suggests that our constructed wetland systems **are robust and resilient to high strength wastewater**. It is worth mentioning that although COD is one of the parameters used in monitoring the performance of wastewater treatment and normally assumed that half of the COD is biodegradable in domestic wastewater. However, the actual biodegradable carbon in the wastewater is unknown, if BOD was measured it would provide a better view in evaluating the biological potential of our constructed wetland systems, and it is recommended that this should be done in future.

Our systems are designed to reuse the treated wastewater to flush the toilet and for gardening including the growing vegetables, as one done for one of the household wetlands. Removal of nitrogen was not the primary goal of our systems and the level of nitrogen after treatment is not regulated in Mexican official standards for reuse treated wastewater (Gutiérrez, 2008). Nitrate and nitrite were reduced in a similar rate as COD, but total nitrogen was not measured, it is unknown how much nitrogen was removed in total. Ammonium stayed same level after treatment, which is desirable as it can be used by the

plants directly. Several potential pathogens were tested in the first three months' study, *E. coli* was removed significantly from all three systems, to potentially meet the standards of reuse treated wastewater in Mexico. Those systems were not capable of removing *Legionella* species from the biomass, however, the biomass in the effluent would be much lower than influent where the *Legionella* species would expect to be lower. Furthermore, emerging contaminants such as AMR was not measured, particularly our household systems are using treated wastewater for irrigation, it is unknown whether the crops pick up AMR from water or not. Although Gentile et al. (2024) reported that no ARG found in lettuce leaves irrigated with wastewater treated with pilot scale constructed wetland, this study only included lettuce from one harvest, it is still not clear about the long-term impact on the soil and corps irrigated with treated wastewater.

6.1.2 The microbial community in small-scale constructed wetland systems stabilised four months after pollutant removal stabilised

As summarised before, despite the performance of wetlands being largely driven by microbial activity, the majority of full-scale constructed wetland studies published focus mostly on the performance, in the small portion of the studies including the underpinning microbial community, these mainly only take samples from one or a few time points. Lab-scale constructed wetland studies are more likely to have microbial community analysis, but the vast majority of them are short-term studies with synthetic wastewater. Our 16 months operational study coupled with a detailed microbial community study fills this gap informing changes of microbial communities from the beginning of the operation. Microbial communities took longer to achieve a less variable microbial community than a less variable pollutant removals (9 months vs 13 months), and despite different microbial communities being found in these two systems they achieved comparable pollutant removal rates, indicating functional redundancy in the microbial community structure.

The microbial communities in our first constructed wetland (horizontal constructed wetland, CW1) of both systems were more similar to the influent, while the microbial communities in the second constructed wetland (vertical

constructed wetland, CW2) were more similar to the effluent. This suggests that more biological processes happened in CW2, the pollutant removal rates of CW2 were higher than CW1, but CW1 removed more pollutants than CW in terms of concentrations. It is not clear whether the lower concentration of pollutants contributed to the change of microbial communities in CW2 or the structure of CW2 as a vertical constructed wetland. Biofilters with similar structure as our vertical constructed wetland has observed a shift of microbial communities between top and bottom (Quinn, 2022). Although the analysis of global microbial communities in full-scale WWTPs summarised that microbial communities in the sludge are more closer to local fresh water (Wu et al., 2019), it would be interesting to see the how the pollutant level affects the microbial communities.

Comamonadaceae family is the most abundant family and some OTUs belonging to this family are core OTUs across global WWTPs, and those core OTUs are significantly associated with BOD, COD, NH4-N, total nitrogen and phosphate removal (Wu et al., 2019). As for our study, the abundance of *Comamonadaceae* was seen to increase overtime, where in the first three months of operation, only one out of top 25 most abundance ASVs came from *Comamonadaceae* family. But in our long-term study, 8 out of 46 most abundance ASVs shared by two systems came from *Comamonadaceae* family. This suggests that *Comamonadaceae* family may be used as indicator to monitor the microbial community in the constructed wetland in terms of microbial maturation and performance.

6.1.3 OD may be a potential method to measure antimicrobial resistance through the length of lag phase

Our preliminary experiment of measuring microbial community growth under a range of amoxicillin concentrations via OD revealed that lag phase and growth rates were changing with amoxicillin concentrations. Community growth rate was further compared with cell count via FCM with selected amoxicillin concentrations, but the results were not enough to conclude OD is suitable for measuring growth rate of mixed communities. The initial hypothesis was to use growth rate measured through OD as proxy to estimate antimicrobial resistance in the whole community. Our ARG abundance measurements suggest that growth rate may not have a direct link to antimicrobial resistance, whilst ARG

abundance is positively associated with amoxicillin concentration. Meanwhile, our preliminary experiment also found that the length of lag phase is positively associated with amoxicillin concentration, the length of lag phase may provide a better approach than growth rate in our study. Li et al. (2016) also observed that the extended lag phase is associated with resistance in both pure strain and mixed strains from activated sludge under some antibiotics and suggested that extended lag phase can be an indicator for antimicrobial resistance. Interestingly, linear relationship of lag phase length with amoxicillin was observed in our study, but Li et al. (2016) found almost constant lag phase in two bacterial community under all tested amoxicillin concentrations. As the resistance of the microbial communities was not tested by Li et al. (2016), it is unknown what the cause of this phenomena is. Meanwhile, it also indicates that microbial communities behave differently with different antibiotics, as antibiotic only works for its targeted microorganisms. More work needs to be done to know if the proportion of antibiotic susceptible microorganisms in a mixed microbial community would affect the reliability of evaluating antibiotic resistance through the length of lag phase.

Only a few studies focused on the length of lag phase in microbial communities, but both Li et al. (2016) and Jindal et al. (2019) as well as our study revealed that the length of lag phase is partially associated with antimicrobial resistance. Nutrients in the culturing media, initial cell density, pH, temperature and the stage of cell growth before inoculation affect the length of lag phase (Jindal et al., 2019). The length of lag phase measured by OD only reflects the amount of cells that reaches the minimum cell number that could be detected by OD. If the initial cell concentration is known, lag phase measured with OD would reveal the time required to reach the cell concentration that is detectable by OD. In this way, the change of the length of lag phase when exposed to antibiotics could be associated with the antimicrobial resistance. However, more research needs to be done to validate the relationship between antimicrobial resistance and the length of lag phase and investigate the microbial activities in the lag phase.

6.1.4 Microbial communities shifted after acute exposure to amoxicillin

The shift of microbial community was observed after amoxicillin exposure, where the diversity and evenness in both DNA and RNA in microbial communities after amoxicillin exposure were higher than the microbial communities without amoxicillin exposure. However, the microbial communities with amoxicillin were cultured for long periods of time due to their longer lag phase, our data is insufficient to conclude that whether culturing time affected the microbial communities or not. Furthermore, the diversity of microbial communities was much lower than expected. The change of culturing condition from anaerobic to aerobic could be a reason, the culture media would be another one. The selection of culture media is barely discussed, although synthetic wastewater is widely used in lab-scale experiments. Three different sources of influent were tested by Gibson et al. (2024) in lab-scale bioreactors seeded with activated sludge from full-scale WWTP and run for at least five months, found out that only up to 14% of the sequencing reads from the activated sludge were coming from the influent. Meanwhile, a study conducted by Goldford et al. (2018) cultured environmental microbiomes from soil and plant leaf surfaces using different carbon sources and found that the diversity of the microbial communities reduced from 110-1290 ASVs to 4-22 ASVs, with the reduced ASVs being diverse at species level but converged at family level. The reduction of ASVs is very similar to what was observed in this study. The same community used in this work was previously used and sequenced by Bruns-Moore (2022) and had 1,491 ASVs while in our study, where it was cultured with LB aerobically, the community was reduced to 41 ASVs and the carbon source was switched from glucose to LB. Likely this is due to selective factors used here – aerobic conditions, LB and temperature. Likely culturing the community on synthetic wastewater would be a better culture media than LB.

6.1.5 HT-qPCR is not always reliable for ARG detecting

Two rounds of HT-qPCR were performed to screen and detect the change of ARGs in the microbial communities after amoxicillin exposure. The second round's result showed inconsistent quality, which resulted in only ARG expression data available. HT-qPCR as a high sensitivity method developed in

recent years and gained popularity in environmental ARG studies, but the reliability of this method has never been discussed. After intense research through the literature involved HT-qPCR methods, we found that two major primer sets used in current ARG studies were validated by Zhu et al. (2013) and Stedtfeld et al. (2018). The detection limit and potentially over estimating the ARGs was tested and discussed by Stedtfeld et al. (2018), whilst it was not mentioned by Zhu et al. (2013). But our issue of various results and poor quality in the *16S rRNA* gene between two rounds are never discussed. Hence, we suggest that the HT-qPCR for ARG detection must be used with caution.

6.1.6 Higher antibiotic concentration or/and longer exposure time triggers more ARG expressions

Higher amoxicillin concentration associated with higher ARG expression was found after ARGs in the microbial community detected using HT-qPCR. However, similar to the microbial community composition results our experiment and data is not enough to conclude that amoxicillin exposure is the sole reason for increased ARG expression, longer exposure time may also trigger ARG expression. Also, as only beta-lactam antibiotic amoxicillin was used in our study, ARGs conferring other antibiotic classes were seen with higher expression after amoxicillin exposure. Due to data quality, it is unknown what the absolute abundance of those expressed ARGs was. Whilst many antibiotic exposure studies only test the ARG paired to the antibiotic used in the study, our study provides an insight into the co-selection of ARGs. However, it is also unknown how this influences and changes ARGs in long term. In human gut studies, co-selection of ARGs are observed but those ARGs often disappeared later on, but enriched ARG due to prolonged exposure are normally long lasting (Fishbein et al., 2023). It is worth studying further the long-term impacts of ARGs after acute exposure, especially in those decentralised WWTPs, and the role they play in disseminating AMR genes to the environment. As a start, the ARG across our constructed wetlands could be quantified and characterised using our suite of DNA samples.

6.2 Future work

- Our study demonstrated small-scale constructed wetland system is capable of treating high strength wastewater from single household.

Whilst the effluent may not always meet the discharge standard, additional treatment unit or change of operation is needed to improvement the treatment. Also, as a household system operated by the users, it is important to listen to user's feedback before making the change. This constructed wetland system is not one solution for all, the application of this system will depend on the need of the user, current sanitation, the goal of wastewater treatment and land available etc.

- In our short-term study, *E. coli* was removed after treatment, although *Legionella* species were not removed from the biomass and were detected in the effluent. Total biomass in treated wastewater was largely reduced in the first three months' operation. However, the long-term performance of potential pathogen removal still needs to be tested. Meanwhile, the potential risk of ARG carrier pathogens within the system and their risk associated with reclaimed water needs to be investigated.

- It is interesting to see that the microbial communities in two household systems were different but they achieved comparable pollutant removal. Also, we have noticed that microbial communities were still changing when pollutants removal reached a relatively stable state. It would be worth looking into the microbes who contributed to pollutants removal, particularly during the time that microbial communities were still changing but pollutants removal were already stable. With a better understanding of the functional microbes in both systems, it may be possible to improve treatment efficiency even further.

- A method to detect antimicrobial resistance via OD could potentially be developed based on the length of lag phase when microbial community are exposed to antibiotics. Our lab-based ARG study did not find any connection between growth rate and ARG expression, but the length of the lag phase and ARG expression both increased with increasing amoxicillin concentrations. Although ARG abundance was unknown, the increased ARG expressions indicating higher potential of spreading multi drug resistance after a single antibiotic. OD is less sensitive method in detecting cell concentrations compared to FCM, but it can be automated

to run multi assays and low in cost, which makes it suitable for routine monitoring. Despite the lag phase measured by OD is the growth time of microbe required to reach the lowest detection limit of OD, which represents the ability of growth under the presence of antibiotic. The change of time required to be detected by OD would correlate with the resistance when expose to the same antibiotic concentration.

- Long-term impact of microbial communities and ARGs after acute antibiotic exposure in small-scale constructed wetland treating domestic wastewater. Our batch experiment is not enough to conclude if acute antibiotic is the only reason for higher ARG expressions, as the longer exposure time may also affect ARG expression. Whilst acute antibiotic exposure is one of the most common routes of antibiotic entering domestic wastewater as the large percentage of antibiotic would still be intact in human excrete. Long-term fate of ARGs within constructed wetland and associated risk with irrigation in the garden will be necessary in assessing the ARG spreading risk in constructed wetland.

7 Appendix

7.1 Chapter 2 – Appendix

Table 7-1 qPCR standard curve parameter for all primers.

Primers	Efficiency (%)	Slope	Intercept	R^2	NTC
Total bacteria (*16S rRNA*)	100.08	-3.32	38.51	0.99	33.68
Escherichia coli	102.65	-3.26	36.6	1.00	NA
Pseudomonas aeruginosa	101.35	-3.29	37.08	0.99	NA
Legionella spp.	105.82	-3.19	37.90	1.00	NA
Legionella pneumophila	104.44	-3.22	37.00	1.00	NA
Legionella pneumophila serogroup 1	105.82	-3.19	37.68	1.00	NA

7.2 Chapter 3 - Appendix

Table 7-2 Faecal coliform (CFU/100ml) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Aug 2020	Sep 2020	Oct 2020	Nov 2020	Feb 2021	Mar 2021			Apr 2021
Sample date		27/08/20	29/09/20	24/10/20	24/11/20	24/02/21	08/03/21	15/03/21	22/03/21	12/04/21
WS1	INF	75 ±63.6	385 ±21.2	550 ±0	515 ±63.6	2500 ±1153.3	5600 ±0	3900 ±1555.6	4050 ±495.0	2733 ±1167.6
	CW1					53.3 ±15.3	10 ±0	80 ±14.1	150 ±14.1	177 ±20.6
	CW2					66.7 ±47.3	85 ±35.4	255 ±219.2	255 ±91.9	103 ±17.8
	EFF	30 ±28.3	45 ±21.2	180 ±113.1	330 ±84.9	40 ±28.3	55 ±35.4	65 ±7.1	140 ±0	
WS2	INF	850 ±28.3	255 ±35.4	550 ±0	545 ±106.1	633.3 ±57.7		2400 ±264.6		
	CW1					33.3 ±23.1			110 ±56.6	
	CW2					105 ±49.5		245 ±7.1		
	EFF	35 ±21.2	40 ±28.3					160 ±0	60 ±0	

Table 7-3 Total Suspended Solids (TSS) (g/L) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Jul 2020	Aug 2020	Sep 2020	Nov 2020	Feb 2021	Mar 2021				Apr 2021		
Sample date		27/07/20	27/08/20	29/09/20	24/11/20	24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	100.0 ±0	540.0 ±0	370.0 ±0	220.0 ±0	210.0 ±0	120.0 ±0	133.3 ±18.9	130.0 ±28.3	245.0 ±63.6	344.4 ±0	212.5 ±17.7	
	CW1					285.0 ±0		59.0 ±12.7	60.0 ±0	31.7 ±7.1	104.4 ±15.6	80.0 ±0	
	CW2					37.5 ±0		24.0 ±2.8	8.6 ±0	16.3 ±8.8	38.7 ±1.8	28.0 ±11.3	
	EFF	120.0 ±0	5.0 ±0	5.0 ±0	60.0 ±0	35.0 ±0		43.3 ±9.4	4.3 ±2	3.9 ±0.8	36.2 ±5.3	32.1 ±20.6	
WS2	INF	580.0 ±0	390.0 ±0	1670.0 ±0	480.0 ±0	260.0 ±0	115.0 ±7.1	365.0 ±35.4	280.0 ±14.1	156.2 ±61.9	260.0 ±56.6		210.0 ±28.3
	CW1					116.7 ±23.6	28.3 ±2.4	153.3 ±0	128.7 ±21.7	160.0 ±0	145.2 ±16.8		77.3 ±5.6
	CW2					40.0 ±0		25.1 ±12.9	22.4 ±12.8	15.0 ±0	19.5 ±6.4		17.6 ±12.8
	EFF	130.0 ±0	46.7 ±0	50.0 ±0	3.0 ±0	35.0 ±0		73.3 ±0	30.0 ±10.1	28.7 ±5.3	46.7 ±23.6		38.3 ±11.8

Table 7-4 Total organic carbon (TOC) (mg/L) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Feb 2021	Mar 2021				Apr 2021		
Sample date		24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	90.7 ±3.8	95.2 ±6.1	75.2 ±2.5	71.7 ±6.2	31.6 ±0	10.5 ±0		
	CW1	38.9 ±1.2	9.4 ±1.5	9.1 ±3.1	14.8 ±0				
	CW2	15.2 ±0.8	10.1 ±0	4.8 ±0			3.0 ±0		
	EFF	21.1 ±0.9	5.7 ±4.6	6.3 ±1.7	5.1 ±2.8				
WS2	INF	177.6 ±1.3	183.3 ±4.9	264.1 ±6.9	231.1 ±4.7	202.3 ±11.2	217.8 ±7.9		128.7 ±2.8
	CW1	40.2 ±3.8	79.2 ±1.7	140.4 ±17.1	98.2 ±4.0	68.3 ±6.0	45.6 ±10.7		
	CW2	18.2 ±1.4	15.9 ±0.9	14.8 ±4.0	11.3 ±6.6		12.1		
	EFF	32.1 ±0.1		39.7 ±0.5	17.9 ±11.8				

Table 7-5 Total carbon (TC) (mg/L) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Feb 2021	Mar 2021				Apr 2021		
Sample date		24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	102.6 ±2.0	173.9 ±4.7	168.4 ±7.8	185.9 ±2.9	145.4 ±81.1	182.8 ±29.1		266.6 ±2.5
	CW1	52.0 ±3.9	107.2 ±2.9	98.6 ±0.4	104.1 ±10.9	181.5 ±10.1	137.1 ±3.9	192.1 ±1.6	174.8 ±6.9
	CW2	27.0 ±1.1	79.3 ±6.3	61.7 ±12.4	91.1 ±5.2	138.2 ±7.2	87.5 ±9.1	161.0 ±19.2	178.9 ±46.9
	EFF	30.5 ±0.1	81.2 ±8.5	70.4 ±2.7	72.9 ±0.7	118.8 ±0.7	78.6 ±12.0	159.5 ±24.5	136.4 ±4.6
WS2	INF	133.1 ±0.8	250.1 ±8.3	324.9 ±5.7	294.4 ±0.7	276.2 ±5.7	320.7 ±4.0		217.8 ±2.8
	CW1	57.9 ±3.3	145.2 ±12.3	213.8 ±25.2	168.5 ±9.0	155.8 ±15.7	152.3 ±9.5		114.7 ±2.8
	CW2	27.2 ±9.6	44.2 ±0.2	60.7 ±3.6	65.8 ±5.8	67.4 ±2.4	60.4 ±16.2		100.6 ±10.3
	EFF	42.8 ±3.8		79.3 ±3.5	83.9 ±12.3	83.8 ±1.0	96.0 ±7.3		116.3 ±3.9

Table 7-6 Total nitrogen (TN) (mg/L) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Feb 2021	Mar 2021				Apr 2021		
Sample date		24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	179.6 ±4.0	178.3 ±6.2	189.3 ±5.8	173.7 ±3.8	123.2 ±4.7	132.5 ±4.2		192.5 ±0.7
	CW1	175.8 ±1.9	172.9 ±0.1	179.9 ±8.5	121.9 ±1.0	117.0 ±1.2	120.6 ±0.1	174.8 ±1.5	161.6 ±0.8
	CW2	166.5 ±3.2	155.9 ±1.6	155.7 ±0.4	90.5 ±6.5	88.0 ±2.7	106.2 ±5.9	167.9 ±2.4	149.6 ±12.2
	EFF	176.8 ±0.2	173.5 ±4.5	168.1 ±2.0	104.1 ±8.3	88.8 ±1.1	92.1 ±2.3	162.0 ±0.3	150.2 ±4.0
WS2	INF	184.6 ±1.8	240.8 ±0.4	219.2 ±3.4	160.2 ±6.7	149.0 ±2.1	161.9 ±2.3	139.1 ±2.3	
	CW1	186.8 ±7.9	180.4 ±1.3	195.1 ±2.3	112.5 ±4.5	114.8 ±7.3	109.1 ±0.4	119.5 ±0	
	CW2	163.5 ±15.5	161.0 ±1.7	136.2 ±3.2	86.7 ±1.5	90.5 ±1.3	109.4 ±1.8	119.5 ±2.2	
	EFF	167.2 ±5.9		179.9 ±3.5	103.9 ±4.9	107.3 ±3.5	100.5 ±11.8	130.8 ±3.0	

Table 7-7 Temperature (℃) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Feb 2021	Mar 2021				Apr 2021		
Sample date		24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	14.25	16.57	18.12	15.75	19.94	18.37	18.50	19.37
	CW1	12.94	14.41	13.94	13.19	14.69	14.81	15.38	15.69
	CW2	14.31	18.19	15.75	15.00	16.56	16.69	16.44	16.75
	EFF	14.81	16.40	16.06	16.19	16.81	16.94	16.75	16.94
WS2	INF	17.44	16.34	16.25	15.13	16.75	17.00		
	CW1	16.19	17.58	17.44	15.81	17.81	19.37		
	CW2	16.12	19.38			17.44	18.75		17.44
	EFF					16.44	15.88		16.62

Table 7-8 pH of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Feb 2021	Mar 2021				Apr 2021		
Sample date		24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	7.15	7.19	7.33	7.46	6.95	6.94	7.33	7.36
	CW1	7.01	6.74	7.37	7.30	7.30	7.37	8.86	9.73
	CW2	6.27	5.13	7.42	7.16	7.55	7.75	8.93	8.36
	EFF	7.64	7.35	7.84	7.19	8.40	7.81	7.12	7.13
WS2	INF	6.67	6.93	6.71	7.01	7.15	7.18		
	CW1		7.05	6.86	7.20	7.36	7.34		
	CW2			7.50	7.22	7.53	7.65		
	EFF		7.85	7.45	7.34	7.85	7.92		

Table 7-9 Dissolved oxygen (DO) (mg/L) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Feb 2021	Mar 2021				Apr 2021		
Sample date		24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	1.84	1.88	2.22	2.22	2.22			
	CW1	3.66	3.33	3.67	3.68	3.70	4.67	4.66	4.87
	CW2		2.83				5.55	3.33	2.22
	EFF	2.22	1.37	2.22	2.22	2.06	2.31	2.22	2.23
WS2	INF								
	CW1		1.11			0.13	0.01		
	CW2		3.82			2.93			
	EFF								

Table 7-10 Oxidation reduction potential (ORP) (mV) of WS1 and WS2 measured in long-term performance monitoring.

Sample month		Feb 2021	Mar 2021				Apr 2021		
Sample date		24/02/21	08/03/21	15/03/21	22/03/21	29/03/21	05/04/21	12/04/21	19/04/21
WS1	INF	-444.83	-458.39	-404.81	-431.97	-440.80	-437.35	-437.45	-417.03
	CW1	-466.27	-479.94	-467.54	-531.57	-537.19	-518.02	-540.38	-569.28
	CW2	-433.64	123.94	-176.09	77.89	-258.48	-258.59	17.71	-218.52
	EFF	-475.11	-525.51	-452.35	-464.85	-464.4	-461.27	-452.67	-454.34
WS2	INF	-963.46	-1474.00	-1405.15	-1438.76				
	CW1	-190.75	-463.82	-435.91	-398.41	-319.82	162.17		
	CW2	150.65	-134.85			24.28	287.33		
	EFF	-498.76				-361.93			

Table 7-11 List of richness and Pielou's evenness of every sample with sample details.

Sample Month	Sample Date	System	Sample Location	Sample type	Replicate	Richness	Pielou's evenness
Jun_2020	02_Jul_2020	WS1	INF	W	R1	110.87	0.19
Jun_2020	02_Jul_2020	WS1	EFF	W	R1	164.60	0.59
Jun_2020	02_Jul_2020	WS2	INF	W	R1	507.59	0.67
Jul_2020	27_Jul_2020	WS1	INF	W	R1	754.13	0.73
Jul_2020	27_Jul_2020	WS1	EFF	W	R1	324.25	0.56
Jul_2020	27_Jul_2020	WS2	INF	W	R1	744.79	0.69
Jul_2020	27_Jul_2020	WS2	EFF	W	R1	722.05	0.67
Aug_2020	27_Aug_2020	WS1	INF	W	R1	471.31	0.62

Aug_2020	27_Aug_2020	WS1	EFF	W	R2	401.89	0.55
Aug_2020	27_Aug_2020	WS2	INF	W	R1	624.40	0.57
Aug_2020	27_Aug_2020	WS2	EFF	W	R2	393.13	0.57
Sep_2020	29_Sep_2020	WS1	INF	W	R1	621.32	0.62
Sep_2020	29_Sep_2020	WS1	EFF	W	R1	416.71	0.63
Sep_2020	29_Sep_2020	WS2	INF	W	R1	818.48	0.67
Sep_2020	29_Sep_2020	WS2	INF	W	R2	918.53	0.65
Sep_2020	29_Sep_2020	WS2	EFF	W	R1	490.15	0.67
Oct_2020	24_Oct_2020	WS1	INF	W	R1	489.60	0.63
Oct_2020	24_Oct_2020	WS1	INF	W	R2	211.30	0.48
Oct_2020	24_Oct_2020	WS1	CW1	W	R1	1060.87	0.75
Oct_2020	24_Oct_2020	WS1	CW1	S	R1	573.93	0.55
Oct_2020	24_Oct_2020	WS1	CW2	W	R2	474.67	0.50
Oct_2020	24_Oct_2020	WS2	CW1	W	R1	928.41	0.73
Oct_2020	24_Oct_2020	WS1	CW2	S	R1	626.89	0.64
Oct_2020	24_Oct_2020	WS1	CW2	S	R2	590.95	0.66
Oct_2020	24_Oct_2020	WS1	EFF	W	R1	390.88	0.63
Oct_2020	24_Oct_2020	WS1	EFF	W	R2	334.06	0.64
Oct_2020	24_Oct_2020	WS2	INF	W	R1	834.24	0.62
Oct_2020	24_Oct_2020	WS2	INF	W	R2	1019.92	0.71
Oct_2020	24_Oct_2020	WS2	CW1	S	R1	549.43	0.57
Oct_2020	24_Oct_2020	WS2	CW2	W	R2	481.42	0.59

Oct_2020	24_Oct_2020	WS2	CW2	S	R2	840.62	0.69
Nov_2020	24_Nov_2020	WS1	INF	W	R1	867.25	0.71
Nov_2020	24_Nov_2020	WS1	INF	W	R2	967.71	0.70
Nov_2020	24_Nov_2020	WS1	CW1	W	R1	877.93	0.70
Nov_2020	24_Nov_2020	WS1	CW1	W	R2	817.63	0.64
Nov_2020	24_Nov_2020	WS1	CW1	S	R1	932.51	0.69
Nov_2020	24_Nov_2020	WS1	CW1	S	R2	829.80	0.63
Nov_2020	24_Nov_2020	WS1	CW2	S	R2	634.09	0.66
Nov_2020	24_Nov_2020	WS1	CW2	S	R1	699.91	0.65
Nov_2020	24_Nov_2020	WS1	EFF	W	R1	363.00	0.57
Nov_2020	24_Nov_2020	WS1	EFF	W	R2	656.80	0.69
Nov_2020	24_Nov_2020	WS2	INF	W	R1	833.77	0.71
Nov_2020	24_Nov_2020	WS2	CW1	W	R1	677.97	0.63
Nov_2020	24_Nov_2020	WS2	CW1	W	R2	952.71	0.72
Nov_2020	24_Nov_2020	WS2	CW1	S	R1	1086.24	0.73
Nov_2020	24_Nov_2020	WS2	CW1	S	R2	463.89	0.53
Nov_2020	24_Nov_2020	WS2	CW2	W	R1	505.81	0.64
Nov_2020	24_Nov_2020	WS2	CW2	W	R2	542.04	0.60
Nov_2020	24_Nov_2020	WS2	CW2	S	R1	896.70	0.67
Nov_2020	24_Nov_2020	WS2	EFF	W	R1	394.91	0.67
Nov_2020	24_Nov_2020	WS2	EFF	W	R2	458.73	0.67
Feb_2021	24_Feb_2021	WS1	INF	W	R1	665.27	0.66
Feb_2021	24_Feb_2021	WS1	INF	W	R2	833.93	0.70
Feb_2021	24_Feb_2021	WS1	CW1	W	R1	733.33	0.69

Feb_2021	24_Feb_2021	WS1	CW1	W	R2	786.89	0.70
Feb_2021	24_Feb_2021	WS1	CW1	S	R1	886.89	0.71
Feb_2021	24_Feb_2021	WS1	CW1	S	R2	731.04	0.73
Feb_2021	24_Feb_2021	WS1	CW2	W	R1	625.67	0.62
Feb_2021	24_Feb_2021	WS1	CW2	W	R2	567.70	0.63
Feb_2021	24_Feb_2021	WS1	CW2	S	R1	1019.76	0.71
Feb_2021	24_Feb_2021	WS1	CW2	S	R2	980.47	0.74
Feb_2021	24_Feb_2021	WS1	EFF	W	R1	456.72	0.67
Feb_2021	24_Feb_2021	WS1	EFF	W	R2	558.56	0.63
Feb_2021	24_Feb_2021	WS2	INF	W	R1	812.52	0.74
Feb_2021	24_Feb_2021	WS2	INF	W	R2	816.87	0.68
Feb_2021	24_Feb_2021	WS2	CW1	W	R1	752.52	0.72
Feb_2021	24_Feb_2021	WS2	CW1	W	R2	943.67	0.72
Feb_2021	24_Feb_2021	WS2	CW1	S	R1	953.00	0.71
Feb_2021	24_Feb_2021	WS2	CW1	S	R2	918.16	0.72
Feb_2021	24_Feb_2021	WS2	CW2	W	R1	886.41	0.70
Feb_2021	24_Feb_2021	WS2	CW2	W	R2	477.84	0.63
Feb_2021	24_Feb_2021	WS2	CW2	S	R1	756.18	0.70
Feb_2021	24_Feb_2021	WS2	CW2	S	R2	774.16	0.71
Feb_2021	24_Feb_2021	WS2	EFF	W	R1	520.85	0.59
Feb_2021	24_Feb_2021	WS2	EFF	W	R2	595.41	0.59
Mar_2021	08_Mar_2021	WS1	INF	W	R1	666.10	0.67
Mar_2021	08_Mar_2021	WS1	INF	W	R2	645.18	0.65
Mar_2021	08_Mar_2021	WS1	CW1	W	R1	732.37	0.68
Mar_2021	08_Mar_2021	WS1	CW1	W	R2	727.51	0.65

Mar_2021	08_Mar_2021	WS1	CW1	S	R1	579.23	0.56
Mar_2021	08_Mar_2021	WS1	CW1	S	R2	596.38	0.55
Mar_2021	08_Mar_2021	WS1	CW2	W	R1	642.72	0.68
Mar_2021	08_Mar_2021	WS1	CW2	W	R2	657.35	0.66
Mar_2021	08_Mar_2021	WS1	CW2	S	R1	851.63	0.65
Mar_2021	08_Mar_2021	WS1	CW2	S	R2	816.25	0.64
Mar_2021	08_Mar_2021	WS1	EFF	W	R1	392.95	0.49
Mar_2021	08_Mar_2021	WS1	EFF	W	R2	433.15	0.52
Mar_2021	08_Mar_2021	WS2	CW1	W	R1	689.87	0.73
Mar_2021	08_Mar_2021	WS2	CW1	W	R2	736.53	0.72
Mar_2021	08_Mar_2021	WS2	CW2	W	R1	541.52	0.68
Mar_2021	08_Mar_2021	WS2	CW2	W	R2	456.76	0.63
Mar_2021	08_Mar_2021	WS2	CW2	S	R1	847.41	0.73
Mar_2021	15_Mar_2021	WS1	INF	W	R1	655.05	0.70
Mar_2021	15_Mar_2021	WS1	INF	W	R2	635.30	0.66
Mar_2021	15_Mar_2021	WS1	CW1	W	R1	643.72	0.61
Mar_2021	15_Mar_2021	WS1	CW1	W	R2	493.75	0.60
Mar_2021	15_Mar_2021	WS1	CW1	S	R1	1132.11	0.73
Mar_2021	15_Mar_2021	WS1	CW1	S	R2	707.64	0.61
Mar_2021	15_Mar_2021	WS1	CW2	W	R1	588.11	0.60
Mar_2021	15_Mar_2021	WS1	CW2	W	R2	316.55	0.52
Mar_2021	15_Mar_2021	WS1	CW2	S	R1	998.43	0.66
Mar_2021	15_Mar_2021	WS1	CW2	S	R2	1209.28	0.74
Mar_2021	15_Mar_2021	WS1	EFF	W	R1	369.49	0.51
Mar_2021	15_Mar_2021	WS1	EFF	W	R2	500.53	0.60

Mar_2021	15_Mar_2021	WS2	INF	W	R1	593.85	0.56
Mar_2021	15_Mar_2021	WS2	INF	W	R2	664.68	0.62
Mar_2021	15_Mar_2021	WS2	CW1	W	R1	785.17	0.61
Mar_2021	15_Mar_2021	WS2	CW1	W	R2	746.45	0.63
Mar_2021	15_Mar_2021	WS2	CW1	S	R1	1034.14	0.72
Mar_2021	15_Mar_2021	WS2	CW1	S	R2	939.58	0.68
Mar_2021	15_Mar_2021	WS2	CW2	W	R1	598.57	0.63
Mar_2021	15_Mar_2021	WS2	CW2	W	R2	537.91	0.64
Mar_2021	15_Mar_2021	WS2	CW2	S	R1	883.34	0.65
Mar_2021	15_Mar_2021	WS2	CW2	S	R2	880.97	0.66
Mar_2021	15_Mar_2021	WS2	EFF	W	R1	713.25	0.62
Mar_2021	15_Mar_2021	WS2	EFF	W	R2	551.90	0.56
Mar_2021	22_Mar_2021	WS1	INF	W	R1	649.70	0.67
Mar_2021	22_Mar_2021	WS1	INF	W	R2	695.60	0.66
Mar_2021	22_Mar_2021	WS1	CW1	W	R1	586.32	0.54
Mar_2021	22_Mar_2021	WS1	CW1	W	R2	619.54	0.60
Mar_2021	22_Mar_2021	WS1	CW1	S	R1	752.93	0.63
Mar_2021	22_Mar_2021	WS1	CW1	S	R2	730.97	0.57
Mar_2021	22_Mar_2021	WS1	CW2	W	R1	711.75	0.68
Mar_2021	22_Mar_2021	WS1	CW2	W	R2	552.99	0.59
Mar_2021	22_Mar_2021	WS1	CW2	S	R1	1072.23	0.70
Mar_2021	22_Mar_2021	WS1	CW2	S	R2	1054.84	0.72
Mar_2021	22_Mar_2021	WS1	EFF	W	R1	476.99	0.60
Mar_2021	22_Mar_2021	WS1	EFF	W	R2	427.45	0.53

Mar_2021	22_Mar_2021	WS2	INF	W	R1	760.41	0.70
Mar_2021	22_Mar_2021	WS2	INF	W	R2	792.81	0.71
Mar_2021	22_Mar_2021	WS2	CW1	W	R1	705.10	0.61
Mar_2021	22_Mar_2021	WS2	CW1	W	R2	683.95	0.62
Mar_2021	22_Mar_2021	WS2	CW1	S	R1	822.48	0.65
Mar_2021	22_Mar_2021	WS2	CW1	S	R2	1065.75	0.72
Mar_2021	22_Mar_2021	WS2	CW2	W	R1	704.57	0.63
Mar_2021	22_Mar_2021	WS2	CW2	W	R2	785.99	0.65
Mar_2021	22_Mar_2021	WS2	CW2	S	R1	834.98	0.66
Mar_2021	22_Mar_2021	WS2	CW2	S	R2	912.62	0.66
Mar_2021	22_Mar_2021	WS2	EFF	W	R1	545.55	0.58
Mar_2021	22_Mar_2021	WS2	EFF	W	R2	539.63	0.58
Mar_2021	29_Mar_2021	WS1	INF	W	R1	602.82	0.68
Mar_2021	29_Mar_2021	WS1	INF	W	R2	632.62	0.66
Mar_2021	29_Mar_2021	WS1	CW1	W	R1	509.38	0.49
Mar_2021	29_Mar_2021	WS1	CW1	W	R2	490.02	0.53
Mar_2021	29_Mar_2021	WS1	CW1	S	R1	663.36	0.59
Mar_2021	29_Mar_2021	WS1	CW1	S	R2	579.86	0.61
Mar_2021	29_Mar_2021	WS1	CW2	W	R1	301.81	0.53
Mar_2021	29_Mar_2021	WS1	CW2	W	R2	572.49	0.57
Mar_2021	29_Mar_2021	WS1	CW2	S	R1	959.19	0.72
Mar_2021	29_Mar_2021	WS1	CW2	S	R2	847.49	0.63
Mar_2021	29_Mar_2021	WS1	EFF	W	R1	451.21	0.64
Mar_2021	29_Mar_2021	WS1	EFF	W	R2	541.02	0.61
Mar_2021	29_Mar_2021	WS2	INF	W	R1	1084.26	0.75

Mar_2021	29_Mar_2021	WS2	INF	W	R2	828.42	0.68
Mar_2021	29_Mar_2021	WS2	CW1	W	R1	1018.93	0.73
Mar_2021	29_Mar_2021	WS2	CW1	W	R2	991.96	0.74
Mar_2021	29_Mar_2021	WS2	CW1	S	R1	621.87	0.54
Mar_2021	29_Mar_2021	WS2	CW1	S	R2	870.23	0.69
Mar_2021	29_Mar_2021	WS2	CW2	W	R1	535.33	0.58
Mar_2021	29_Mar_2021	WS2	CW2	W	R2	512.36	0.63
Mar_2021	29_Mar_2021	WS2	CW2	S	R1	766.77	0.62
Mar_2021	29_Mar_2021	WS2	CW2	S	R2	936.50	0.71
Mar_2021	29_Mar_2021	WS2	EFF	W	R1	674.29	0.60
Mar_2021	29_Mar_2021	WS2	EFF	W	R2	555.96	0.52
Apr_2021	05_Apr_2021	WS1	INF	W	R1	669.89	0.67
Apr_2021	05_Apr_2021	WS1	INF	W	R2	627.46	0.67
Apr_2021	05_Apr_2021	WS1	CW1	W	R1	577.56	0.54
Apr_2021	05_Apr_2021	WS1	CW1	W	R2	543.81	0.51
Apr_2021	05_Apr_2021	WS1	CW1	S	R1	584.44	0.50
Apr_2021	05_Apr_2021	WS1	CW1	S	R2	519.47	0.53
Apr_2021	05_Apr_2021	WS1	CW2	W	R1	659.70	0.61
Apr_2021	05_Apr_2021	WS1	CW2	W	R2	612.15	0.56
Apr_2021	05_Apr_2021	WS1	CW2	S	R1	705.26	0.62
Apr_2021	05_Apr_2021	WS1	CW2	S	R2	740.65	0.59
Apr_2021	05_Apr_2021	WS1	EFF	W	R1	481.46	0.61
Apr_2021	05_Apr_2021	WS1	EFF	W	R2	524.58	0.62
Apr_2021	05_Apr_2021	WS2	INF	W	R1	786.48	0.70
Apr_2021	05_Apr_2021	WS2	INF	W	R2	909.48	0.74

Apr_2021	05_Apr_2021	WS2	CW1	W	R1	875.93	0.69
Apr_2021	05_Apr_2021	WS2	CW1	W	R2	805.14	0.69
Apr_2021	05_Apr_2021	WS2	CW1	S	R1	1032.78	0.71
Apr_2021	05_Apr_2021	WS2	CW1	S	R2	991.17	0.74
Apr_2021	05_Apr_2021	WS2	CW2	W	R1	810.23	0.71
Apr_2021	05_Apr_2021	WS2	CW2	W	R2	607.54	0.57
Apr_2021	05_Apr_2021	WS2	CW2	S	R1	882.53	0.68
Apr_2021	05_Apr_2021	WS2	CW2	S	R2	876.32	0.66
Apr_2021	05_Apr_2021	WS2	EFF	W	R1	637.21	0.69
Apr_2021	05_Apr_2021	WS2	EFF	W	R2	657.89	0.65
Apr_2021	12_Apr_2021	WS1	INF	W	R1	640.16	0.64
Apr_2021	12_Apr_2021	WS1	INF	W	R2	680.79	0.69
Apr_2021	12_Apr_2021	WS1	CW1	W	R1	642.22	0.51
Apr_2021	12_Apr_2021	WS1	CW1	W	R2	646.06	0.52
Apr_2021	12_Apr_2021	WS1	CW1	S	R1	1098.55	0.75
Apr_2021	12_Apr_2021	WS1	CW1	S	R2	569.90	0.49
Apr_2021	12_Apr_2021	WS1	CW2	W	R1	639.08	0.57
Apr_2021	12_Apr_2021	WS1	CW2	W	R2	596.60	0.55
Apr_2021	12_Apr_2021	WS1	CW2	S	R1	672.05	0.56
Apr_2021	12_Apr_2021	WS1	CW2	S	R2	611.53	0.57
Apr_2021	12_Apr_2021	WS1	EFF	W	R1	628.28	0.60
Apr_2021	12_Apr_2021	WS1	EFF	W	R2	662.17	0.60
Apr_2021	19_Apr_2021	WS2	INF	W	R1	898.47	0.71
Apr_2021	19_Apr_2021	WS2	INF	W	R2	833.58	0.71
Apr_2021	19_Apr_2021	WS2	CW1	W	R1	1019.63	0.70

Apr_2021	19_Apr_2021	WS2	CW1	S	R1	1088.24	0.79
Apr_2021	19_Apr_2021	WS2	CW1	S	R2	1136.45	0.76
Apr_2021	19_Apr_2021	WS2	CW2	W	R1	891.56	0.67
Apr_2021	19_Apr_2021	WS2	CW2	W	R2	944.22	0.66
Apr_2021	19_Apr_2021	WS2	CW2	S	R1	841.98	0.64
Apr_2021	19_Apr_2021	WS2	CW2	S	R2	894.28	0.65
Apr_2021	19_Apr_2021	WS2	EFF	W	R1	829.10	0.65
Apr_2021	19_Apr_2021	WS2	EFF	W	R2	844.65	0.67

Table 7-12 PERMANOVA results of beta diversity analysis with all samples from both systems.

PERMANOVA	Bray-Curtis			UniFrac		
Parameters	R^2	P		R^2	P	
System (WS1 vs WS2)	0.08	0.001	***	**0.07**	0.001	***
Location (Inf, CW1, CW2, Eff)	0.12	0.001	***	**0.09**	0.001	***
Type (Water vs sediment)	0.01	0.003	**	**0.01**	0.001	***
Month	0.12	0.001	***	**0.09**	0.001	***

Table 7-13 PERMANOVA results of beta diversity analysis with all samples from single system.

Samples	PERMANOVA	Bray-Curtis dissimilarity		Unweighted UniFrac	
	Parameters	R^2	P	R^2	P
WS1	Location	0.17	0.001	0.14	0.001
	Type	0.02	0.001	0.02	0.001
	Month	0.27	0.001	0.20	0.001
WS2	Location	0.16	0.001	0.14	0.001
	Type	0.01	0.088	0.01	0.007
	Month	0.24	0.001	0.19	0.001

Table 7-14 PERMANOVA results of beta diversity analysis with samples within each system.

	PERMANOVA	Bray-Curtis dissimilarity		Unweighted UniFrac	
Samples	Parameters	R^2	P	R^2	P
WS1 Influent and effluent	Location	0.14	0.001	0.13	0.001
	Month	0.42	0.001	0.36	0.001
WS2 Influent and effluent	Location	0.10	0.001	0.09	0.001
	Month	0.36	0.001	0.34	0.001
WS1 Water overall	Location	0.20	0.001	0.17	0.001
	Month	0.31	0.001	0.25	0.001
WS2 Water overall	Location	0.16	0.001	0.14	0.001
	Month	0.26	0.001	0.23	0.001
WS1 Wetlands	Location	0.11	0.001	0.08	0.001
	Type	0.04	0.001	0.04	0.001
	Month	0.26	0.001	0.17	0.001
WS2 Wetlands	Location	0.14	0.001	0.10	0.001
	Type	0.02	0.079	0.03	0.005
	Month	0.21	0.001	0.14	0.001

7.3 Chapter 4 - Appendix

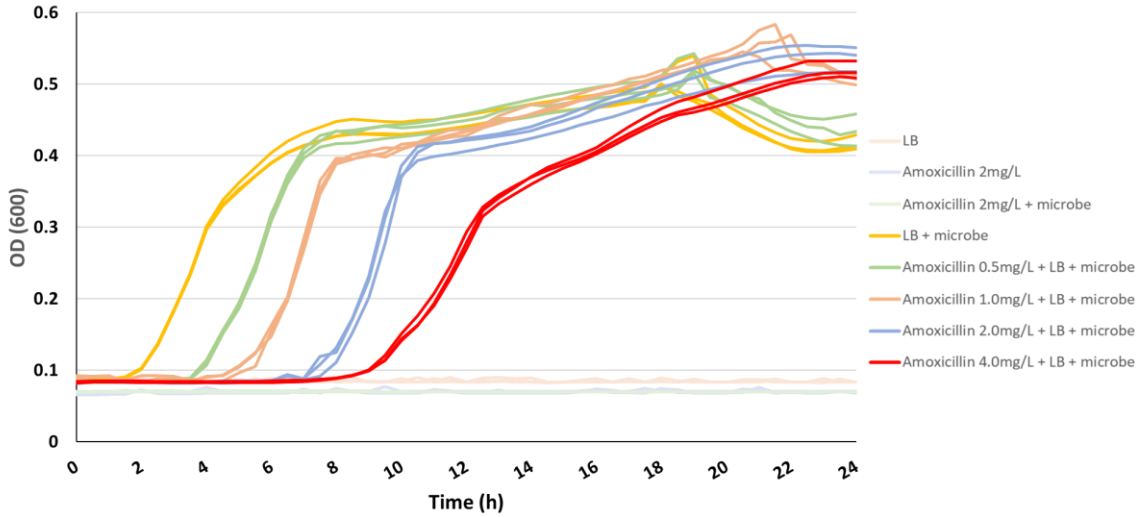

Figure 7-1 Community growth curve measured by OD via plate reader at amoxicillin concentration of 0, 0.5, 1.0, 2.0 and 4.0mg/L for 24h. Negative controls of culture media (LB), 2mg/L amoxicillin, microbe with 2mg/L amoxicillin were included. Each line represents the change of OD value from single well, all conditions were run triplicated.

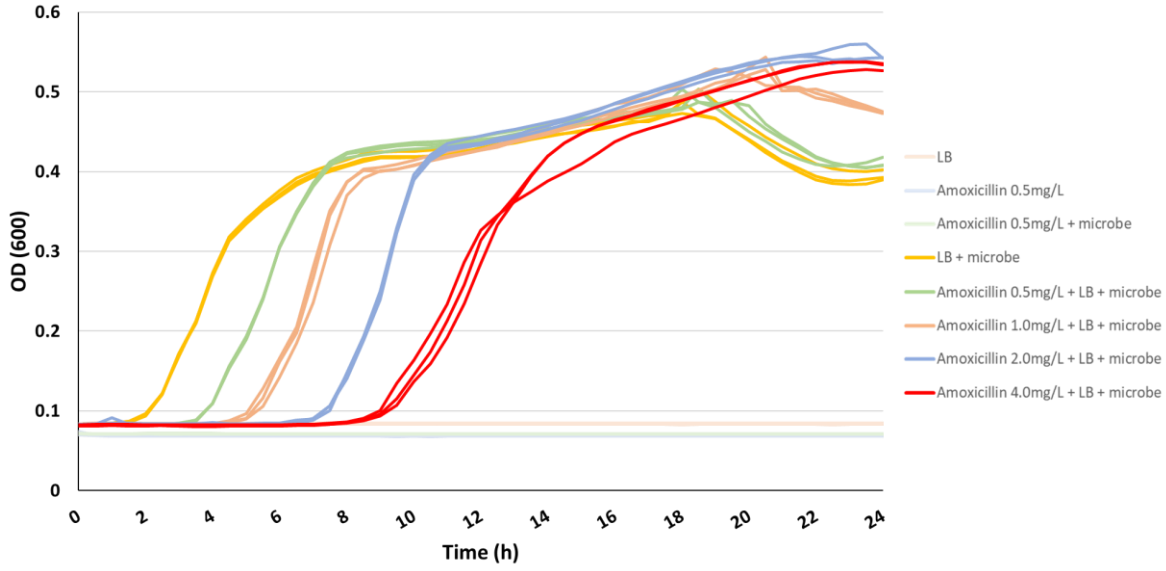

Figure 7-2 Community growth curve measured by OD via plate reader at amoxicillin concentration of 0, 0.5, 1.0, 2.0 and 4.0 mg/L for 24h. Negative controls of culture media (LB), 0.5 mg/L amoxicillin, microbe with 0.5 mg/L amoxicillin were included. Each line represents the change of OD value from single well, all conditions were run triplicated.

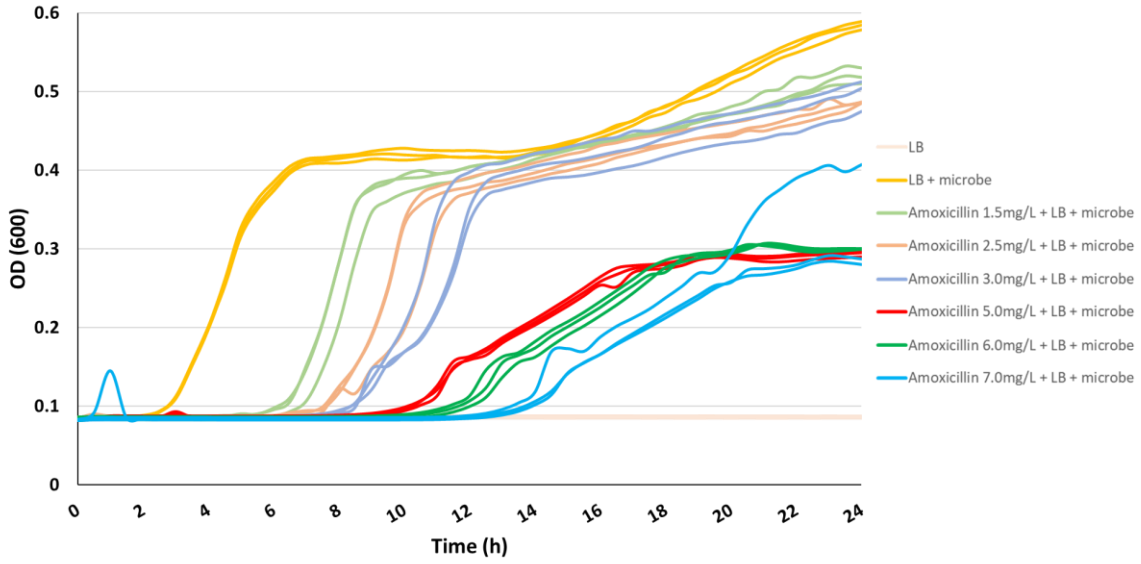

Figure 7-3 Community growth curve measured by OD via plate reader at amoxicillin concentration of 0, 1.5, 2.5, 3.0, 5.0, 6.0 and 7.0 mg/L for 24h. Negative control of culture media (LB) was included. Each line represents the change of OD value from single well, all conditions were run triplicated.

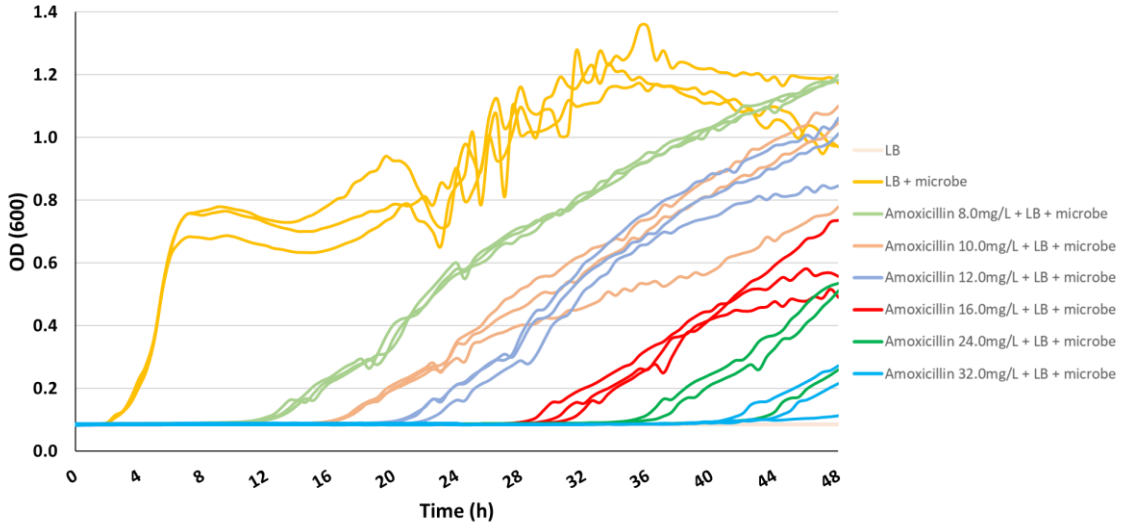

Figure 7-4 Community growth curve measured by OD via plate reader at amoxicillin concentration of 0, 8.0, 10.0, 12.0, 16.0, 24.0 and 32.0 mg/L for 48h. Negative control of culture media (LB) was included. Each line represents the change of OD value from single well, all conditions were run triplicated.

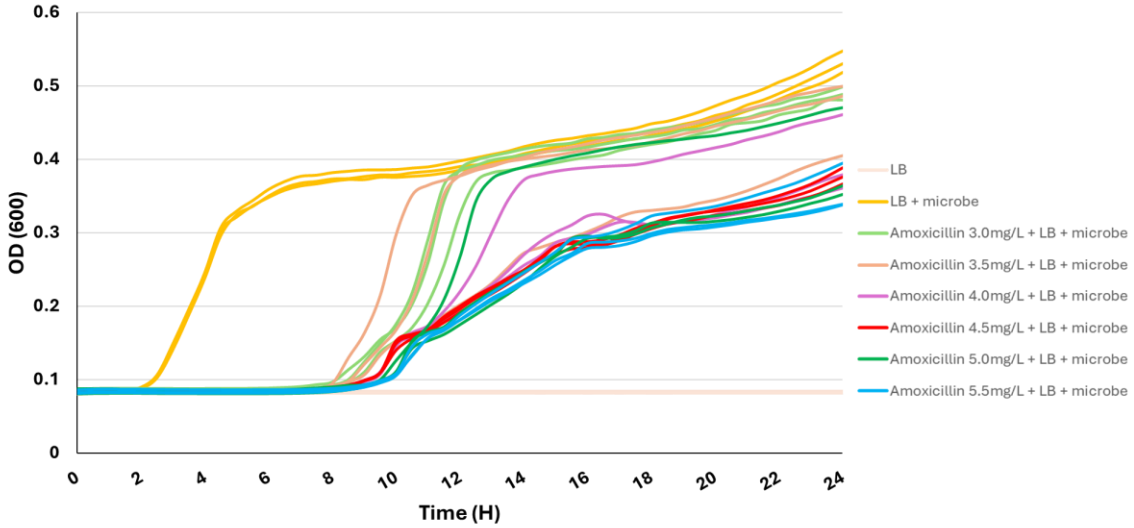

Figure 7-5 Community growth curve measured by OD via plate reader at amoxicillin concentration of 0, 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5 mg/L for 24h. Negative control of culture media (LB) was included. Each line represents the change of OD value from single well, all conditions were run triplicated.

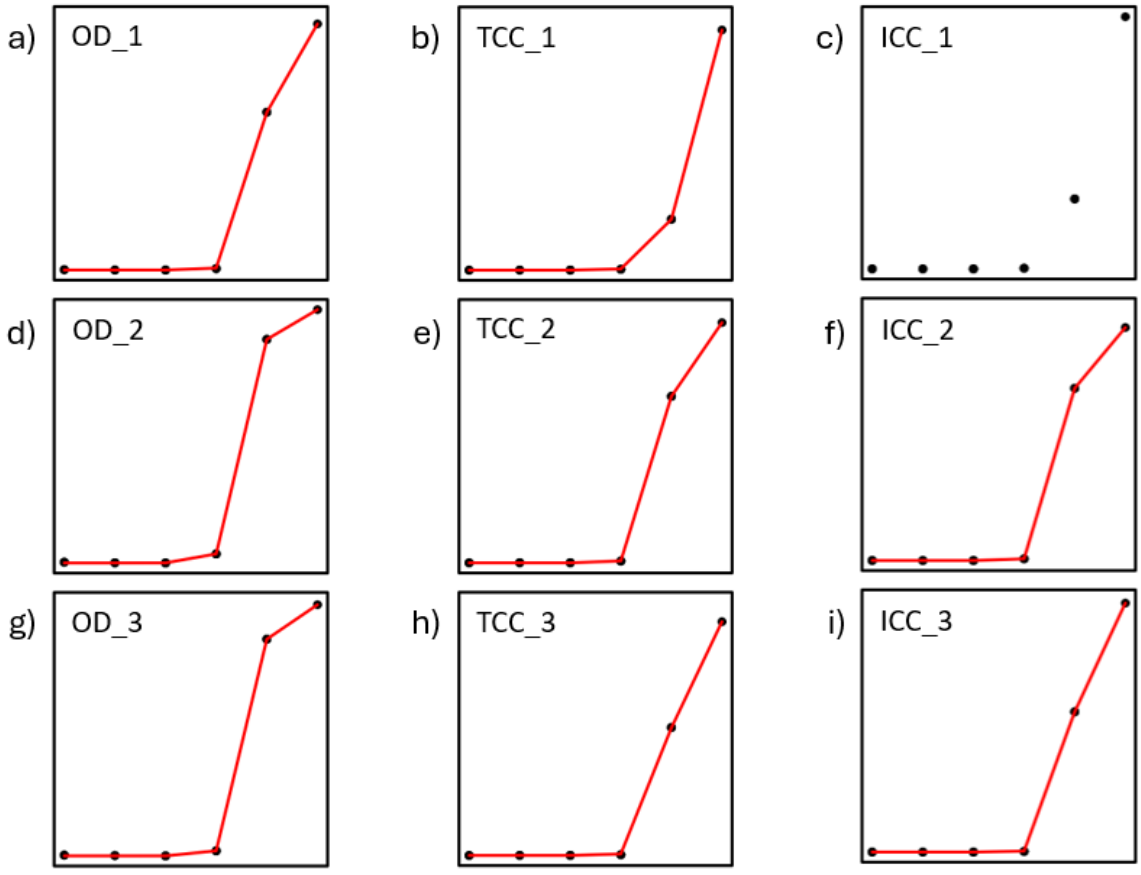

Figure 7-6 Growth curve of microbial communities with 3.0 mg/L amoxicillin measured with OD, TCC and ICC. a), b), c); d), e), f); and g), h), i) were measured from replicate 1; 2; and 3, respectively. a), d) and g) are growth curve measured through OD; b), e) and h) are growth curve measured through TCC; c), f) and i) are growth curve measured through ICC.

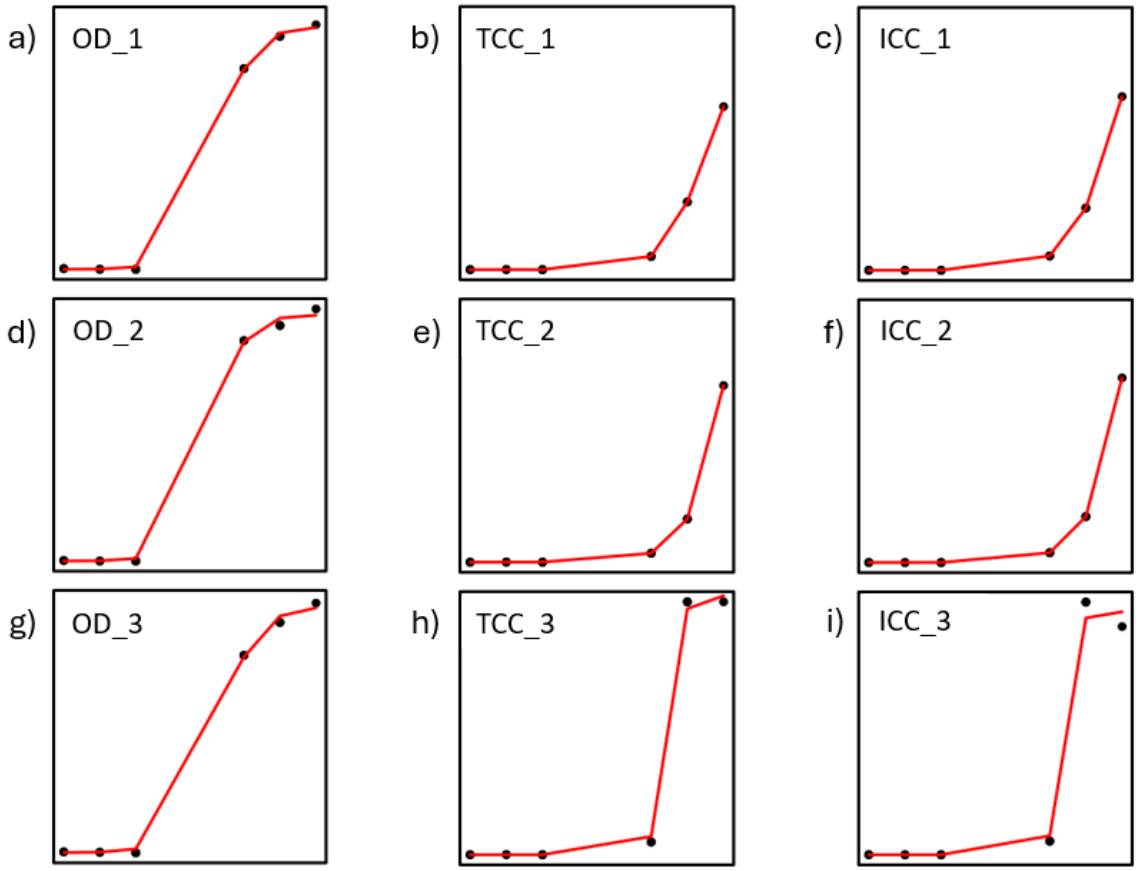

Figure 7-7 Growth curve of microbial communities with 4.5 mg/L amoxicillin measured with OD, TCC and ICC. a), b), c); d), e), f); and g), h), i) were measured from replicate 1; 2; and 3, respectively. a), d) and g) are growth curve measured through OD; b), e) and h) are growth curve measured through TCC; c), f) and i) are growth curve measured through ICC.

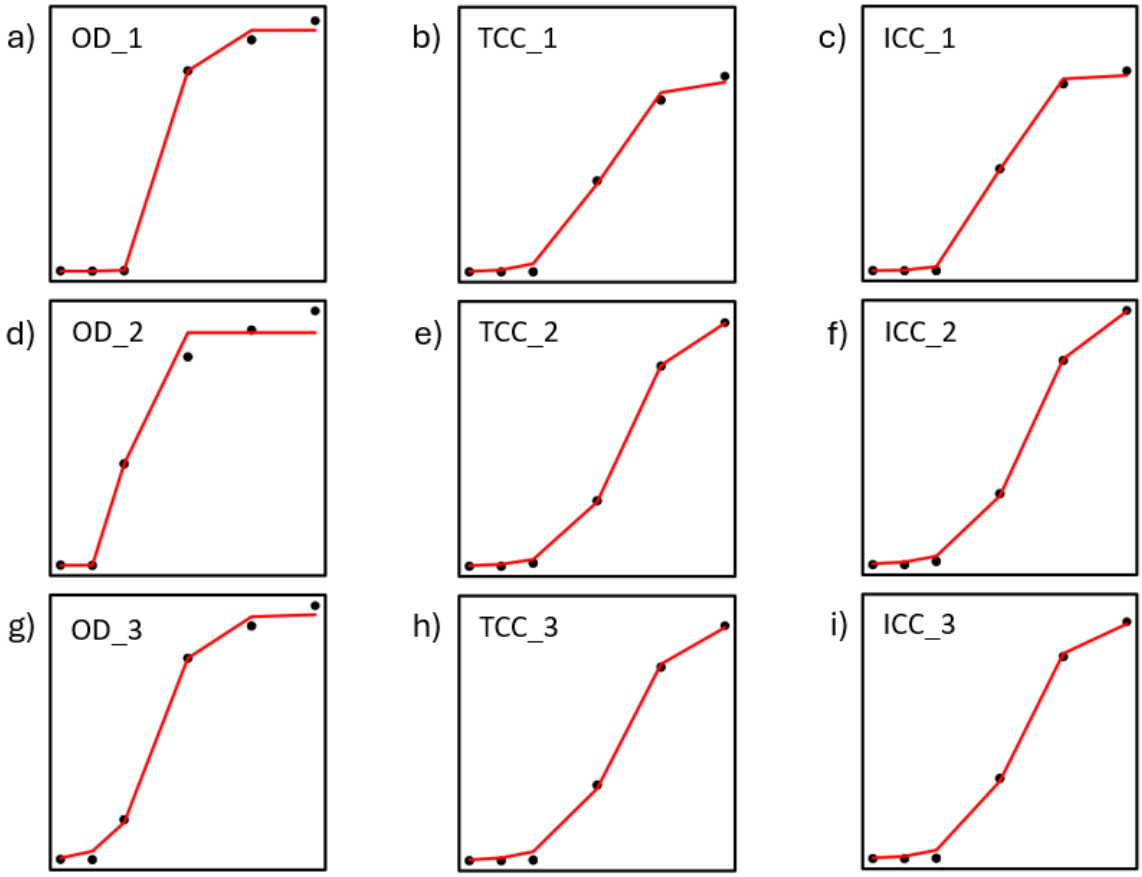

Figure 7-8 Growth curve of microbial communities with 8.0 mg/L amoxicillin measured with OD, TCC and ICC. a), b), c); d), e), f); and g), h), i) were measured from replicate 1; 2; and 3, respectively. a), d) and g) are growth curve measured through OD; b), e) and h) are growth curve measured through TCC; c), f) and i) are growth curve measured through ICC.

7.4 Chapter 5 - Appendix

Table 7-15 List of 216 primer sets and gene targets used in first round's (pooled sample) HT-qPCR.

Assay	Gene	Target antibiotics (major)	Forward Primer	Reverse Primer
AY1	16S rRNA	16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG
AY392	aac(3)-id_ie	Aminoglycoside	AGATAGTTATGCCCGCAACAAG	ACGCGCTGCGCCTATA
AY388	aac3-IVa	Aminoglycoside	CCAACACGACGCTGCATC	GCTGTCGCCACAATGTCG
AY408	aadA10	Aminoglycoside	ACAGGCACTCAACGTCATCG	CGCGGAGAACTCTGCTTTGA
AY15	aadA9_1	Aminoglycoside	CGCGGCAAGCCTATCTTG	CAAATCAGCGACCGCAGACT
AY414	ant4-ib	Aminoglycoside	GATGGCCGCTGACACATG	TCAACATTGCGCCATAGTGG
AY430	ampC_cefa	Beta Lactam	CAGGATCTGATGTGGGAGAACTA	TCGGGAACCATTTGTTGGC
AY117	ampC/blaDHA	Beta Lactam	TGGCCGCAGCAGAAAGA	CCGTTTTATGCACCCAGGAA
AY443	beta_B2	Beta Lactam	GTAACGCCTACTGGAAGTCCA	CAGCTTCTCCTTGAGAATGCAG
AY441	beta_ccra	Beta Lactam	CACTGGCACGGCGATTGTA	CGGCAGCCAAACCACGATA
AY442	bl1acc	Beta Lactam	TGTTATCCGTGATTACCTGTCTGG	CTCAGCGAGCCAACTTCAAATA
AY113	bla-L1	Beta Lactam	CACCGGGTTACCAGCTGAAG	GCGAAGCTGCGCTTGTAGTC
AY338	bla1	Beta Lactam	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA
AY336	blaACC	Beta Lactam	CACACAGCTGATGGCTTATCTAAAA	AATAAACGCGATGGGTTCCA
AY444	blaACT	Beta Lactam	AAGCCGCTCAAGCTGGA	GCCATATCCTGCACGTTGG
AY454	blaADC-nonmobile	Beta Lactam	GGTATGGCTGTGGGTGTTATTCA	AGGCAAGGTTACCACTTGTATACG
AY445	blaB	Beta Lactam	CGTGCCGGAGGTCTTGAATA	GGGATAGTAAACCTGAAACTCGGA
AY453	blaBEL-nonmobile	Beta Lactam	ATGTCCATGGCACAGACTGTG	CCTGTCTTGTCACCCGTTACC
AY446	blaCARB	Beta Lactam	TGATTTGAGGGATACGACAACTCC	CTGTAATACTCCGAGCACCAA
AY339	blaCMY_2	Beta Lactam	AAAGCCTCAT GGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA
AY432	blaCTX-M	Beta Lactam	CGTACCGAGCCGACGTTAA	CAACCCAGGAAGCAGGCA
AY134	blaCTX-M_5	Beta Lactam	GCGATAACGTGGCGATGAAT	GTCGAGACGGAACGTTTCGT

AY147	blaCTX-M_8	Beta Lactam	CGTCACGCTGTTGTTAGGAA	CGCTCATCAGCACGATAAAG
AY433	blaFOX	Beta Lactam	CCTACGGCTATTCGAAGGAAGATAAG	CCGGATTGGCCTGGAAGC
AY125	blaGES	Beta Lactam	GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG
AY447	blaGOB	Beta Lactam	CTTGGGCTTGAATGCTCAGGTA	TGTATGGTCGTAGTGAGCCTGA
AY448	blaHERA	Beta Lactam	GGGCAACCGCATTCTGAC	GCATCTCCCACTTTATCGTCAC
AY449	blaIMI	Beta Lactam	ACATCTACACCTGCAGCAGTAG	AATCGCTTGGTACGCTAGCA
AY450	blaIND	Beta Lactam	CGCCTGTTAAACCCAACCTGTA	CGCTCTGTCATCATGAGAGTGG
AY440	blaKPC	Beta Lactam	GCCGCCAATTTGTTGCTGAA	GCCGGTCGTGTTTCCCTTT
AY451	blaLEN	Beta Lactam	TGTTCGCCTGTGTGTTATCTCC	GCAGCACTTTAAAGGTGCTCAC
AY452	blaMIR	Beta Lactam	CGGTCTGCCGTTACAGGTG	AAAGACCCGCGTCGTCATG
AY101	blaMOX/blaCMY	Beta Lactam	CTATGTCAATGTGCCGAAGCA	GGCTTGTCCTCTTTCGAATAGC
AY152	blaNDM	Beta Lactam	GGCCACACCAGTGACAATATCA	CAGGCAGCCACCAAAAGC
AY102	blaOCH	Beta Lactam	GGCGACTTGCGCCGTAT	TTTTCTGCTCGGCCATGAG
AY601	blaOXA48	Beta Lactam	TGTTTTTGGTGGCATCGAT	GTAAMRATGCTTGGTTCGC
AY435	blaOXA51	Beta Lactam	CGACCGAGTATGTACCTGCTTC	TCAAGTCCAATACGACGAGCTA
AY108	blaOXY	Beta Lactam	CGTTCAGGCGGCAGGTT	GCCGCGATATAAGATTTGAGAATT
AY436	blaOXY1	Beta Lactam	AAAGGTGACCGCATTCGC	CCAGCGTCAGCTTGCG
AY103	blaPAO	Beta Lactam	CGCCGTACAACCGGTGAT	GAAGTAATGCGGTTCTCCTTTCA
AY437	blaPER	Beta Lactam	GCAAATGAAGCGCAGATGC	GACCACAGTACCAGCTGGTA
AY109	blaPSE	Beta Lactam	TTGTGACCTATTCCCCTGTAATAGAA	TGCGAAGCACGCATCATC
AY107	blaROB	Beta Lactam	GCAAAGGCATGACGATTGC	CGCGCTGTTGTCGCTAAA
AY126	blaSFO	Beta Lactam	CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT
AY438	blaSHV11	Beta Lactam	TTGACCGCTGGGAAACGG	TCCGGTCTTATCGGCGATAAAC
AY431	blaSME	Beta Lactam	GAGGAAGACTTTGATGGGAGGATTG	CGCTATATTGCAATGCAGCAGAAG
AY439	blaTEM	Beta Lactam	CGCCGCATACACTATTCTCAG	GCTTCATTCAGCTCCGGTTC
AY127	blaTLA	Beta Lactam	ACACTTTGCCATTGCTGTTTATGT	TGCAAATTTCGGCAATAATCTTT
AY105	blaVEB	Beta Lactam	CCCGATGCAAAGCGTTATG	GAAAGATTCCCTTTATCTATCTCAGACAA

AY129	blaVIM	Beta Lactam	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT
AY128	blaZ	Beta Lactam	GGAGATAAAGTAACAAATCCAGTTAGATATGA	TGCTTAATTTTCCATTTGCGATAAG
AY115	cepA	Beta Lactam	AGTTGCGCAGAACAGTCCTCTT	TCGTATCTTGCCCGTCGATAAT
AY97	cfiA	Beta Lactam	GCAGCGTTGCTGGACACA	GTTCGGGATAAACGTGGTGACT
AY114	cfxA	Beta Lactam	TCATTCCTCGTTCAAGTTTTCAGA	TGCAGCACCAAGAGGAGATGT
AY111	cphA_1	Beta Lactam	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC
AY434	imiR_2	Beta Lactam	AGCCGGACTAGAGCTTCATG	GGCAGAACTCATCATCTGCAAA
AY133	mecA	Beta Lactam	GGTTACGGACAAGGTGAAATACTGAT	TGTCTTTTAATAAGTGAGGTGCGTTAATA
AY132	pbp	Beta Lactam	CCGGTGCCATTGGTTTAGA	AAAATAGCCGCCCCAAGATT
AY131	pbp5	Beta Lactam	GGCGAACTTCTAATTAATCCTATCCA	CGCCGATGACATTCTTCTTATCTT
AY138	penA	Beta Lactam	AGACGGTAACGTATAACTTTTTGAAAGA	GCGTGTAGCCGGCAATG
AY293	intI1_1	Integrons	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA
AY289	intI1_2	Integrons	CGAAGTCGAGGCATTTCTGTC	GCCTTCCAGAAAACCGAGGA
AY294	intI2_2	Integrons	TGCTTTTCCCACCCTTACC	GACGGCTACCCTCTGTTATCTC
AY500	intI3	Integrons	CAGGTGCTGGGCATGGA	CCTGGGCAGCATCACCA
AY207	acrA_1	MDR	GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC
AY199	acrB_1	MDR	AGTCGGTGTTCGCCGTTAAC	CAAGGAAACGAACGCAATACC
AY201	acrF	MDR	GCGGCCAGGCACAAAA	TACGCTCTTCCCACGGTTTC
AY355	acrR_1	MDR	GCGCTGGAGACACGACAAC	GCCTTGCTGCGAGAACAAA
AY202	adeA	MDR	CAGTTCGAGCGCCTATTTCTG	CGCCCTGACCGACCAAT
AY483	adeI	MDR	CAGTCTGGTTTGCAGTAACCA	CACTCCTACAACAACAGGCAA
AY490	arsA	MDR	CAGGTCAGCCGCATCAACC	GCCTGAAACACGGCAATTTCTTC
AY484	bexA/norM	MDR	TCGGGCATCCCGTTTATGATC	GTAGGCTGCGCATAATACCCA
AY491	cadC	MDR	CGCTCTGTGTCAGGATGAAGAG	CTTTCTTATGTGCTAGGGCGATCA
AY487	cefa_qacelta	MDR	TAGTTGGCGAAGTAATCGCAAC	TGCGATGCCATAACCGATTATG
AY234	cfr	MDR	GCAAAATTCAGAGCAAGTTACGAA	AAAATGACTCCCAACCTGCTTTAT
AY206	cmr	MDR	CGGCATCGTCAGTGGAATT	CGGTTCCGAAAAAGATGGAA

AY492	copA	MDR	TGCACCTGACVGGSCAYAT	GVACTTCRCGGAACATRCC
AY493	czcA	MDR	GCCTTGTTCATCGGCGAAC	GGCAATGTCGCCTTCGTTC
AY219	emrB/qacA_1	MDR	CTTTTCTCTAACCGTACATTATCTACGATAAA	AGAACGTAGCGACTGATAAAATGCT
AY208	emrD_1	MDR	CTCAGCAGTATGGTGGTAAGCATT	ACCAGGCGCCGAAGAAC
AY360	marR_3	MDR	GCTGTTGATGACATTGCTCACA	CGGCGTACTGGTGAAGCTAAC
AY350	mdsA	MDR	CGGAGTCCATCGACCATTTG	ATCGTCGGCAAGGAGAATCA
AY485	mdtA	MDR	ACAAGCCCAGGGCCAAC	CCTTAATGGTGCCTTCGGTTTC
AY211	mdtE	MDR	CGTCGGCGCACTCGTT	TCCAGACGTTGTACGGTAACCA
AY212	mdtG_1	MDR	TGGCACAAAATATCTGGCAGTT	TTGTGTGGCGATAAGAGCATTAG
AY486	mdtH	MDR	ATGCTGGCTGTACAAGTGATG	CACTCCAGCGGGCGATA
AY227	mepA	MDR	ATCGGTCGCTCTTCGTTCAC	ATAAATAGGATCGAGCTGCTGGAT
AY215	mexA	MDR	AGGACAACGCTATGCAACGAA	CCGGAAAGGGCCGAAAT
AY240	mexB	MDR	CTGGAGATCGACGACGAGAAG	GAAATCGTTGACGTAGCTGGAA
AY228	mexE	MDR	GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC
AY222	mtrE	MDR	CGATGTGTCGTTTTGGAAGGT	CCTGCACCATGATTCCTCAATA
AY224	oprD	MDR	ATGAAGTGGAGCGCCATTG	GGCCACGGCGAACTGA
AY482	oqxA	MDR	GAGTCAACCTACCTCCACTATCA	GCTGCGAGTTATCCAGCAG
AY494	pbrT	MDR	GATGCGCACTGGGCTTG	TCGGAATATGCGGAAATGCG
AY495	pcoA	MDR	TGGCGTATGGAGTTTCAATGC	GAATAATGCCGTGCCAGTGAA
AY42	pmrA	MDR	TTTGCAGGTTTTGTTCCTAATGC	GCAGAGCCTGATTTCTCCTTTG
AY488	qacA/B	MDR	AAGGGCCACTGCATTAGCTG	CCAGTCCAATCATGCCTGCA
AY489	qacF/H	MDR	CTGAAGTCTAGCCATGGATTCACTAG	CAAGCAATAGCTGCCACAAGC
AY497	sugE	MDR	CTTAGTTATTGCTGGTCTGCTGGA	GCATCGGGTTAGCGGACTC
AY498	tcrB	MDR	GTGCCGGAACTCAAGTAGCA	GCACCGACTGCTGGACTTAA
AY499	terW	MDR	TCAAAGAGCTACGCGAGTCATA	CCTTCCCTGTGGACTCACC
AY353	tolC_2	MDR	CAGGCAGAGAACCTGATGCA	CGCAATTCCGGGTTGCT
AY226	ttgA	MDR	ACGCCAATGCCAAACGATT	GTCACGGCGCAGCTTGA

AY501	cro	MGE	AGATGTTATCGACCACTTCGGA	CCGCTTGGCGATAAGCG
AY502	EAE_05855	MGE	CCCATCACCGCTGAACTGG	TGGGCGCTGCCATCTAAAC
AY503	IncHI2-smr0018	MGE	ATAATGATTCACCGGGGTAG	CTTCAGGCTATCGTTTCG
AY504	IncI1_repI1	MGE	CGAAAGCCGGACGGCAGAA	TCGTCGTTCCGCCAAGTTCGT
AY505	IncN_korA	MGE	GGAACGTTTGTAYCTTGTATTG	ACTCACTATCTTCTGTTGATTG
AY317	IncN_oriT	MGE	TTGGGCTTCATAGTACCC	GTGTGATAGCGTGATTTATGC
AY316	IncN_rep	MGE	AGTTCACCACCTACTCGCTCCG	CAAGTTCTTCTGTTGGGATTCCG
AY318	IncP_oriT	MGE	CAGCCTCGCAGAGCAGGAT	CAGCCGGGCAGGATAGGTGAAGT
AY319	IncQ_oriT	MGE	TTCGCGCTCGTTGTTCTTCGAGC	GCCGTTAGGCCAGTTTCTCG
AY320	IncW_trwAB	MGE	AGCGTATGAAGCCCGTGAAGGG	AAAGATAAGCGGCAGGACAATAACG
AY313	IS1111	MGE	GTCTTAAGGTGGGCTGCGTG	CCCCGAATCTCATTGATCAGC
AY314	IS1133	MGE	GCAGCGTCGGGTTGGA	ACGCGTTCGAACAACTGTAATG
AY506	IS1247_1	MGE	CGGCCGTCACTGACCAA	TCGGCAGGTTGGTGACG
AY310	IS1247_2	MGE	TGGATCGACCGGTTCCAT	GCTGACCGAGCTGTCCATGT
AY508	IS200_1	MGE	CCAAATACCGAAGACAAGCGTTC	CCAAACTGCTCGTAAAGCATCAG
AY509	IS200_2	MGE	GCACACCCGATGGAACTGTAAA	TCGGCGGGATCTCCAGAAG
AY510	IS21-ISAs29	MGE	GGTCCGTCAGGCACAAGTC	GGGATCGTATCGGCAAGCC
AY511	IS256	MGE	CTTGCGCATCATTGGATGATGG	AAGAACGGCTCCAATTAAGCGA
AY512	IS26_1	MGE	ATGGATGAAACCTACGTGAAGGTC	CGGTACTTAATCTGTCGGTGTTCA
AY513	IS3	MGE	CGGTCTGAGCTTCGGGAA	AGAACTGTCACTCCGGTCTG
AY514	IS5/IS1182	MGE	TTCTCGAAGAATCGCCATGGC	GCTTTGGATCGCTCCAATCGA
AY515	IS6/257	MGE	ATATCGTGCCATTGATGCAGAG	ACCATTGCTACCTTCGTTGAAG
AY516	IS6100	MGE	CGCACCGGCTTGATCAGTA	CTGCCACGCTCAATACCGA
AY298	IS613	MGE	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT
AY517	IS630	MGE	CCGCCACCAGTGTGATGG	TTGGCGCTGACTGGATGC
AY311	ISAba3	MGE	TCAGAGGCAGCGGTATACGA	GGTTGATTCAGTTAAAGTACGTAAAACTTT
AY519	ISCR1	MGE	ATGGTTTCATGCGGGTT	CTGAGGGTGTGAGCGAG

AY520	ISEcp1	MGE	CATGCTCTGCGGTCACTTC	GACGCACCTTCTTGATGACC
AY312	ISEfm1	MGE	AGGTGTCCATGACGTGAAAGTG	TCCTTTGTCCCCTAGGATATTGG
AY309	ISPps	MGE	CACACTGCAAAAACGCATCCT	TGTCTTTGGCGTCACAGTTCTC
AY521	lncF_FIC	MGE	GTGAACTGGCAGATGAGGAAGG	TTCTCCTCGTCGCCAAACTAGAT
AY307	orf37-IS26	MGE	GCCGGGTTGTGCAAATAGAC	TGGCAATCTGTCGCTGCTG
AY324	pAKD1	MGE	GGTAAGATTACCGATAAACT	GTTCGTGAAGAAGATGTA
AY321	pAMBL	MGE	CAGGCTCTTAATGTGATA	TTATGCTCAATACTCGTG
AY523	Tn3	MGE	GCTGAGGTGTTCAGCTACATCC	GCTGAGGTAGTCACAGGCATTC
AY315	Tn5	MGE	TCAGAGGCAGCGGTATACGA	GGTTGATTCAGTTAAAGTACGTAAAACTTT
AY524	Tn5403	MGE	AAGCGAATGGCGCGAAC	CGCGCAGGGTAAACTGC
AY299	tnpA_1	MGE	GCCGCACTGTCGATTTTTATC	GCGGGATCTGCCACTTCTT
AY300	tnpA_2	MGE	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC
AY301	tnpA_3	MGE	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT
AY302	tnpA_4	MGE	CATCATCGGACGGACAGAATT	GTCGGAGATGTGGGTGTAGAAAGT
AY303	tnpA_5	MGE	GAAACCGATGCTACAATATCCAATTT	CAGCACCGTTTGCAGTGTAAG
AY304	tnpA_6	MGE	TGCAGATGGTTTAACCTTGGATATTT	TCGGTTCATCAAACTGCTTCAC
AY305	tnpA_7	MGE	AATTGATGCGGACGGCTTAA	TCACCAAACTGTTTATGGAGTCGTT
AY297	Tp614	MGE	GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT
AY526	traN	MGE	GCTTGGCGGTCAGCAATT	TTAGGAATAACAATCGCTACACCTTTA
AY527	trbC	MGE	CGGYATWCCGSCSACRCTGCG	GCCACCTGYSBGCAGTCMCC
AY306	trfA	MGE	ACGAAGAAATGGTTGTCCTGTTC	CGTCAGCTTGCGGTACTTCTC
AY530	erm34	MLSB	AAAGCGGTTTACAAGCGTTTCG	GGGTGCTCTAGGGTTGTTTAGTG
AY531	erm35	MLSB	CCTTCAGTCAGAACCGGCAA	GCTGATTTGACAGTTGGTGGTG
AY90	ermA/ermTR	MLSB	ACATTTTACCAAGGAACTTGTGGAA	GTGGCATGACATAAACCTTCATCA
AY545	ermC_2	MLSB	CCCTTGAATTAGTACAGAGGTG	GCAAACTCGTATTCCACGA
AY44	ermD/K	MLSB	GAGCCGCAAGCCCCTTT	GTGTTTCATTTGACGCGGAGTAA
AY57	ermT_1	MLSB	GTTCACTAGCACTATTTTTAATGACAGAAGT	GAAGGGTGTCTTTTTAATACAATTAACGA

AY68	ermX_1	MLSB	GCTCAGTGGTCCCCATGGT	ATCCCCCCGTCAACGTTT
AY53	lmrA_1	MLSB	TTCAGATGCAATGGCGTTTG	ATAATCGGGAACATAATGAGCATAACTAC
AY536	lnuB	MLSB	GGATCGTTTACCAAAGGAGAAGG	AGCATAGCCTTCGTATCAGGAA
AY537	lnuC	MLSB	GGGTGTAGATGCTCTTCTTGGA	CTTTACCCGAAAGAGTTTCTACCG
AY538	mefA	MLSB	TAATTATCGCAGCAGCTGGTTC	GTTCCCAAACGGAGTATAAGAGTG
AY61	mphB	MLSB	CGCAGCGCTTGATCTTGTAG	TTACTGCATCCATACGCTGCTT
AY66	msrA_1	MLSB	CTGCTAACACAAGTACGATTCCAAAT	TCAAGTAAAGTTGTCTTACCTACACCATT
AY58	msrC_1	MLSB	TCAGACCGGATCGGTTGTC	CCTATTTTTTGGAGTCTTCTCTCTAATGTT
AY91	oleC	MLSB	CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG
AY73	pncA	MLSB	GCAATCGAGGCGGTGTTC	TTGCCGCAGCCAATTCA
AY77	vatE_2	MLSB	GACCGTCCTACCAGGCGTAA	TTGGATTGCCACCGACAATT
AY541	vga(A)LC_1	MLSB	GTGAAGATGTCTCGGGTACAATTG	GAAATACCAGGATTCCCATGCAC
AY71	vgaA_1	MLSB	CGAGTATTGTGGAAAGCAGCTAGTT	CCCGTACCGTTAGAGCCGATA
AY555	cat	Phenicol	ATCGGCCAGACTGGATATCGA	CACAGCTCCAGTTGCAACAAC
AY35	cmlA_2	Phenicol	TAGGAAGCATCGGAACGTTGAT	CAGACCGAGCACGACTGTTG
AY33	yidY/mdtL	Phenicol	GCAGTTGCATATCGCCTTCTC	CTTCCCGGCAAACAGCAT
AY96	qnrB	Quinolone	GCGACGTTCAGTGGTTCAGA	GCTGCTCGCCAGTCGAA
AY462	qnrVC1_VC3_VC6	Quinolone	CTCACATCAGGACTTGCAAGAA	ATGAAGCATCTCGAAGATCAGC
AY247	folA_1	Sulfonamide	CGAGCAGTTCCTGCCAAAG	CCCAGTCATCCGGTTCATAATC
AY361	folP_2	Sulfonamide	CAGGCTCGTAAATTGATAGCAGAAG	CTTTCCTTGCGAATCGCTTT
AY245	sul1_2	Sulfonamide	GCCGATGAGATCAGACGTATTG	CGCATAGCGCTGGGTTTC
AY365	sul2_2	Sulfonamide	TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT
AY241	sul4	Sulfonamide	TCAACGTCACTCCAGACAGC	TGGAAATAACGACGTCCACA
AY250	tet32	Tetracycline	CCATTACTTCGGACAACGGTAGA	CAATCTCTGTGAGGGCATTTAACA
AY249	tet36_1	Tetracycline	AGAATACTCAGCAGAGGTCAGTTCCT	TGGTAGGTCGATAACCCGAAAAT
AY570	tet38	Tetracycline	AAGCGACATTAGCCGGTTTAG	CTGCTCGTACTTAAGCCAAGG
AY568	tet39	Tetracycline	TATAGCGGGTCCGGTAATAGGTG	CCATAACGATCCTGCCCATAGATAAC

AY576	tet44	Tetracycline	CTCATGTAGATGCAGGAAAGACG	GTAACTGCTGCCTGAATTGTGA
AY254	tetA_2	Tetracycline	CTCACCAGCCTGACCTCGAT	CACGTTGTTATAGAAGCCGCATAG
AY255	tetA/B_1	Tetracycline	AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA
AY268	tetC_2	Tetracycline	ACTGGTAAGGTAAACGCCATTGTC	ATGCATAAACCAGCCATTGAGTAAG
AY571	tetD	Tetracycline	AATTGCACTGCCTGCATTGC	GACAGATTGCCAGCAGCAGA
AY273	tetE	Tetracycline	TTGGCGCTGTATGCAATGAT	CGACGACCTATGCGATCTGA
AY572	tetG	Tetracycline	TCGCGTTCCTGCTTGCC	CCGCGAGCGACAAACCA
AY260	tetH	Tetracycline	TTTGGGTCATCTTACCAGCATTAA	TTGCGCATTATCATCGACAGA
AY573	tetJ	Tetracycline	CAGCGCCCATACGCCATTTA	CCTACTTCAGTAGTGTGCCAAGC
AY258	tetK	Tetracycline	CAGCAGTCATTGGAAAATTATCTGATTATA	CCTTGTACTAACCTACCAAAAATCAAAATA
AY367	tetL_2	Tetracycline	ATGGTTGTAGTTGCGCGCTATAT	ATCGCTGGACCGACTCCTT
AY574	tetM	Tetracycline	GGAGCGATTACAGAATTAGGAAGC	TCCATATGTCCTGGCGTGTC
AY264	tetO_2	Tetracycline	CAACATTAACGGAAAGTTTATTGTATACCA	TTGACGCTCCAAATTCATTGTATC
AY575	tetPA	Tetracycline	GGAAACCTTAGTTCAGTGACTTGG	CCCATTTAACCACGCACTGAA
AY274	tetPB_1	Tetracycline	TGGGCGACAGTAGGCTTAGAA	TGACCCTACTGAAACATTAGAAATATACCT
AY259	tetQ	Tetracycline	CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT
AY577	tetR	Tetracycline	CCGTCAATGCGCTGATGAC	GCCAATCCATCGACAATCACC
AY325	tetR_1	Tetracycline	CAATCCATCGACAATCAC	GACAATCAGCTACTTCAC
AY269	tetS	Tetracycline	TTAAGGACAAACTTTCTGACGACATC	TGTCTCCCATTGTTCTGGTTCA
AY276	tetT	Tetracycline	CCATATAGAGGTTCCACCAAATCC	TGACCCTATTGGTAGTGGTTCTATTG
AY263	tetW	Tetracycline	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC
AY267	tetX	Tetracycline	AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT
AY284	dfrA1_1	Trimethoprim	GGAATGGCCCTGATATTCCA	AGTCTTGCGTCCAACCAACAG
AY580	dfrA15	Trimethoprim	AGGCCGAAAGACTTTCGAGTC	TCACCTTCTGGCTCAATGTCG
AY584	dfrA22	Trimethoprim	CAGCCGAACACGGCAAAG	CGGAGTGCGTGTACGTGA
AY588	dfrA7	Trimethoprim	GTAATCGGTAGTGGTCCTGA	ATCAGGACCACTACCGATTAC
AY590	dfrAB4	Trimethoprim	CGGTTCGCATTCCCATCAAA	CGCAGTCATGGGATAAATCTGG

AY595	vanA	Vancomycin	GGGCTGTGAGGTCGGTTG	TTCAGTACAATGCGGCCGTTA
AY159	vanB_1	Vancomycin	TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT
AY596	vanC2	Vancomycin	TGACTGTCGGTGCTTGTGA	GATAGAGCAGCTGAGCTTGTTC
AY161	vanD	Vancomycin	CAGAGGAACATAATGTTTCGATAAAATCT	GCCGGATTTTGTGATTCCAA
AY176	vanRC4	Vancomycin	AGTGCTTTGGCTTATCTCGAAAA	TCCGGCAGCATCACATCTAA

Table 7-16 List of 54 primer sets and gene targets used in second round's HT-qPCR.

Assay	Gene	Target antibiotics (major)	Forward Primer	Reverse Primer
AY1	16S rRNA	16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG
AY117	ampC/blaDHA	Beta Lactam	TGGCCGCAGCAGAAAGA	CCGTTTTATGCACCCAGGAA
AY442	bl1acc	Beta Lactam	TGTTATCCGTGATTACCTGTCTGG	CTCAGCGAGCCAACTTCAAATA
AY113	bla-L1	Beta Lactam	CACCGGGTTACCAGCTGAAG	GCGAAGCTGCGCTTGTAGTC
AY338	bla1	Beta Lactam	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA
AY445	blaB	Beta Lactam	CGTGCCGGAGGTCTTGAATA	GGGATAGTAAACCTGAAACTCGGA
AY446	blaCARB	Beta Lactam	TGATTTGAGGGATACGACAACTCC	CTGTAATACTCCGAGCACCAA
AY125	blaGES	Beta Lactam	GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG
AY440	blaKPC	Beta Lactam	GCCGCCAATTTGTTGCTGAA	GCCGGTCGTGTTTCCCTTT
AY451	blaLEN	Beta Lactam	TGTTCGCCTGTGTGTTATCTCC	GCAGCACTTTAAAGGTGCTCAC
AY109	blaPSE	Beta Lactam	TTGTGACCTATTCCCCTGTAATAGAA	TGCGAAGCACGCATCATC
AY438	blaSHV11	Beta Lactam	TTGACCGCTGGGAAACGG	TCCGGTCTTATCGGCGATAAAC
AY207	acrA_1	MDR	GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC
AY199	acrB_1	MDR	AGTCGGTGTTCGCCGTTAAC	CAAGGAAACGAACGCAATACC
AY355	acrR_1	MDR	GCGCTGGAGACACGACAAC	GCCTTGCTGCGAGAACAAA
AY206	cmr	MDR	CGGCATCGTCAGTGGAATT	CGGTTCCGAAAAAGATGGAA
AY219	emrB/qacA_1	MDR	CTTTTCTCTAACCGTACATTATCTACGATAAA	AGAACGTAGCGACTGATAAAATGCT
AY212	mdtG_1	MDR	TGGCACAAAATATCTGGCAGTT	TTGTGTGGCGATAAGAGCATTAG

AY228	mexE	MDR	GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC
AY222	mtrE	MDR	CGATGTGTCGTTTTGGAAGGT	CCTGCACCATGATTCCTCAATA
AY495	pcoA	MDR	TGGCGTATGGAGTTTCAATGC	GAATAATGCCGTGCCAGTGAA
AY499	terW	MDR	TCAAAGAGCTACGCGAGTCATA	CCTTCCCTGTGGACTCACC
AY503	IncHI2-smr0018	MGE	ATAATGATTCACCGGGGTAG	CTTCAGGCTATCGTTTCG
AY504	IncI1_repI1	MGE	CGAAAGCCGGACGGCAGAA	TCGTCGTTCCGCCAAGTTCGT
AY317	IncN_oriT	MGE	TTGGGCTTCATAGTACCC	GTGTGATAGCGTGATTTATGC
AY509	IS200_2	MGE	GCACACCCGATGGAACTGTAAA	TCGGCGGGATCTCCAGAAG
AY512	IS26_1	MGE	ATGGATGAAACCTACGTGAAGGTC	CGGTACTTAATCTGTCGGTGTTCA
AY298	IS613	MGE	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT
AY311	ISAba3	MGE	TCAGAGGCAGCGGTATACGA	GGTTGATTCAGTTAAAGTACGTAAAACTTT
AY315	Tn5	MGE	TCAGAGGCAGCGGTATACGA	GGTTGATTCAGTTAAAGTACGTAAAACTTT
AY299	tnpA_1	MGE	GCCGCACTGTCGATTTTTATC	GCGGGATCTGCCACTTCTT
AY305	tnpA_7	MGE	AATTGATGCGGACGGCTTAA	TCACCAAACTGTTTATGGAGTCGTT
AY306	trfA	MGE	ACGAAGAAATGGTTGTCCTGTTC	CGTCAGCTTGCGGTACTTCTC
AY531	erm35	MLSB	CCTTCAGTCAGAACCGGCAA	GCTGATTTGACAGTTGGTGGTG
AY53	lmrA_1	MLSB	TTCAGATGCAATGGCGTTTG	ATAATCGGGAACATAATGAGCATAACTAC
AY536	lnuB	MLSB	GGATCGTTTACCAAAGGAGAAGG	AGCATAGCCTTCGTATCAGGAA
AY66	msrA_1	MLSB	CTGCTAACACAAGTACGATTCCAAAT	TCAAGTAAAGTTGTCTTACCTACACCATT
AY58	msrC_1	MLSB	TCAGACCGGATCGGTTGTC	CCTATTTTTTGGAGTCTTCTCTCTAATGTT
AY33	yidY/mdtL	Phenicol	GCAGTTGCATATCGCCTTCTC	CTTCCCGGCAAACAGCAT
AY247	folA_1	Sulfonamide	CGAGCAGTTCCTGCCAAAG	CCCAGTCATCCGGTTCATAATC
AY361	folP_2	Sulfonamide	CAGGCTCGTAAATTGATAGCAGAAG	CTTTCCTTGCGAATCGCTTT
AY241	sul4	Sulfonamide	TCAACGTCACTCCAGACAGC	TGGAAATAACGACGTCCACA
AY250	tet32	Tetracycline	CCATTACTTCGGACAACGGTAGA	CAATCTCTGTGAGGGCATTTAACA
AY249	tet36_1	Tetracycline	AGAATACTCAGCAGAGGTCAGTTCCT	TGGTAGGTCGATAACCCGAAAAT
AY568	tet39	Tetracycline	TATAGCGGGTCCGGTAATAGGTG	CCATAACGATCCTGCCCATAGATAAC

AY273	tetE	Tetracycline	TTGGCGCTGTATGCAATGAT	CGACGACCTATGCGATCTGA
AY574	tetM	Tetracycline	GGAGCGATTACAGAATTAGGAAGC	TCCATATGTCCTGGCGTGTC
AY274	tetPB_1	Tetracycline	TGGGCGACAGTAGGCTTAGAA	TGACCCTACTGAAACATTAGAAATATACCT
AY325	tetR_1	Tetracycline	CAATCCATCGACAATCAC	GACAATCAGCTACTTCAC
AY276	tetT	Tetracycline	CCATATAGAGGTTCCACCAAATCC	TGACCCTATTGGTAGTGGTTCTATTG
AY284	dfrA1_1	Trimethoprim	GGAATGGCCCTGATATTCCA	AGTCTTGCGTCCAACCAACAG
AY590	dfrAB4	Trimethoprim	CGGTTCGCATTCCCATCAAA	CGCAGTCATGGGATAAATCTGG
AY161	vanD	Vancomycin	CAGAGGAACATAATGTTTCGATAAAATCT	GCCGGATTTTGTGATTCCAA
AY176	vanRC4	Vancomycin	AGTGCTTTGGCTTATCTCGAAAA	TCCGGCAGCATCACATCTAA

Table 7-17 PERMANOVA results of Bray-Curtis beta diversity analysis with all samples. Asterisk represents P value, where "*" p≤0.001, "**" 0.001 < p ≤ 0.01, "*" 0.01< p ≤ 0.05.**

Parameters	R^2	P	
Condition (T0, T-end control, T-end amoxicillin)	0.20	0.001	***
Amoxicillin concentration	0.08	0.004	**
Sample type (DNA vs cDNA)	0.05	0.012	*

8 References

A, D., DENG, Y.-Y., GUO, Q.-M., JIANG, Y. & CHEN, C.-X. 2023. A three-year study on the treatment of domestic-industrial mixed wastewater using a full-scale hybrid constructed wetland. *Environmental Science and Pollution Research,* 30**,** 31256-31267.

ABAY, A., SIMIONATO, G., CHACHANIDZE, R., BOGDANOVA, A., HERTZ, L., BIANCHI, P., VAN DEN AKKER, E., VON LINDERN, M., LEONETTI, M., MINETTI, G., WAGNER, C. & KAESTNER, L. 2019. Glutaraldehyde – A Subtle Tool in the Investigation of Healthy and Pathologic Red Blood Cells. *Frontiers in Physiology,* 10.

ABRAHAM, E. P. & CHAIN, E. 1940. An Enzyme from Bacteria able to Destroy Penicillin. *Nature,* 146**,** 837-837.

ADAMSKI, P., BYCZKOWSKA-ROSTKOWSKA, Z., GAJEWSKA, J., ZAKRZEWSKI, A. J. & KŁĘBUKOWSKA, L. 2023. Prevalence and Antibiotic Resistance of Bacillus sp. Isolated from Raw Milk. *Microorganisms,* 11.

AHMAD, V., MUHAMMAD ZAFAR IQBAL, A. N., HASEEB, M. & KHAN, M. S. 2014. Antimicrobial potential of bacteriocin producing Lysinibacillus jx416856 against foodborne bacterial and fungal pathogens, isolated from fruits and vegetable waste. *Anaerobe,* 27**,** 87-95.

ALCOCK, B. P., HUYNH, W., CHALIL, R., SMITH, K. W., RAPHENYA, A. R., WLODARSKI, M. A., EDALATMAND, A., PETKAU, A., SYED, S. A., TSANG, K. K., BAKER, S. J. C., DAVE, M., MCCARTHY, M. C., MUKIRI, K. M., NASIR, J. A., GOLBON, B., IMTIAZ, H., JIANG, X., KAUR, K., KWONG, M., LIANG, Z. C., NIU, K. C., SHAN, P., YANG, J. Y. J., GRAY, K. L., HOAD, G. R., JIA, B., BHANDO, T., CARFRAE, L. A., FARHA, M. A., FRENCH, S., GORDZEVICH, R., RACHWALSKI, K., TU, M. M., BORDELEAU, E., DOOLEY, D., GRIFFITHS, E., ZUBYK, H. L., BROWN, E. D., MAGUIRE, F., BEIKO, R. G., HSIAO, W. W. L., BRINKMAN, F. S. L., VAN DOMSELAAR, G. & MCARTHUR, A. G. 2023. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Res,* 51**,** D690-d699.

ALFIYA, Y., GROSS, A., SKLARZ, M. & FRIEDLER, E. 2013. Reliability of on-site greywater treatment systems in Mediterranean and arid environments – a case study. *Water Science and Technology,* 67**,** 1389-1395.

AMÁBILE-CUEVAS, C. 2010. Antibiotic resistance in Mexico: a brief overview of the current status and its causes. *J Infect Dev Ctries,* 4**,** 126-31.

AMABILE-CUEVAS, C. F. 2021. Antibiotic usage and resistance in Mexico: an update after a decade of change. *J Infect Dev Ctries,* 15**,** 442-449.

AMIR, A., MCDONALD, D., NAVAS-MOLINA JOSE, A., KOPYLOVA, E., MORTON JAMES, T., ZECH XU, Z., KIGHTLEY ERIC, P., THOMPSON LUKE, R., HYDE EMBRIETTE, R., GONZALEZ, A. & KNIGHT, R. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *mSystems,* 2**,** e00191-16.

ANDERSSON, D. I. & HUGHES, D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology,* 8**,** 260-271.

ANUJ, S. N., WHILEY, D. M., KIDD, T. J., BELL, S. C., WAINWRIGHT, C. E., NISSEN, M. D. & SLOOTS, T. P. 2009. Identification of Pseudomonas aeruginosa by a duplex real-time polymerase chain reaction assay targeting the ecfX and the gyrB genes. *Diagn Microbiol Infect Dis,* 63**,** 127-31.

APPLING, K. C., SOBSEY, M. D., DURSO, L. M. & FISHER, M. B. 2023. Environmental monitoring of antimicrobial resistant bacteria in North Carolina water and

wastewater using the WHO Tricycle protocol in combination with membrane filtration and compartment bag test methods for detecting and quantifying ESBL E. coli. *PLOS Water,* 2**,** e0000117.

ARLIYANI, I., TANGAHU, B. V. & MANGKOEDIHARDJO, S. 2021. Plant Diversity in a Constructed Wetland for Pollutant Parameter Processing on Leachate: A Review. *Journal of Ecological Engineering,* 22**,** 240-255.

AYAZ, S. Ç., AKTAŞ, Ö., AKÇA, L. & FıNDıK, N. 2015. Effluent quality and reuse potential of domestic wastewater treated in a pilot-scale hybrid constructed wetland system. *Journal of Environmental Management,* 156**,** 115-120.

BAIRD, R. B., EATON, A. D., RICE, E. W., BRIDGEWATER, L. L., AMERICAN PUBLIC HEALTH ASSOCIATION, I. B., AMERICAN WATER WORKS ASSOCIATION, I. B. & WATER ENVIRONMENT FEDERATION, I. B. 2017. *Standard methods for the examination of water and wastewater*, Washington, DC : American Public Health Association.

BARTLETT, A., PADFIELD, D., LEAR, L., BENDALL, R. & VOS, M. 2022. A comprehensive list of bacterial pathogens infecting humans. *Microbiology,* 168.

BEAL, J., FARNY, N. G., HADDOCK-ANGELLI, T., SELVARAJAH, V., BALDWIN, G. S., BUCKLEY-TAYLOR, R., GERSHATER, M., KIGA, D., MARKEN, J., SANCHANIA, V., SISON, A., WORKMAN, C. T., PEHLIVAN, M., ROIGE, B. B., AARNIO, T., KIVISTO, S., KOSKI, J., LEHTONEN, L., PEZZUTTO, D., RAUTANEN, P., BIAN, W., HU, Z., LIU, Z., LIU, Z., MA, L., PAN, L., QIN, Z., WANG, H., WANG, X., XU, H., XU, X., EL MOUBAYED, Y., DONG, S., FANG, C., HE, H., HE, H., HUANG, F., SHI, R., TANG, C., TANG, C., XU, S., YAN, C., BARTZOKA, N., KANATA, E., KAPSOKEFALOU, M., KATOPODI, X.-L., KOSTADIMA, E., KOSTOPOULOS, I. V., KOTZASTRATIS, S., KOUTELIDAKIS, A. E., KROKOS, V., LITSA, M., NTEKAS, I., SPATHARAS, P., TSITSILONIS, O. E., ZERVA, A., ANNEM, V., CONE, E., ELIAS, N., GUPTA, S., LAM, K., TUTUIANU, A., MISHLER, D. M., TORO, B., AKINFENWA, A., BURNS, F., HERBERT, H., JONES, M., LAUN, S., MORRISON, S., SMITH, Z., PENG, Z., ZIWEI, Z., DENG, R., HUANG, Y., LI, T., MA, Y., SHEN, Z., WANG, C., WANG, Y., ZHAO, T., LANG, Y., LIANG, Y., WANG, X., WU, Y., AIZIK, D., ANGEL, S., FARHI, E., KEIDAR, N., OSER, E., PASI, M., KALINOWSKI, J., OTTO, M., RUHNAU, J., CUBUKCU, H., HOSKAN, M. A., SENYUZ, I., CHI, J., SAUTER, A. P., SIMONA, M. F., et al. 2020. Robust estimation of bacterial cell count from optical density. *Communications Biology,* 3**,** 512.

BELMONT, M. A., CANTELLANO, E., THOMPSON, S., WILLIAMSON, M., SÁNCHEZ, A. & METCALFE, C. D. 2004. Treatment of domestic wastewater in a pilot-scale natural treatment system in central Mexico. *Ecological Engineering,* 23**,** 299-311.

BENITEZ, A. J. & WINCHELL, J. M. 2013. Clinical application of a multiplex real-time PCR assay for simultaneous detection of Legionella species, Legionella pneumophila, and Legionella pneumophila serogroup 1. *J Clin Microbiol,* 51**,** 348-51.

BENVENUTI, T., HAMERSKI, F., GIACOBBO, A., BERNARDES, A. M., ZOPPAS-FERREIRA, J. & RODRIGUES, M. A. S. 2018. Constructed floating wetland for the treatment of domestic sewage: A real-scale study. *Journal of Environmental Chemical Engineering,* 6**,** 5706-5711.

BERKNER, S., KONRADI, S. & SCHÖNFELD, J. 2014. Antibiotic resistance and the environment—there and back again. *EMBO reports,* 15**,** 740.

BERNEY, M., HAMMES, F., BOSSHARD, F., WEILENMANN, H. U. & EGLI, T. 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. *Appl Environ Microbiol,* 73**,** 3283-90.

BRUNS-MOORE, M. 2022. *The effect of acute amoxicillin exposure on anaerobic microbial communities - activity, ecology and resistome.* PhD, University of Glasgow.

BURGMANN, H., FRIGON, D., GAZE, W. H., MANAIA, C. M., PRUDEN, A., SINGER, A. C., SMETS, B. F. & ZHANG, T. 2018. Water and sanitation: an essential battlefront in the war on antimicrobial resistance. *FEMS Microbiology Ecology,* 94.

CACACE, D., FATTA-KASSINOS, D., MANAIA, C. M., CYTRYN, E., KREUZINGER, N., RIZZO, L., KARAOLIA, P., SCHWARTZ, T., ALEXANDER, J., MERLIN, C., GARELICK, H., SCHMITT, H., DE VRIES, D., SCHWERMER, C. U., MERIC, S., OZKAL, C. B., PONS, M.-N., KNEIS, D. & BERENDONK, T. U. 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. *Water Research,* 162**,** 320-330.

CALLAHAN, B. J., MCMURDIE, P. J., ROSEN, M. J., HAN, A. W., JOHNSON, A. J. A. & HOLMES, S. P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods,* 13**,** 581-583.

CAPORASO, J. G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F. D., COSTELLO, E. K., FIERER, N., PEÑA, A. G., GOODRICH, J. K., GORDON, J. I., HUTTLEY, G. A., KELLEY, S. T., KNIGHTS, D., KOENIG, J. E., LEY, R. E., LOZUPONE, C. A., MCDONALD, D., MUEGGE, B. D., PIRRUNG, M., REEDER, J., SEVINSKY, J. R., TURNBAUGH, P. J., WALTERS, W. A., WIDMANN, J., YATSUNENKO, T., ZANEVELD, J. & KNIGHT, R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods,* 7**,** 335-336.

CHEN, G., MO, Y., GU, X., JEPPESEN, E., XIE, T., NING, Z., LI, Y., LI, D., CHEN, C., CUI, B. & WU, H. 2024. Sustainability of global small-scale constructed wetlands for multiple pollutant control. *npj Clean Water,* 7**,** 45.

CHEN, G., VAN LOOSDRECHT, M. C., EKAMA, G. A. & BRDJANOVIC, D. 2020. Wastewater treatment development. *Biological wastewater treatment: principles, modelling and design*.

CHEN, H. & CHANG, S. 2017. Impact of temperatures on microbial community structures of sewage sludge biological hydrolysis. *Bioresource Technology,* 245**,** 502-510.

CHEN, J., WEI, X.-D., LIU, Y.-S., YING, G.-G., LIU, S.-S., HE, L.-Y., SU, H.-C., HU, L.-X., CHEN, F.-R. & YANG, Y.-Q. 2016a. Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: Optimization of wetland substrates and hydraulic loading. *Science of The Total Environment,* 565**,** 240-248.

CHEN, J., ZHANG, J., ZHAN, L., CHEN, H., ZHANG, Z., HUANG, C. & YUE, M. 2022. Prevalence and antimicrobial-resistant characterization of Bacillus cereus isolated from ready-to-eat rice products in Eastern China. *Front Microbiol,* 13**,** 964823.

CHEN, Q., AN, X., LI, H., SU, J., MA, Y. & ZHU, Y.-G. 2016b. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. *Environment International,* 92-93**,** 1-10.

CHERN, E. C., SIEFRING, S., PAAR, J., DOOLITTLE, M. & HAUGLAND, R. A. 2011. Comparison of quantitative PCR assays for Escherichia coli targeting ribosomal RNA and single copy genes. *Lett Appl Microbiol,* 52**,** 298-306.

CHOI, H., GERONIMO, F. K., JEON, M. & KIM, L.-H. 2022. Evaluation of bacterial community in constructed wetlands treating different sources of wastewater. *Ecological Engineering,* 182**,** 106703.

CONAGUA. 2015. *Inventario Nacional de Plantas Municipales de Potabilización y de Tratamiento de Aguas Residuales en Operación* [Online]. Ciudad de México: Comisión Nacional del Agua. Available: https://sswm.info/sites/default/files/reference_attachments/CONAGUA%202015.%20Inventario%20Nacional%20de%20Plantas%20Municipales.pdf [Accessed 7 July 2023].

CONAGUA. 2017. *Statistics on Water in Mexico, 2017 edition* [Online]. Mexico: National Water Commission. Available: https://sina.conagua.gob.mx/publicaciones/EAM_i_2017.pdf [Accessed 3 May 2023].

CONAGUA. 2020a. *Inventario Nacional de Plantas Municipales de Potabilización y de Tratamiento de Aguas Residuales en Operación* [Online]. Ciudad de México: Comisión Nacional del Agua. Available: https://files.conagua.gob.mx/conagua/publicaciones/Publicaciones/SGAPDS-2-22-a.pdf [Accessed 6 July 2023].

CONAGUA. 2020b. *Temperatura Media por Entidad Federativa y Nacional* [Online]. Ciudad de México: 10 August. Available: https://smn.conagua.gob.mx/tools/DATA/Climatología/Pronóstico%20climático/Temperatura%20y%20Lluvia/TMED/2020.pdf [Accessed 2023].

CONAGUA. 2022. *Estadisticas del Agua en Mexico 2021* [Online]. Mexico: Comisión Nacional del Agua. Available: https://sina.conagua.gob.mx/publicaciones/EAM_2021.pdf [Accessed 12 June 2023].

CONAGUA. 2023. *Numeragua, edición 2022* [Online]. Mexico: Comisión Nacional del Agua. Available: https://sina.conagua.gob.mx/publicaciones/Numeragua_2022.pdf [Accessed 12 June 2023].

DE ANDA, J., LÓPEZ-LÓPEZ, A., VILLEGAS-GARCÍA, E. & VALDIVIA-AVIÑA, K. 2018. High-Strength Domestic Wastewater Treatment and Reuse with Onsite Passive Methods. *Water* [Online], 10.

DE ANDA, J. & SHEAR, H. 2021. Sustainable Wastewater Management to Reduce Freshwater Contamination and Water Depletion in Mexico. *Water* [Online], 13.

DI CAPRIO, F. 2020. Methods to quantify biological contaminants in microalgae cultures. *Algal Research,* 49**,** 101943.

DOMAŃSKA, M., HAMAL, K., JASIONOWSKI, B. & ŁOMOTOWSKI, J. 2019. Bacteriological Contamination Detection in Water and Wastewater Samples Using OD600. *Polish Journal of Environmental Studies,* 28**,** 4503-4509.

DOS SANTOS, C. E. D., COSTA, R. B., RABELO, C. A. B. S., FERRAZ JÚNIOR, A. D. N., PERSINOTI, G. F., POZZI, E., FORESTI, E. & DAMIANOVIC, M. H. R. Z. 2021. Hacking biofilm developed in a structured-bed reactor (SBRRIA) with integrated processes of nitrogen and organic matter removal. *Bioprocess and Biosystems Engineering,* 44**,** 1841-1851.

EHLING-SCHULZ, M., LERECLUS, D. & KOEHLER, T. M. 2019. The Bacillus cereus Group: Bacillus Species with Pathogenic Potential. *Microbiol Spectr,* 7.

EKAKORO, J. E., GUPTILL, L. F., HENDRIX, G. K., DORSEY, L. & RUPLE, A. 2023. Antimicrobial Susceptibility of Bacteria Isolated from Freshwater Mussels in the Wildcat Creek Watershed, Indiana, United States. *Antibiotics (Basel),* 12.

EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING. 2024. *Breakpoint tables for interpretation of MICs and zone diameters Version 14.0* [Online]. Available: http://www.eucast.org [Accessed 15 August 2024].

FAN, J., ZHANG, J., GUO, W., LIANG, S. & WU, H. 2016. Enhanced long-term organics and nitrogen removal and associated microbial community in intermittently aerated subsurface flow constructed wetlands. *Bioresource Technology,* 214**,** 871-875.

FAN, X.-Y., GAO, J.-F., PAN, K.-L., LI, D.-C. & DAI, H.-H. 2017. Temporal dynamics of bacterial communities and predicted nitrogen metabolism genes in a full-scale wastewater treatment plant. *Rsc Advances,* 7**,** 56317-56327.

FANG, H., TIAN, L., YE, N. & ZHANG, S. 2023. Alizarin enhancement of the abundance of ARGs and impacts on the microbial community in water. *Water Science and Technology,* 87**,** 2250-2264.

FARINA, D., BIANCO, A., MANZULLI, V., CASTELLANA, S., PARISI, A., CARUSO, M., FRACCALVIERI, R., SERRECCHIA, L., RONDINONE, V., PACE, L., FASANELLA, A., VETRITTO, V., DIFATO, L. M., CIPOLLETTA, D., IATAROLA, M. & GALANTE, D. 2024. Antimicrobial and Phylogenomic Characterization of Bacillus cereus Group Strains Isolated from Different Food Sources in Italy. *Antibiotics (Basel),* 13.

FENSELAU, C., HAVEY, C., TEERAKULKITTIPONG, N., SWATKOSKI, S., LAINE, O. & EDWARDS, N. 2008. Identification of beta-lactamase in antibiotic-resistant Bacillus cereus spores. *Appl Environ Microbiol,* 74**,** 904-6.

FERNANDES, J. P., ALMEIDA, C. M. R., PEREIRA, A. C., RIBEIRO, I. L., REIS, I., CARVALHO, P., BASTO, M. C. P. & MUCHA, A. P. 2015. Microbial community dynamics associated with veterinary antibiotics removal in constructed wetlands microcosms. *Bioresource Technology,* 182**,** 26-33.

FERNÁNDEZ-CALVIÑO, D. & BÅÅTH, E. 2010. Growth response of the bacterial community to pH in soils differing in pH. *FEMS Microbiology Ecology,* 73**,** 149-156.

FIEDLER, G., SCHNEIDER, C., IGBINOSA, E. O., KABISCH, J., BRINKS, E., BECKER, B., STOLL, D. A., CHO, G.-S., HUCH, M. & FRANZ, C. M. A. P. 2019. Antibiotics resistance and toxin profiles of Bacillus cereus-group isolates from fresh vegetables from German retail markets. *BMC Microbiology,* 19**,** 250.

FISHBEIN, S. R. S., MAHMUD, B. & DANTAS, G. 2023. Antibiotic perturbations to the gut microbiome. *Nature Reviews Microbiology,* 21**,** 772-788.

FITZGERALD, C. M., CAMEJO, P., OSHLAG, J. Z. & NOGUERA, D. R. 2015. Ammonia-oxidizing microbial communities in reactors with efficient nitrification at low-dissolved oxygen. *Water Research,* 70**,** 38-51.

FRANKLIN, A. M., BRINKMAN, N. E., JAHNE, M. A. & KEELY, S. P. 2021. Twenty-first century molecular methods for analyzing antimicrobial resistance in surface waters to support One Health assessments. *Journal of Microbiological Methods,* 184**,** 106174.

FU, W., WANG, Y., WEI, W. & LI, P. 2019. Species Diversity and Functional Prediction of Soil Bacterial Communities in Constructed Wetlands with Different Plant Conditions. *Current Microbiology,* 76**,** 338-345.

GALLARDO-ALTAMIRANO, M. J., MAZA-MÁRQUEZ, P., MONTEMURRO, N., PÉREZ, S., RODELAS, B., OSORIO, F. & POZO, C. 2021. Insights into the removal of pharmaceutically active compounds from sewage sludge by two-stage mesophilic anaerobic digestion. *Science of The Total Environment,* 789**,** 147869.

GAO, P., MUNIR, M. & XAGORARAKI, I. 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Science of The Total Environment,* 421-422**,** 173-183.

GAO, Y., ZHU, J., WANG, K., MA, Y., FANG, J. & LIU, G. 2023. Discovery of a heterotrophic aerobic denitrification Pseudomonas sp. G16 and its unconventional nitrogen metabolic pathway. *Bioresource Technology,* 387**,** 129670.

GENTILE, A., PICCOLO, P., IANNECE, P., CICATELLI, A., CASTIGLIONE, S. & GUARINO, F. 2024. Reduction of antimicrobial resistance: Advancements in nature-based wastewater treatment. *Journal of Hazardous Materials,* 471**,** 134330.

GHAITIDAK, D. M. & YADAV, K. D. 2013. Characteristics and treatment of greywater—a review. *Environmental Science and Pollution Research,* 20**,** 2795-2809.

GIBSON, C., JAUFFUR, S., GUO, B. & FRIGON, D. 2024. Activated sludge microbial community assembly: the role of influent microbial community immigration. *Applied and Environmental Microbiology,* 90.

GOLDFORD, J. E., LU, N., BAJIĆ, D., ESTRELA, S., TIKHONOV, M., SANCHEZ-GOROSTIAGA, A., SEGRÈ, D., MEHTA, P. & SANCHEZ, A. 2018. Emergent simplicity in microbial community assembly. *Science,* 361**,** 469-474.

GONZALEZ-FLO, E., ROMERO, X. & GARCÍA, J. 2023. Nature based-solutions for water reuse: 20 years of performance evaluation of a full-scale constructed wetland system. *Ecological Engineering,* 188**,** 106876.

GRADY, E. N., MACDONALD, J., LIU, L., RICHMAN, A. & YUAN, Z.-C. 2016. Current knowledge and perspectives of Paenibacillus: a review. *Microbial Cell Factories,* 15**,** 203.

GRAF, J. 2014. The Family Rikenellaceae. *In:* ROSENBERG, E., DELONG, E. F., LORY, S., STACKEBRANDT, E. & THOMPSON, F. (eds.) *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea.* Berlin, Heidelberg: Springer Berlin Heidelberg.

GULLBERG, E., ALBRECHT LISA, M., KARLSSON, C., SANDEGREN, L. & ANDERSSON DAN, I. 2014. Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals. *mBio,* 5**,** 10.1128/mbio.01918-14.

GULLBERG, E., CAO, S., BERG, O. G., ILBÄCK, C., SANDEGREN, L., HUGHES, D. & ANDERSSON, D. I. 2011. Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog,* 7**,** e1002158.

GUNES, K., TUNCSIPER, B., AYAZ, S. & DRIZO, A. 2012. The ability of free water surface constructed wetland system to treat high strength domestic wastewater: A case study for the Mediterranean. *Ecological Engineering,* 44**,** 278-284.

GUO, J., LI, J., CHEN, H., BOND, P. L. & YUAN, Z. 2017. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Research,* 123**,** 468-478.

GUTIÉRREZ, C. 2008. Standards and Thresholds for Waste Water Discharges in Mexico. *In:* SCHMIDT, M., GLASSON, J., EMMELIN, L. & HELBRON, H. (eds.) *Standards and Thresholds for Impact Assessment.* Berlin, Heidelberg: Springer Berlin Heidelberg.

HAMMES, F., BERNEY, M., WANG, Y., VITAL, M., KÖSTER, O. & EGLI, T. 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Research,* 42**,** 269-277.

HARIRCHI, S., SAR, T., RAMEZANI, M., HABIBU, A., ETEMADIFAR, Z., NOJOUMI, S., YAZDIAN, F., AWASTHI, M. K. & TAHERZADEH, M. 2022. Bacillales: From Taxonomy to Biotechnological and Industrial Perspectives. *Microorganisms,* 10**,** 2355.

HASHMI, I., BINDSCHEDLER, S. & JUNIER, P. 2020. Chapter 18 - Firmicutes. *In:* AMARESAN, N., SENTHIL KUMAR, M., ANNAPURNA, K., KUMAR, K. & SANKARANARAYANAN, A. (eds.) *Beneficial Microbes in Agro-Ecology.* Academic Press.

HENCH, K. R., BISSONNETTE, G. K., SEXSTONE, A. J., COLEMAN, J. G., GARBUTT, K. & SKOUSEN, J. G. 2003. Fate of physical, chemical, and microbial contaminants in domestic wastewater following treatment by small constructed wetlands. *Water Research,* 37**,** 921-927.

HENDRIKSEN, R. S., MUNK, P., NJAGE, P., VAN BUNNIK, B., MCNALLY, L., LUKJANCENKO, O., RÖDER, T., NIEUWENHUIJSE, D., PEDERSEN, S. K., KJELDGAARD, J., KAAS, R. S., CLAUSEN, P. T. L. C., VOGT, J. K., LEEKITCHAROENPHON, P., VAN DE SCHANS, M. G. M., ZUIDEMA, T., DE RODA HUSMAN, A. M., RASMUSSEN, S., PETERSEN, B., BEGO, A., REES, C., CASSAR, S., COVENTRY, K., COLLIGNON, P., ALLERBERGER, F., RAHUBE, T. O., OLIVEIRA, G., IVANOV, I., VUTHY, Y., SOPHEAK, T., YOST, C. K., KE, C., ZHENG, H., BAISHENG, L., JIAO, X., DONADO-GODOY, P., COULIBALY, K. J., JERGOVIĆ, M.,

HRENOVIC, J., KARPÍŠKOVÁ, R., VILLACIS, J. E., LEGESSE, M., EGUALE, T., HEIKINHEIMO, A., MALANIA, L., NITSCHE, A., BRINKMANN, A., SABA, C. K. S., KOCSIS, B., SOLYMOSI, N., THORSTEINSDOTTIR, T. R., HATHA, A. M., ALEBOUYEH, M., MORRIS, D., CORMICAN, M., O'CONNOR, L., MORAN-GILAD, J., ALBA, P., BATTISTI, A., SHAKENOVA, Z., KIIYUKIA, C., NG'ENO, E., RAKA, L., AVSEJENKO, J., BĒRZIŅŠ, A., BARTKEVICS, V., PENNY, C., RAJANDAS, H., PARIMANNAN, S., HABER, M. V., PAL, P., JEUNEN, G.-J., GEMMELL, N., FASHAE, K., HOLMSTAD, R., HASAN, R., SHAKOOR, S., ROJAS, M. L. Z., WASYL, D., BOSEVSKA, G., KOCHUBOVSKI, M., RADU, C., GASSAMA, A., RADOSAVLJEVIC, V., WUERTZ, S., ZUNIGA-MONTANEZ, R., TAY, M. Y. F., GAVAČOVÁ, D., PASTUCHOVA, K., TRUSKA, P., TRKOV, M., ESTERHUYSE, K., KEDDY, K., CERDÀ-CUÉLLAR, M., PATHIRAGE, S., NORRGREN, L., ÖRN, S., LARSSON, D. G. J., HEIJDEN, T. V. D., KUMBURU, H. H., et al. 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nature Communications,* 10**,** 1124.

HIJOSA-VALSERO, M., SIDRACH-CARDONA, R. & BÉCARES, E. 2012. Comparison of interannual removal variation of various constructed wetland types. *Science of The Total Environment,* 430**,** 174-183.

HILEY, P. D. 1990. WETLANDS TREATMENT REVIVAL IN YORKSHIRE. *In:* COOPER, P. F. & FINDLATER, B. C. (eds.) *Constructed Wetlands in Water Pollution Control.* Pergamon.

HILEY, P. D. 1995. The reality of sewage treatment using wetlands. *Water Science and Technology,* 32**,** 329-338.

HOLLSTEIN, M., COMERFORD, M., UHL, M., ABEL, M., EGAN, S. P. & STADLER, L. B. 2023. Impact of a natural disturbance on the performance and microbial communities in a full-scale constructed wetland for industrial wastewater treatment. *Frontiers in Environmental Science,* 11.

HUA, M., HUANG, W., CHEN, A., REHMET, M., JIN, C. & HUANG, Z. 2020. Comparison of Antimicrobial Resistance Detected in Environmental and Clinical Isolates from Historical Data for the US. *Biomed Res Int,* 2020**,** 4254530.

HUANG, L., WANG, N., DENG, C., LIANG, Y., WANG, Q., LIU, M. & CHEN, Y. 2019. Interactive effect of carbon source with influent COD/N on nitrogen removal and microbial community structure in subsurface flow constructed wetlands. *Journal of Environmental Management,* 250**,** 109491.

HUANG, M.-Q., CUI, Y.-W., HUANG, J.-L., SUN, F.-L. & CHEN, S. 2022. A novel Pseudomonas aeruginosa strain performs simultaneous heterotrophic nitrification-aerobic denitrification and aerobic phosphate removal. *Water Research,* 221**,** 118823.

HUANG, Y., RAGUSH, C. M., JOHNSTON, L. H., HALL, M. W., BEIKO, R. G., JAMIESON, R. C. & TRUELSTRUP HANSEN, L. 2021. Changes in Bacterial Communities During Treatment of Municipal Wastewater in Arctic Wastewater Stabilization Ponds. *Frontiers in Water,* 3.

HULTMAN, J., TAMMINEN, M., PARNANEN, K., CAIRNS, J., KARKMAN, A., VIRTA, M., INSTITUTIONEN FÖR BIOMEDICIN, A. F. I., GÖTEBORGS, U., GOTHENBURG, U., SAHLGRENSKA, A., INSTITUTE OF BIOMEDICINE, D. O. I. M., SAHLGRENSKA, A., ANTIBIOTIKARESISTENSFORSKNING, C. A.-C. F. & CENTRE FOR ANTIBIOTIC RESISTANCE RESEARCH, C. 2018. Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent. *FEMS MICROBIOLOGY ECOLOGY,* 94.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION 2019. ISO-20776-1: 2019 susceptibility testing of infectious agents and evaluation of performance of

antimicrobial susceptibility test devices—part 1: broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. International Organization for Standardization Geneva, Switzerland.

JAŁOWIECKI, Ł., ŻUR, J., CHOJNIAK, J., EJHED, H. & PŁAZA, G. 2018. Properties of Antibiotic-Resistant Bacteria Isolated from Onsite Wastewater Treatment Plant in Relation to Biofilm Formation. *Current Microbiology,* 75**,** 639-649.

JECHALKE, S., BROSZAT, M., LANG, F., SIEBE, C., SMALLA, K. & GROHMANN, E. 2015. Effects of 100 years wastewater irrigation on resistance genes, class 1 integrons and IncP-1 plasmids in Mexican soil. *Front Microbiol,* 6**,** 163.

JIANG, X.-T., YE, L., JU, F., LI, B., MA, L.-P. & ZHANG, T. 2018. Temporal dynamics of activated sludge bacterial communities in two diversity variant full-scale sewage treatment plants. *Applied Microbiology and Biotechnology,* 102**,** 9379-9388.

JINDAL, S., THAMPY, H., DAY, P. J. R. & KELL, D. B. 2019. Very rapid flow cytometric assessment of antimicrobial susceptibility during the apparent lag phase of microbial (re)growth. *Microbiology,* 165**,** 439-454.

JU, F., BECK, K., YIN, X. L., MACCAGNAN, A., MCARDELL, C. S., SINGER, H. P., JOHNSON, D. R., ZHANG, T. & BURGMANN, H. 2019. Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. *ISME Journal,* 13**,** 346-360.

JU, F. & ZHANG, T. 2015. Bacterial assembly and temporal dynamics in activated sludge of a full-scale municipal wastewater treatment plant. *The ISME Journal,* 9**,** 683-695.

KAMIYA, E., IZUMIYAMA, S., NISHIMURA, M., MITCHELL, J. G. & KOGURE, K. 2007. Effects of fixation and storage on flow cytometric analysis of marine bacteria. *Journal of Oceanography,* 63**,** 101-112.

KASUGA, I., NAGASAWA, K., SUZUKI, M., KURISU, F. & FURUMAI, H. 2022. High-Throughput Screening of Antimicrobial Resistance Genes and Their Association With Class 1 Integrons in Urban Rivers in Japan. *Frontiers in Environmental Science,* 10.

KIHILA, J. M. & BALENGAYABO, J. G. 2020. Adaptable improved onsite wastewater treatment systems for urban settlements in developing countries. *Cogent Environmental Science,* 6**,** 1823633.

KIM, C., RILEY, A., SRIHARAN, S., NARTEA, T., NDEGWA, E., DHAKAL, R., ZHENG, G. & BAFFAUT, C. 2024. Examining Antimicrobial Resistance in Escherichia coli: A Case Study in Central Virginia's Environment. *Antibiotics* [Online], 13.

KLEIN, E. Y., VAN BOECKEL, T. P., MARTINEZ, E. M., PANT, S., GANDRA, S., LEVIN, S. A., GOOSSENS, H. & LAXMINARAYAN, R. 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proceedings of the National Academy of Sciences,* 115**,** E3463-E3470.

KROISS, H., MATSCHÉ, N. & KRAMPE, J. 2021. 50 years of design and operation of large wastewater treatment plant conferences. A history of innovation and development. *Water Science and Technology,* 84**,** 263-273.

KRUGLOVA, A., MIKOLA, A., GONZALEZ-MARTINEZ, A. & VAHALA, R. 2019. Effect of sulfadiazine and trimethoprim on activated sludge performance and microbial community dynamics in laboratory-scale membrane bioreactors and sequencing batch reactors at 8°C. *Biotechnology Progress,* 35.

KRUSONG, W., LA CHINA, S., POTHIMON, R. & GULLO, M. 2022. Defining Paenibacillus azoreducens (P8) and Acetobacter pasteurianus (UMCC 2951) strains performances in producing acetic acid. *Frontiers in Microbiology,* 13.

KÜMMERER, K. 2009a. Antibiotics in the aquatic environment – A review – Part I. *Chemosphere,* 75**,** 417-434.

KÜMMERER, K. 2009b. Antibiotics in the aquatic environment – A review – Part II. *Chemosphere,* 75**,** 435-441.

KÜMMERER, K. & HENNINGER, A. 2003. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clinical Microbiology and Infection,* 9**,** 1203-1214.

LALUCAT, J., GOMILA, M., MULET, M., ZARUMA, A. & GARCÍA-VALDÉS, E. 2022. Past, present and future of the boundaries of the Pseudomonas genus: Proposal of Stutzerimonas gen. Nov. *Systematic and Applied Microbiology,* 45**,** 126289.

LARSSON, D. G. J. & FLACH, C.-F. 2022. Antibiotic resistance in the environment. *Nature Reviews Microbiology,* 20**,** 257-269.

LEE, J.-W., KIM, Y.-E., KANG, M.-S., LEE, K.-E., LEE, E.-Y. & PARK, S.-J. 2018. Paenibacillus albilobatus sp. nov., isolated from acidic soil on Jeju Island. *Journal of Microbiology,* 56**,** 393-398.

LEIVA, A. M., PIÑA, B. & VIDAL, G. 2021. Antibiotic resistance dissemination in wastewater treatment plants: a challenge for the reuse of treated wastewater in agriculture. *Reviews in Environmental Science and Bio/Technology,* 20**,** 1043-1072.

LI, B., QIU, Y., SHI, H. & YIN, H. 2016. The importance of lag time extension in determining bacterial resistance to antibiotics. *Analyst,* 141**,** 3059-3067.

LI, C., YANG, J., WANG, X., WANG, E., LI, B., HE, R. & YUAN, H. 2015. Removal of nitrogen by heterotrophic nitrification–aerobic denitrification of a phosphate accumulating bacterium Pseudomonas stutzeri YG-24. *Bioresource Technology,* 182**,** 18-25.

LI, H., LIU, F., LUO, P., CHEN, X., CHEN, J., HUANG, Z., PENG, J., XIAO, R. & WU, J. 2019. Stimulation of optimized influent C:N ratios on nitrogen removal in surface flow constructed wetlands: Performance and microbial mechanisms. *Science of The Total Environment,* 694**,** 133575.

LIU, G. J., ZHENG, D., DENG, L. W., WEN, Q. & LIU, Y. 2014. Comparison of constructed wetland and stabilization pond for the treatment of digested effluent of swine wastewater. *Environ Technol,* 35**,** 2660-9.

LIU, J., WEI, Z., XU, S. & TIAN, H. 2023a. A novel enhanced bio-ecological combined reactor for rural wastewater treatment: Operational performance and microbial communities. *Biochemical Engineering Journal,* 198**,** 108991.

LIU, L., SU, J.-Q., GUO, Y., WILKINSON, D. M., LIU, Z., ZHU, Y.-G. & YANG, J. 2018. Large-scale biogeographical patterns of bacterial antibiotic resistome in the waterbodies of China. *Environment International,* 117**,** 292-299.

LIU, L. M., HU, J. D., TENG, Y. G., WANG, J. S., CHEN, H. Y., GUO, X. R. & ZHAI, Y. Z. 2023b. Response of microbial community to different media in start-up period of Annan constructed wetland in Beijing of China. *Environmental Pollution,* 337.

LIU, Y., BAILEY, K. E., DYALL-SMITH, M., MARENDA, M. S., HARDEFELDT, L. Y., BROWNING, G. F., GILKERSON, J. R. & BILLMAN-JACOBE, H. 2021. Faecal microbiota and antimicrobial resistance gene profiles of healthy foals. *Equine Veterinary Journal,* 53**,** 806-816.

LOFRANO, G. & BROWN, J. 2010. Wastewater management through the ages: A history of mankind. *Science of The Total Environment,* 408**,** 5254-5264.

LOOFT, T., JOHNSON, T. A., ALLEN, H. K., BAYLES, D. O., ALT, D. P., STEDTFELD, R. D., SUL, W. J., STEDTFELD, T. M., CHAI, B., COLE, J. R., HASHSHAM, S. A., TIEDJE, J. M. & STANTON, T. B. 2012. In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences,* 109**,** 1691-1696.

LOVE, M. I., HUBER, W. & ANDERS, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology,* 15**,** 1-21.

MAJLANDER, J., ANTTILA, V. J., NURMI, W., SEPPÄLÄ, A., TIEDJE, J. & MUZIASARI, W. 2021. Routine wastewater-based monitoring of antibiotic resistance in two Finnish hospitals: focus on carbapenem resistance genes and genes associated with bacteria causing hospital-acquired infections. *J Hosp Infect,* 117**,** 157-164.

MANAIA, C. M., ROCHA, J., SCACCIA, N., MARANO, R., RADU, E., BIANCULLO, F., CERQUEIRA, F., FORTUNATO, G., IAKOVIDES, I. C., ZAMMIT, I., KAMPOURIS, I., VAZ-MOREIRA, I. & NUNES, O. C. 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. *Environment International,* 115**,** 312-324.

MANCUSO, G., LAVRNIĆ, S., CANET-MARTÍ, A., ZAHEER, A., AVOLIO, F., LANGERGRABER, G. & TOSCANO, A. 2023. Performance of lagoon and constructed wetland systems for tertiary wastewater treatment and potential of reclaimed water in agricultural irrigation. *Journal of Environmental Management,* 348**,** 119278.

MARÍN-MUÑIZ, J. L., SANDOVAL HERAZO, L. C., LÓPEZ-MÉNDEZ, M. C., SANDOVAL-HERAZO, M., MELÉNDEZ-ARMENTA, R. Á., GONZÁLEZ-MORENO, H. R. & ZAMORA, S. 2023. Treatment Wetlands in Mexico for Control of Wastewater Contaminants: A Review of Experiences during the Last Twenty-Two Years. *Processes* [Online], 11.

MASHARQA, A., AL-TARDEH, S., MLIH, R. & BOL, R. 2023. Vertical and Hybrid Constructed Wetlands as a Sustainable Technique to Improve Domestic Wastewater Quality. *Water* [Online], 15.

MASSÉ, D. I., SAADY, N. M. C. & GILBERT, Y. 2014. Potential of Biological Processes to Eliminate Antibiotics in Livestock Manure: An Overview. *Animals : an open access journal from MDPI,* 4**,** 146-163.

MCARDELL, C. S., MOLNAR, E., SUTER, M. J. F. & GIGER, W. 2003. Occurrence and Fate of Macrolide Antibiotics in Wastewater Treatment Plants and in the Glatt Valley Watershed, Switzerland. *Environmental Science & Technology,* 37**,** 5479-5486.

MCMURDIE, P. J. & HOLMES, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one,* 8**,** e61217.

MEEHAN, C., BJOURSON, A. J. & MCMULLAN, G. 2001. Paenibacillus azoreducens sp. nov., a synthetic azo dye decolorizing bacterium from industrial wastewater. *Int J Syst Evol Microbiol,* 51**,** 1681-1685.

MÉNDEZ-MENDOZA, A. S., BELLO-MENDOZA, R., HERRERA-LÓPEZ, D., MEJÍA-GONZÁLEZ, G. & CALIXTO-ROMO, A. 2015. Performance of constructed wetlands with ornamental plants in the treatment of domestic wastewater under the tropical climate of South Mexico. *Water Practice and Technology,* 10**,** 110-123.

MILLS, E., SULLIVAN, E. & KOVAC, J. 2022. Comparative Analysis of Bacillus cereus Group Isolates' Resistance Using Disk Diffusion and Broth Microdilution and the Correlation between Antimicrobial Resistance Phenotypes and Genotypes. *Applied and Environmental Microbiology,* 88**,** e02302-21.

MOHAMMADI, B., GORKINA, N., PÉREZ-REYES, M. E. & SMITH, S. A. 2023. Profiling toxin genes and antibiotic resistance in Bacillus cereus isolated from pre-launch spacecraft. *Frontiers in Microbiology,* 14.

MYERS, J. A., CURTIS, B. S. & CURTIS, W. R. 2013. Improving accuracy of cell and chromophore concentration measurements using optical density. *BMC Biophysics,* 6**,** 4.

NAKASAKI, K., NGUYEN, K. K., BALLESTEROS, F. C., MAEKAWA, T. & KOYAMA, M. 2020. Characterizing the microbial community involved in anaerobic digestion of lipid-rich wastewater to produce methane gas. *Anaerobe,* 61**,** 102082.

NIVALA, J., BOOG, J., HEADLEY, T., AUBRON, T., WALLACE, S., BRIX, H., MOTHES, S., VAN AFFERDEN, M. & MÜLLER, R. A. 2019. Side-by-side comparison of 15 pilot-scale conventional and intensified subsurface flow wetlands for treatment of domestic wastewater. *Science of The Total Environment,* 658**,** 1500-1513.

OBARSKA-PEMPKOWIAK, H., GAJEWSKA, M., WOJCIECHOWSKA, E. & OSTOJSKI, A. 2015. Single-Family Treatment Wetlands Progress in Poland. *In:* VYMAZAL, J. (ed.) *The Role of Natural and Constructed Wetlands in Nutrient Cycling and Retention on the Landscape.* Cham: Springer International Publishing.

OGUNDERO, A., VIGNOLA, M., CONNELLY, S. & SLOAN WILLIAM, T. 2022. Validating Flow Cytometry as a Method for Quantifying Bdellovibrio Predatory Bacteria and Its Prey for Microbial Ecology. *Microbiology Spectrum,* 10**,** e01033-21.

OKSANEN, J., KINDT, R., LEGENDRE, P., O'HARA, B., STEVENS, M. H. H., OKSANEN, M. J. & SUGGESTS, M. 2007. The vegan package. *Community ecology package,* 10**,** 719.

OREN, A. 2014. The Family Rhodocyclaceae. *In:* ROSENBERG, E., DELONG, E. F., LORY, S., STACKEBRANDT, E. & THOMPSON, F. (eds.) *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria.* Berlin, Heidelberg: Springer Berlin Heidelberg.

OSIŃSKA, A., KORZENIEWSKA, E., HARNISZ, M., FELIS, E., BAJKACZ, S., JACHIMOWICZ, P., NIESTĘPSKI, S. & KONOPKA, I. 2020. Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. *Journal of Hazardous Materials,* 381**,** 121221.

OTTOSSON, J., JARNHEIMER, P.-Å., AXEL STENSTRÖM, T. & OLSEN, B. 2012. A longitudinal study of antimicrobial resistant faecal bacteria in sediments collected from a hospital wastewater system. *Infection Ecology & Epidemiology,* 2**,** 7438.

PARADA, A. E., NEEDHAM, D. M. & FUHRMAN, J. A. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology,* 18**,** 1403-1414.

PARDE, D., PATWA, A., SHUKLA, A., VIJAY, R., KILLEDAR, D. J. & KUMAR, R. 2021. A review of constructed wetland on type, treatment and technology of wastewater. *Environmental Technology & Innovation,* 21**,** 101261.

PÄRNÄNEN, K. M. M., NARCISO-DA-ROCHA, C., KNEIS, D., BERENDONK, T. U., CACACE, D., DO, T. T., ELPERS, C., FATTA-KASSINOS, D., HENRIQUES, I., JAEGER, T., KARKMAN, A., MARTINEZ, J. L., MICHAEL, S. G., MICHAEL-KORDATOU, I., O'SULLIVAN, K., RODRIGUEZ-MOZAZ, S., SCHWARTZ, T., SHENG, H., SØRUM, H., STEDTFELD, R. D., TIEDJE, J. M., GIUSTINA, S. V. D., WALSH, F., VAZ-MOREIRA, I., VIRTA, M. & MANAIA, C. M. 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Science Advances,* 5**,** eaau9124.

PAULUS, G. K., HORNSTRA, L. M., ALYGIZAKIS, N., SLOBODNIK, J., THOMAIDIS, N. & MEDEMA, G. 2019. The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. *International Journal of Hygiene and Environmental Health,* 222**,** 635-644.

PEACH, K. C., BRAY, W. M., WINSLOW, D., LININGTON, P. F. & LININGTON, R. G. 2013. Mechanism of action-based classification of antibiotics using high-content bacterial image analysis. *Molecular Biosystems,* 9**,** 1837-1848.

PÉDRON, J., GUYON, L., LECOMTE, A., BLOTTIÈRE, L., CHANDEYSSON, C., ROCHELLE-NEWALL, E., RAYNAUD, X., BERGE, O. & BARNY, M.-A. 2020. Comparison of Environmental and Culture-Derived Bacterial Communities through 16S Metabarcoding: A Powerful Tool to Assess Media Selectivity and Detect Rare Taxa. *Microorganisms,* 8**,** 1129.

PÉREZ, Y., VARGAS, E., GARCÍA-CORTÉS, D., HERNÁNDEZ, W., CHECO, H. & JÁUREGUI-HAZA, U. 2024. Efficiency and effectiveness of systems for the treatment of domestic wastewater based on subsurface flow constructed wetlands in Jarabacoa, Dominican Republic. *Water Science and Engineering,* 17**,** 118-128.

PERRY, M. R., LEPPER, H. C., MCNALLY, L., WEE, B. A., MUNK, P., WARR, A., MOORE, B., KALIMA, P., PHILIP, C., DE RODA HUSMAN, A. M., AARESTRUP, F. M., WOOLHOUSE, M. E. J. & VAN BUNNIK, B. A. D. 2021. Secrets of the Hospital Underbelly: Patterns of Abundance of Antimicrobial Resistance Genes in Hospital Wastewater Vary by Specific Antimicrobial and Bacterial Family. *Front Microbiol,* 12**,** 703560.

PETRILLI, R., FABBRETTI, A., CERRETANI, A., PUCCI, K., PAGLIARETTA, G., PICCIOLINI, M., NAPOLIONI, V. & FALCONI, M. 2023. Selection, Identification and Functional Performance of Ammonia-Degrading Microbial Communities from an Activated Sludge for Landfill Leachate Treatment. *Microorganisms* [Online], 11.

PFRUNDER, S., GROSSMANN, J., HUNZIKER, P., BRUNISHOLZ, R., GEKENIDIS, M.-T. & DRISSNER, D. 2016. Bacillus cereus Group-Type Strain-Specific Diagnostic Peptides. *Journal of Proteome Research,* 15**,** 3098-3107.

POLIANCIUC, S. I., GURZĂU, A. E., KISS, B., ŞTEFAN, M. G. & LOGHIN, F. 2020. Antibiotics in the environment: causes and consequences. *Med Pharm Rep,* 93**,** 231-240.

PU, Y., LI, Y., ZHU, L., CHENG, Y., NUAMAH, L. A., ZHANG, H., CHEN, H., DU, G., WANG, L. & SONG, C. 2023. Long-term assessment on performance and seasonal optimal operation of a full-scale integrated multiple constructed wetland-pond system. *Science of The Total Environment,* 862**,** 161219.

QUAST, C., PRUESSE, E., YILMAZ, P., GERKEN, J., SCHWEER, T., YARZA, P., PEPLIES, J. & GLÖCKNER, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res,* 41**,** D590-6.

QUINCE, C., LANZEN, A., DAVENPORT, R. J. & TURNBAUGH, P. J. 2011. Removing Noise From Pyrosequenced Amplicons. *BMC Bioinformatics,* 12**,** 38.

QUINN, D. 2022. *The effect of filter bed length on the microbial communities within GAC biofilters and treated effluent water.* PhD, University of Glasgow.

QUINTELA-BALUJA, M., ABOUELNAGA, M., ROMALDE, J., SU, J.-Q., YU, Y., GOMEZ-LOPEZ, M., SMETS, B., ZHU, Y.-G. & GRAHAM, D. W. 2019. Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. *Water Research,* 162**,** 347-357.

RAJER, F. & SANDEGREN, L. 2022. The Role of Antibiotic Resistance Genes in the Fitness Cost of Multiresistance Plasmids. *mBio,* 13**,** e03552-21.

RAM, Y., DELLUS-GUR, E., BIBI, M., KARKARE, K., OBOLSKI, U., FELDMAN, M. W., COOPER, T. F., BERMAN, J. & HADANY, L. 2019. Predicting microbial growth in a mixed culture from growth curve data. *Proceedings of the National Academy of Sciences,* 116**,** 14698-14707.

RODRIGUEZ-DOMINGUEZ, M. A., KONNERUP, D., BRIX, H. & ARIAS, C. A. 2020. Constructed Wetlands in Latin America and the Caribbean: A Review of Experiences during the Last Decade. *Water* [Online], 12.

RODRIGUEZ-MOZAZ, S., CHAMORRO, S., MARTI, E., HUERTA, B., GROS, M., SÀNCHEZ-MELSIÓ, A., BORREGO, C. M., BARCELÓ, D. & BALCÁZAR, J. L. 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Research,* 69**,** 234-242.

ROSAS-BAÑOS, M. & GARCÍA-SALAZAR, E. M. 2024. Wastewater and agriculture in Mezquital valley (Mexico): analyzing alternatives from circular economy and ecological economy using bibliometric review. *Discover Water,* 4**,** 61.

SABRI, N. A., SCHMITT, H., VAN DER ZAAN, B., GERRITSEN, H. W., ZUIDEMA, T., RIJNAARTS, H. H. M. & LANGENHOFF, A. A. M. 2020a. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. *Journal of Environmental Chemical Engineering,* 8**,** 102245.

SABRI, N. A., VAN HOLST, S., SCHMITT, H., VAN DER ZAAN, B. M., GERRITSEN, H. W., RIJNAARTS, H. H. M. & LANGENHOFF, A. A. M. 2020b. Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants. *Science of The Total Environment,* 741**,** 140199.

SACRISTÁN-SORIANO, O., JARMA, D., SÁNCHEZ, M. I., ROMERO, N., ALONSO, E., GREEN, A. J., SÀNCHEZ-MELSIÓ, A., HORTAS, F., BALCÁZAR, J. L., PERALTA-SÁNCHEZ, J. M. & BORREGO, C. M. 2024. Winged resistance: Storks and gulls increase carriage of antibiotic resistance by shifting from paddy fields to landfills. *Science of The Total Environment,* 914**,** 169946.

SAEED, T. & SUN, G. 2011. The removal of nitrogen and organics in vertical flow wetland reactors: Predictive models. *Bioresource Technology,* 102**,** 1205-1213.

SAFFORD, H. R. & BISCHEL, H. N. 2019. Flow cytometry applications in water treatment, distribution, and reuse: A review. *Water Research,* 151**,** 110-133.

SÁNCHEZ-HUESCA, R., LERMA, A., GUZMÁN-SALDAÑA, R. M. E. & LERMA, C. 2020. Prevalence of Antibiotics Prescription and Assessment of Prescribed Daily Dose in Outpatients from Mexico City. *Antibiotics (Basel),* 9.

SANDEGREN, L. 2014. Selection of antibiotic resistance at very low antibiotic concentrations. *Ups J Med Sci,* 119**,** 103-7.

SANTOSANINGSIH, D., FADRIYANA, A. P., DAVID, N. I. & RATRIDEWI, I. 2023. Prevalence and Abundance of Beta-Lactam Resistance Genes in Hospital Wastewater and Enterobacterales Wastewater Isolates. *Tropical Medicine and Infectious Disease* [Online], 8.

SAYERS, E. W., BOLTON, E. E., BRISTER, J. R., CANESE, K., CHAN, J., COMEAU, D. C., CONNOR, R., FUNK, K., KELLY, C., KIM, S., MADEJ, T., MARCHLER-BAUER, A., LANCZYCKI, C., LATHROP, S., LU, Z., THIBAUD-NISSEN, F., MURPHY, T., PHAN, L., SKRIPCHENKO, Y., TSE, T., WANG, J., WILLIAMS, R., TRAWICK, B. W., PRUITT, K. D. & SHERRY, S. T. 2022. Database resources of the national center for biotechnology information. *Nucleic Acids Res,* 50**,** D20-d26.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols,* 3**,** 1101-1108.

SECRETARIA DE MEDIO AMBIENTE Y RECURSOS NATURALES. 2022. *NORMA Oficial Mexicana NOM-001-SEMARNAT-2021, Que establece los límites permisibles de contaminantes en las descargas de aguas residuales en cuerpos receptores propiedad de la nación.* [Online]. Estados Unidos Mexicanos: Secretaría de Medio Ambiente y Recursos Naturales. Available: https://agua.org.mx/wp-content/uploads/2022/03/DOF-NOM-001-SEMARNAT-2021.pdf [Accessed 11 Aug 2023].

SHIAU, Y.-J. & CHANG, E.-H. 2022. Microbial community development in tropical constructed wetland soils in Taiwan. *Science of The Total Environment,* 812**,** 152563.

SHUKLA, A., PARDE, D., GUPTA, V., VIJAY, R. & KUMAR, R. 2022. A review on effective design processes of constructed wetlands. *International Journal of Environmental Science and Technology,* 19**,** 12749-12774.

SI, Z., SONG, X., WANG, Y., CAO, X., ZHAO, Y., WANG, B., CHEN, Y. & AREFE, A. 2018. Intensified heterotrophic denitrification in constructed wetlands using four solid

carbon sources: Denitrification efficiency and bacterial community structure. *Bioresource Technology,* 267**,** 416-425.

SIEBE, C., CHAPELA, M., CAYETANO-SALAZAR, M., PRADO, B. & SIEMENS, J. 2016. Effects of more than 100 years of irrigation with Mexico City's wastewater in the Mezquital Valley (Mexico).

SIRIVEDHIN, T. & GRAY, K. A. 2006. Factors affecting denitrification rates in experimental wetlands: Field and laboratory studies. *Ecological Engineering,* 26**,** 167-181.

SLOBODIUK, S., NIVEN, C., ARTHUR, G., THAKUR, S. & ERCUMEN, A. 2021. Does Irrigation with Treated and Untreated Wastewater Increase Antimicrobial Resistance in Soil and Water: A Systematic Review. *International Journal of Environmental Research and Public Health* [Online], 18.

SMITH, C. J., NEDWELL, D. B., DONG, L. F. & OSBORN, A. M. 2006. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environmental Microbiology,* 8**,** 804-815.

SPROUFFSKE, K. & WAGNER, A. 2016. Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics,* 17**,** 172.

STANAWAY, J. D., AFSHIN, A., GAKIDOU, E., LIM, S. S., ABATE, D., ABATE, K. H., ABBAFATI, C., ABBASI, N., ABBASTABAR, H., ABD-ALLAH, F., ABDELA, J., ABDELALIM, A., ABDOLLAHPOUR, I., ABDULKADER, R. S., ABEBE, M., ABEBE, Z., ABERA, S. F., ABIL, O. Z., ABRAHA, H. N., ABRHAM, A. R., ABU-RADDAD, L. J., ABU-RMEILEH, N. M. E., ACCROMBESSI, M. M. K., ACHARYA, D., ACHARYA, P., ADAMU, A. A., ADANE, A. A., ADEBAYO, O. M., ADEDOYIN, R. A., ADEKANMBI, V., ADEMI, Z., ADETOKUNBOH, O. O., ADIB, M. G., ADMASIE, A., ADSUAR, J. C., AFANVI, K. A., AFARIDEH, M., AGARWAL, G., AGGARWAL, A., AGHAYAN, S. A., AGRAWAL, A., AGRAWAL, S., AHMADI, A., AHMADI, M., AHMADIEH, H., AHMED, M. B., AICHOUR, A. N., AICHOUR, I., AICHOUR, M. T. E., AKBARI, M. E., AKINYEMIJU, T., AKSEER, N., AL-ALY, Z., AL-EYADHY, A., AL-MEKHLAFI, H. M., ALAHDAB, F., ALAM, K., ALAM, S., ALAM, T., ALASHI, A., ALAVIAN, S. M., ALENE, K. A., ALI, K., ALI, S. M., ALIJANZADEH, M., ALIZADEH-NAVAEI, R., ALJUNID, S. M., ALKERWI, A. A., ALLA, F., ALSHARIF, U., ALTIRKAWI, K., ALVIS-GUZMAN, N., AMARE, A. T., AMMAR, W., ANBER, N. H., ANDERSON, J. A., ANDREI, C. L., ANDROUDI, S., ANIMUT, M. D., ANJOMSHOA, M., ANSHA, M. G., ANTÓ, J. M., ANTONIO, C. A. T., ANWARI, P., APPIAH, L. T., APPIAH, S. C. Y., ARABLOO, J., AREMU, O., ÄRNLÖV, J., ARTAMAN, A., ARYAL, K. K., ASAYESH, H., ATARO, Z., AUSLOOS, M., AVOKPAHO, E. F. G. A., AWASTHI, A., AYALA QUINTANILLA, B. P., AYER, R., AYUK, T. B., AZZOPARDI, P. S., et al. 2018. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet,* 392**,** 1923-1994.

STANTON, I. C., MURRAY, A. K., ZHANG, L., SNAPE, J. & GAZE, W. H. 2020. Evolution of antibiotic resistance at low antibiotic concentrations including selection below the minimal selective concentration. *Communications Biology,* 3**,** 467.

STEDTFELD, R. D., GUO, X., STEDTFELD, T. M., SHENG, H., WILLIAMS, M. R., HAUSCHILD, K., GUNTURU, S., TIFT, L., WANG, F., HOWE, A., CHAI, B., YIN, D., COLE, J. R., TIEDJE, J. M. & HASHSHAM, S. A. 2018. Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. *FEMS Microbiol Ecol,* 94.

STEER, D., FRASER, L., BODDY, J. & SEIBERT, B. 2002. Efficiency of small constructed wetlands for subsurface treatment of single-family domestic effluent. *Ecological Engineering,* 18**,** 429-440.

STEFANOWICZ, A. 2006. The Biolog Plates Technique as a Tool in Ecological Studies of Microbial Communities. *Polish Journal of Environmental Studies,* 15**,** 669-676.

SULTANA, M. Y., AKRATOS, C. S., VAYENAS, D. V. & PAVLOU, S. 2015. Constructed wetlands in the treatment of agro-industrial wastewater: A review. *Hemijska Industrija,* 69**,** 127-142.

SUN, G. & SAEED, T. 2009. Kinetic modelling of organic matter removal in 80 horizontal flow reed beds for domestic sewage treatment. *Process Biochemistry,* 44**,** 717-722.

SUN, H., ZHANG, H., WU, D., DING, J., NIU, Y., JIANG, T., YANG, X. & LIU, Y. 2023. Deciphering the antibiotic resistome and microbial community in municipal wastewater treatment plants at different elevations in eastern and western China. *Water Research,* 229**,** 119461.

SUTRADHAR, I., CHING, C., DESAI, D., HEINS, Z., KHALIL, A. S. & ZAMAN, M. H. 2023. Effects of antibiotic interaction on antimicrobial resistance development in wastewater. *Scientific Reports,* 13**,** 7801.

SUTTON, S. 2011. Measurement of microbial cells by optical density. *Journal of Validation technology,* 17**,** 46-49.

SUZUKI, M. T., TAYLOR, L. T. & DELONG, E. F. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. *Appl Environ Microbiol,* 66**,** 4605-14.

SZEKERES, S., KISS, I., KALMAN, M. & SOARES, M. I. M. 2002. Microbial population in a hydrogen-dependent denitrification reactor. *Water Research,* 36**,** 4088-4094.

TABLA-VÁZQUEZ, C. G., CHÁVEZ-MEJÍA, A. C., ORTA LEDESMA, M. T. & RAMÍREZ-ZAMORA, R. M. 2020. Wastewater Treatment in Mexico. *In:* RAYNAL-VILLASENOR, J. A. (ed.) *Water Resources of Mexico.* Cham: Springer International Publishing.

TANNER, C. C., CRAGGS, R. J., SUKIAS, J. P. & PARK, J. B. 2005. Comparison of maturation ponds and constructed wetlands as the final stage of an advanced pond system. *Water Sci Technol,* 51**,** 307-14.

TIAN, T., TAM, N. F. Y., ZAN, Q., CHEUNG, S. G., SHIN, P. K. S., WONG, Y. S., ZHANG, L. & CHEN, Z. 2017. Performance and bacterial community structure of a 10-years old constructed mangrove wetland. *Marine Pollution Bulletin,* 124**,** 1096-1105.

TIAN, W., LI, Q., LUO, Z., WU, C., SUN, B., ZHAO, D., CHI, S., CUI, Z., XU, A. & SONG, Z. 2024. Microbial community structure in a constructed wetland based on a recirculating aquaculture system: Exploring spatio-temporal variations and assembly mechanisms. *Marine Environmental Research,* 197**,** 106413.

TORRENTÓ, C., URMENETA, J., OTERO, N., SOLER, A., VIÑAS, M. & CAMA, J. 2011. Enhanced denitrification in groundwater and sediments from a nitrate-contaminated aquifer after addition of pyrite. *Chemical Geology,* 287**,** 90-101.

TRENBERTH, K. E., SMITH, L., QIAN, T., DAI, A. & FASULLO, J. 2007. Estimates of the Global Water Budget and Its Annual Cycle Using Observational and Model Data. *Journal of Hydrometeorology,* 8**,** 758-769.

TRUU, M., JUHANSON, J. & TRUU, J. 2009. Microbial biomass, activity and community composition in constructed wetlands. *Science of The Total Environment,* 407**,** 3958-3971.

TULCHINSKY, T. H. 2018. Chapter 5 - John Snow, Cholera, the Broad Street Pump; Waterborne Diseases Then and Now. *In:* TULCHINSKY, T. H. (ed.) *Case Studies in Public Health.* Academic Press.

TYRRELL, C., DO, T. T., LEIGH, R. J., BURGESS, C. M., BRENNAN, F. P. & WALSH, F. 2023. Differential impact of swine, bovine and poultry manure on the microbiome and resistome of agricultural grassland. *Science of The Total Environment,* 886**,** 163926.

UNITED NATIONS. 2015. *Sustainable Development Goals* [Online]. New York. Available: https://sdgs.un.org/goals [Accessed 14 Auguest 2024].

UNITED NATIONS CHILDREN'S FUND (UNICEF) & WORLD HEALTH ORGANIZATION (WHO) 2023. *Progress on household drinking water, sanitation and hygiene 2000–2022: special focus on gender,* New York.

VERDUZCO GARIBAY, M., FERNÁNDEZ DEL CASTILLO, A., DÍAZ TORRES, O., DE ANDA, J., YEBRA-MONTES, C., SENÉS-GUERRERO, C. & GRADILLA-HERNÁNDEZ, M. S. 2021. Characterization of the Spatial Variation of Microbial Communities in a Decentralized Subtropical Wastewater Treatment Plant Using Passive Methods. *Water* [Online], 13.

VETERINARY MEDICINES DIRECTORATE 2023. *Third UK One Health Report - Joint report on antibiotic use, antibiotic sales and antibiotic resistance.,* New Haw, Addlestone: Veterinary Medicines Directorate.

VĚTROVSKÝ, T. & BALDRIAN, P. 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS One,* 8**,** e57923.

VIGNOLA, M., WERNER, D., HAMMES, F., KING, L. C. & DAVENPORT, R. J. 2018. Flow-cytometric quantification of microbial cells on sand from water biofilters. *Water Research,* 143**,** 66-76.

VON SPERLING, M., DORNELAS, F. L., ASSUNÇÃO, F. A., DE PAOLI, A. C. & MABUB, M. O. 2010. Comparison between polishing (maturation) ponds and subsurface flow constructed wetlands (planted and unplanted) for the post-treatment of the effluent from UASB reactors. *Water Sci Technol,* 61**,** 1201-9.

VYMAZAL, J. 2007. Removal of nutrients in various types of constructed wetlands. *Science of The Total Environment,* 380**,** 48-65.

VYMAZAL, J. 2009. The use constructed wetlands with horizontal sub-surface flow for various types of wastewater. *Ecological Engineering,* 35**,** 1-17.

VYMAZAL, J. 2011a. Constructed Wetlands for Wastewater Treatment: Five Decades of Experience. *Environmental Science & Technology,* 45**,** 61-69.

VYMAZAL, J. 2011b. Plants used in constructed wetlands with horizontal subsurface flow: a review. *Hydrobiologia,* 674**,** 133-156.

VYMAZAL, J. 2013. The use of hybrid constructed wetlands for wastewater treatment with special attention to nitrogen removal: A review of a recent development. *Water Research,* 47**,** 4795-4811.

VYMAZAL, J. 2014. Constructed wetlands for treatment of industrial wastewaters: A review. *Ecological Engineering,* 73**,** 724-751.

VYMAZAL, J., ZHAO, Y. & MANDER, Ü. 2021. Recent research challenges in constructed wetlands for wastewater treatment: A review. *Ecological Engineering,* 169**,** 106318.

WANG, F.-H., QIAO, M., SU, J.-Q., CHEN, Z., ZHOU, X. & ZHU, Y.-G. 2014. High Throughput Profiling of Antibiotic Resistance Genes in Urban Park Soils with Reclaimed Water Irrigation. *Environmental Science & Technology,* 48**,** 9079-9085.

WANG, J., LONG, Y., YU, G., WANG, G., ZHOU, Z., LI, P., ZHANG, Y., YANG, K. & WANG, S. 2022. A Review on Microorganisms in Constructed Wetlands for Typical Pollutant Removal: Species, Function, and Diversity. *Frontiers in Microbiology,* 13.

WANG, Y., HAO, A., QUAN, Y., JIN, M. & PIAO, W. 2023a. Analysis of the Degradation Characteristics of Chlorpyrifos in an Electrochemically Constructed Wetland Coupled System under Different Pesticide Exposure Conditions and Microbial Community Analysis. *Sustainability* [Online], 15.

WANG, Y., THOMPSON, K. N., YAN, Y., SHORT, M. I., ZHANG, Y., FRANZOSA, E. A., SHEN, J., HARTMANN, E. M. & HUTTENHOWER, C. 2023b. RNA-based amplicon sequencing is ineffective in measuring metabolic activity in environmental microbial communities. *Microbiome,* 11**,** 131.

WANNER, J. 2021. The development in biological wastewater treatment over the last 50 years. *Water Science and Technology,* 84**,** 274-283.

WASEEM, H., JAMEEL, S., ALI, J., SALEEM UR REHMAN, H., TAUSEEF, I., FAROOQ, U., JAMAL, A. & ALI, M. I. 2019. Contributions and Challenges of High Throughput qPCR for Determining Antimicrobial Resistance in the Environment: A Critical Review. *Molecules,* 24.

WATKINSON, A. J., MURBY, E. J., KOLPIN, D. W. & COSTANZO, S. D. 2009. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. *Science of The Total Environment,* 407**,** 2711-2723.

WEBER, K. P. & LEGGE, R. L. 2011. Dynamics in the bacterial community-level physiological profiles and hydrological characteristics of constructed wetland mesocosms during start-up. *Ecological Engineering,* 37**,** 666-677.

WHELAN, M. J., LINSTEAD, C., WORRALL, F., ORMEROD, S. J., DURANCE, I., JOHNSON, A. C., JOHNSON, D., OWEN, M., WIIK, E., HOWDEN, N. J. K., BURT, T. P., BOXALL, A., BROWN, C. D., OLIVER, D. M. & TICKNER, D. 2022. Is water quality in British rivers "better than at any time since the end of the Industrial Revolution"? *Science of The Total Environment,* 843**,** 157014.

WHO. 2017. *WHO publishes list of bacteria for which new antibiotics are urgently needed* [Online]. GENEVA: World Health Organization. Available: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed) [Accessed 2 June 2023].

WILLEMS, A. 2014. The Family Comamonadaceae. *In:* ROSENBERG, E., DELONG, E. F., LORY, S., STACKEBRANDT, E. & THOMPSON, F. (eds.) *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria.* Berlin, Heidelberg: Springer Berlin Heidelberg.

WOLFF, D., ABOU-KANDIL, A., AZAIZEH, H., WICK, A. & JADOUN, J. 2024. Influence of vegetation and substrate type on removal of emerging organic contaminants and microbial dynamics in horizontal subsurface constructed wetlands. *Science of The Total Environment,* 927**,** 172346.

WORLD HEALTH ORGANIZATION (WHO) 2014. Briefing note: antimicrobial resistance: an emerging water, sanitation and hygiene Issue. Geneva: World Health Organization.

WORLD HEALTH ORGANIZATION (WHO) 2023. *Burden of disease attributable to unsafe drinking-water, sanitation and hygiene,* Geneva, World Health Organization.

WORLD HEALTH ORGANIZATION (WHO) & UNITED NATIONS CHILDREN'S FUND (UNICEF) 2015. *Progress on sanitation and drinking water – 2015 update and MDG assessment,* Geneva, World Health Organization.

WORLD HEALTH ORGANIZATION (WHO) & UNITED NATIONS CHILDREN'S FUND (UNICEF) 2019. *Progress on household drinking water, sanitation and hygiene 2000-2017: special focus on inequalities,* Geneva, World Health Organization.

WORLD HEALTH ORGANIZATION (WHO) & UNITED NATIONS CHILDREN'S FUND (UNICEF) 2020. *State of the world's sanitation: an urgent call to transform sanitation for*

better health, environments, economies and societies, Geneva, World Health Organization.

WORLD HEALTH ORGANIZATION (WHO) & UNITED NATIONS CHILDREN'S FUND (UNICEF) 2021. *Progress on household drinking water, sanitation and hygiene 2000-2020: five years into the SDGs,* Geneva, World Health Organization.

WU, H., MA, W., KONG, Q. & LIU, H. 2018. Spatial-temporal dynamics of organics and nitrogen removal in surface flow constructed wetlands for secondary effluent treatment under cold temperature. *Chemical Engineering Journal,* 350**,** 445-452.

WU, H., WANG, R., YAN, P., WU, S., CHEN, Z., ZHAO, Y., CHENG, C., HU, Z., ZHUANG, L., GUO, Z., XIE, H. & ZHANG, J. 2023. Constructed wetlands for pollution control. *Nature Reviews Earth & Environment,* 4**,** 218-234.

WU, L., NING, D., ZHANG, B., LI, Y., ZHANG, P., SHAN, X., ZHANG, Q., BROWN, M. R., LI, Z., VAN NOSTRAND, J. D., LING, F., XIAO, N., ZHANG, Y., VIERHEILIG, J., WELLS, G. F., YANG, Y., DENG, Y., TU, Q., WANG, A., ACEVEDO, D., AGULLO-BARCELO, M., ALVAREZ, P. J. J., ALVAREZ-COHEN, L., ANDERSEN, G. L., DE ARAUJO, J. C., BOEHNKE, K. F., BOND, P., BOTT, C. B., BOVIO, P., BREWSTER, R. K., BUX, F., CABEZAS, A., CABROL, L., CHEN, S., CRIDDLE, C. S., DENG, Y., ETCHEBEHERE, C., FORD, A., FRIGON, D., SANABRIA, J., GRIFFIN, J. S., GU, A. Z., HABAGIL, M., HALE, L., HARDEMAN, S. D., HARMON, M., HORN, H., HU, Z., JAUFFUR, S., JOHNSON, D. R., KELLER, J., KEUCKEN, A., KUMARI, S., LEAL, C. D., LEBRUN, L. A., LEE, J., LEE, M., LEE, Z. M. P., LI, Y., LI, Z., LI, M., LI, X., LING, F., LIU, Y., LUTHY, R. G., MENDONÇA-HAGLER, L. C., DE MENEZES, F. G. R., MEYERS, A. J., MOHEBBI, A., NIELSEN, P. H., NING, D., OEHMEN, A., PALMER, A., PARAMESWARAN, P., PARK, J., PATSCH, D., REGINATTO, V., DE LOS REYES, F. L., RITTMANN, B. E., NOYOLA, A., ROSSETTI, S., SHAN, X., SIDHU, J., SLOAN, W. T., SMITH, K., DE SOUSA, O. V., STAHL, D. A., STEPHENS, K., TIAN, R., TIEDJE, J. M., TOOKER, N. B., TU, Q., VAN NOSTRAND, J. D., DE LOS COBOS VASCONCELOS, D., VIERHEILIG, J., WAGNER, M., WAKELIN, S., WANG, A., WANG, B., WEAVER, J. E., et al. 2019. Global diversity and biogeography of bacterial communities in wastewater treatment plants. *Nature Microbiology,* 4**,** 1183-1195.

WU, Y., HAN, R., YANG, X., FANG, X., CHEN, X., YANG, D. & ZHANG, R. 2016. Correlating microbial community with physicochemical indices and structures of a full-scale integrated constructed wetland system. *Applied Microbiology and Biotechnology,* 100**,** 6917-6926.

WU, Y., HAN, R., YANG, X., ZHANG, Y. & ZHANG, R. 2017. Long-term performance of an integrated constructed wetland for advanced treatment of mixed wastewater. *Ecological Engineering,* 99**,** 91-98.

XIAO, J., HUANG, J., HUANG, M., CHEN, M. & WANG, M. 2020. Application of basalt fiber in vertical flow constructed wetland for different pollution loads wastewater: Performance, substrate enzyme activity and microorganism community. *Bioresource Technology,* 318**,** 124229.

XUE, J., SCHMITZ, B. W., CATON, K., ZHANG, B., ZABALETA, J., GARAI, J., TAYLOR, C. M., ROMANCHISHINA, T., GERBA, C. P., PEPPER, I. L. & SHERCHAN, S. P. 2019. Assessing the spatial and temporal variability of bacterial communities in two Bardenpho wastewater treatment systems via Illumina MiSeq sequencing. *Science of The Total Environment,* 657**,** 1543-1552.

YAN, Q., XU, Y., YU, Y., ZHU, Z. W. & FENG, G. 2018. Effects of pharmaceuticals on microbial communities and activity of soil enzymes in mesocosm-scale constructed wetlands. *Chemosphere,* 212**,** 245-253.

YANG, Y., LI, B., ZOU, S., FANG, H. H. P. & ZHANG, T. 2014. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Research,* 62**,** 97-106.

ZHANG, N., LU, D., SHENG, H., XIA, J., KAN, P., YAO, Z., CHEN, H., LI, G., ZHU, D. Z. & LIU, H. 2023a. Constructed wetlands as hotspots of antibiotic resistance genes and pathogens: Evidence from metagenomic analysis in Chinese rural areas. *Journal of Hazardous Materials,* 447**,** 130778.

ZHANG, W., CHU, H., YANG, L., YOU, X., YU, Z., ZHANG, Y. & ZHOU, X. 2023b. Technologies for pollutant removal and resource recovery from blackwater: a review. *Frontiers of Environmental Science & Engineering,* 17**,** 83.

ZHANG, X., XIA, Y., ZENG, Y., SUN, X., TAO, R., MEI, Y. & QU, M. 2022. Simultaneous nitrification and denitrification by Pseudomonas sp. Y-5 in a high nitrogen environment. *Environmental Science and Pollution Research,* 29**,** 69491-69501.

ZHAO, Y.-J., LI, J.-H., WANG, Z.-F., YAN, C., WANG, S.-B. & ZHANG, J.-B. 2012. Influence of the plant development on microbial diversity of vertical-flow constructed wetlands. *Biochemical Systematics and Ecology,* 44**,** 4-12.

ZHOU, X.-Y., LI, H., ZHOU, S.-Y.-D., ZHANG, Y.-S. & SU, J.-Q. 2023. City-scale distribution of airborne antibiotic resistance genes. *Science of The Total Environment,* 856**,** 159176.

ZHU, L., YUAN, L., SHUAI, X.-Y., LIN, Z.-J., SUN, Y.-J., ZHOU, Z.-C., MENG, L.-X., JU, F. & CHEN, H. 2023. Deciphering basic and key traits of antibiotic resistome in influent and effluent of hospital wastewater treatment systems. *Water Research,* 231**,** 119614.

ZHU, Y.-G., JOHNSON, T. A., SU, J.-Q., QIAO, M., GUO, G.-X., STEDTFELD, R. D., HASHSHAM, S. A. & TIEDJE, J. M. 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences,* 110**,** 3435-3440.

ZHU, Y., CUI, L., LI, J., WANG, R., VYMAZAL, J., LI, W., LEI, Y., ZHANG, M., HAO, T. & WEI, J. 2021. Long-term performance of nutrient removal in an integrated constructed wetland. *Science of The Total Environment,* 779**,** 146268.

ZURITA, F., ROY, E. D. & WHITE, J. R. 2012. Municipal wastewater treatment in Mexico: current status and opportunities for employing ecological treatment systems. *Environmental Technology,* 33**,** 1151-1158.