THE UNIVERSITY OF MIYAZAKI

JAPAN

The Effect of Antibiotic-Resistant Bacteria and Antibiotic-Resistant Genes Released from Wastewater Treatment Plants on the Receiving River

下水処理場から放出された薬剤耐性菌や薬剤耐性遺伝子が放流河川に及ぼす影響

Xie Hui

Master of Engineering

University of Miyazaki

A thesis submitted for the degree of Doctor of Philosophy at
The University of Miyazaki in July 2023

Department of Environment and Resource Science
Course of Environmentally Harmonized Technology and Science

Table of Contents

Abstract

Antibiotic resistance (AR) is a growing public health threat worldwide, and the aquatic environment is recognized as a reservoir and a vehicle for the transmission of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (AGRs). Treated effluent from wastewater treatment plants (WWTPs) is often discharged into aquatic environment, where it can come into contact with natural microbial communities and promote the dissemination of AR. It has been well recognized that antibiotics could stimulate the dissemination of AR through horizontal gene transfer (HGT). However, the pathways of acquisition and transmission of ARB and ARGs in wastewater treatment processes and their dissemination in aquatic environments, including rivers, remain poorly understood. The aim of this thesis was to conduct a three-part experiment to investigate the prevalence of AR *E. coli* under aerobic conditions, residual ESBL-resistant *E. coli*/coliforms during treatment and then flow into the receiving river, and the potential spread of ESBL-producing *E. coli* from the effluent transfer AR to STEC bacteria in the river. The surviving AR *E. coli* predominantly included MRD and ESBL-producing *E. coli* strains of phylogroup B2. Throughout the treatment process, the numbers of *E. coli* and coliforms were significantly reduced, while ESBL-producing *E. coli* and coliforms were detected in each treatment process (even after chlorination). In addition, ESBL-*E. coli* that persists in effluent from WWTPs, can potentially disseminate resistance to STEC bacteria in rivers, even at low concentrations. The results confirm that WWTPs serve as reservoirs of ARB and ARGs and pose a potential risk to human. Further research is needed to identify the specific mechanisms underlying the spread of ARB and ARGs in the environment and to develop targeted interventions to prevent the transmission of AR and safeguard public health.

Keywords

Water environment; *Escherichia coli* (*E. coli*); coliforms; extended-spectrum β-lactamases (ESBLs); antimicrobial resistance (AMR); antibiotic resistance (AR); antibiotic resistance genes (ARGs); horizontal gene transfer (HGT); conjugation.

Declaration by author

This thesis is entirely my own original work and contains no material previously published or composed by anyone except as appropriate references in the paper. The content of my thesis is the result of work undertaken after the research candidature for a higher degree has been commenced and excludes substantial parts of work submitted for the award of any other degree or diploma from any university or other institution of higher education.

I acknowledge that an electronic copy of my thesis must be submitted to the University Library and that it will be used for research and study, in accordance with the policies and procedures of University of Miyazaki.

I acknowledge that the copyright in all material contained in my thesis belongs to the copyright holder who owns that material. In appropriate situations, I have obtained copyright permission from the copyright owner to reproduce material from this thesis and have obtained the permission of the co-authors for any jointly created works included in the thesis.

Research Involving Human or Animal Subjects

No animal or human subjects were involved in this research.

Financial support

This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI; Grant Number: JP 21H0362021.

List of Abbreviations

ABPC, AMP	Ampicillin
AMR	Antimicrobial resistance
AMO	Amoxicillin
AR	Antibiotic resistance
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic resistance genes
BHI	Brain heart infusion
CAZ	Ceftazidime
CAZ/CVA	Ceftazidime/Clavulanic acid
CFU	Colony-forming unit
CFZ	Cefazolin
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CPM	Cefepime
CTX	Cefotaxime
CTX/CVA	Cefotaxime/Clavulanic acid
DAEC	Diffusely Adherent
EAEC	Enteroaggregative *E. coli*
EC	Electrical conductivity
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended spectrum β-lactamases
ETEC	Enterotoxigenic *E. coli*
FQREC	Fluoroquinolone-resistant *Escherichia coli*
GEN	Gentamicin

GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
HGT	Horizontal gene transfer
HUS	Hemolytic uremic syndrome
IMP	Imipenem
LB	Luria-Bertani
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MEP	Meropenem
MIC	Minimum inhibitory concentration
MDR	Multi-drug resistance
MH	Mueller–Hinton
MGEs	Mobile genetic elements
MSRA	Methicillin-resistant *Staphylococcus aureus*
NA	Nalidixic acid
nMDS	Non-metric multidimensional scaling
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
RFP	Rifampicin
STEC	Shiga toxin-producing *Escherichia coli*
TET, TC	Tetracycline
TOC	Total organic carbon
TU	Turbidity
UV	Ultraviolet
VGT	Vertical gene transfer
WHO	World health organization

List of Figures

List of Tables

Chapter 1. Introduction

1.1 Background

1.1.1 Occurrence and issues of antibiotic resistance

Human beings have been plagued by a variety of diseases over the course of history, whether it was malaria caused by hunting, influenza caused by animal breeding, or even cholera caused by trade after the Great Voyage. Infectious diseases have accompanied the development of mankind from the pre-historic period to modern society, and they have accounted for a large proportion of the illnesses of mankind. The discovery and introduction of antimicrobial agents in the last century has made a tremendous influence on the human health. With the discovery of penicillin, the establishment of serotherapy and many other treatments for infectious diseases, the number of deaths from infectious diseases has decreased globally.

Antimicrobial agents cover a wide range of pharmaceutical products (antibiotics, antivirals, antifungals, antimalarials) which are used against bacterial, viral, fungal and parasitic infections [1, 2]. Antimicrobial resistance (AMR) refers to the acquisition of

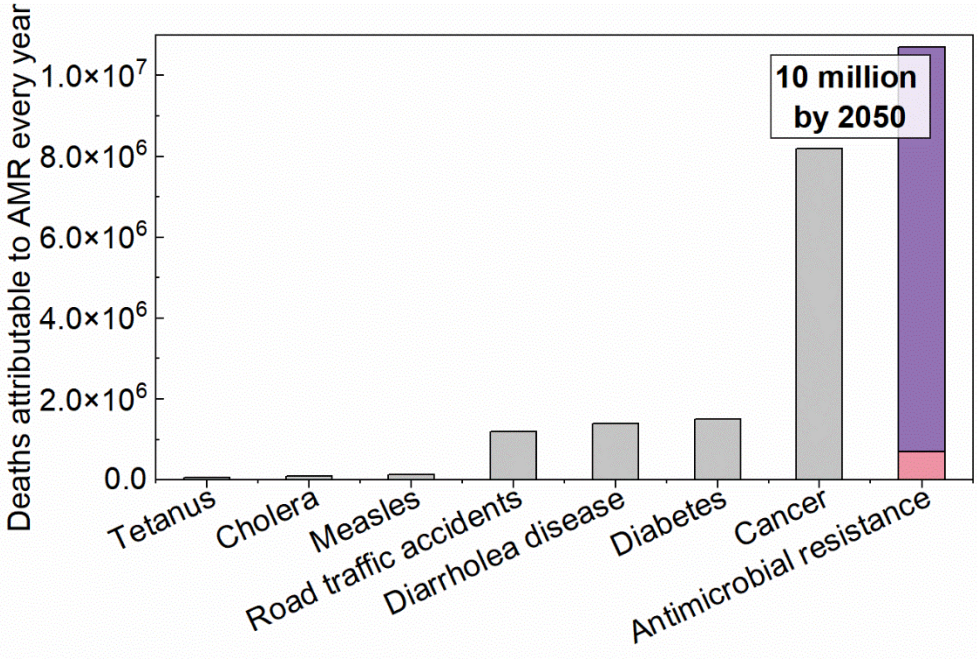

Figure 1.1 The total deaths projected by 2050 attributable to antimicrobial resistance (AMR) every year compared to other major causes of death [4].

resistance by previously susceptible microorganisms through various routes, which may lead to the failure of antimicrobials to be effective as time goes on, causing infections to be more difficult to treat and increasing the risk of disease spread [3]. The emergence and spread of AMR pose a serious threat to animal and human health, as antimicrobial resistant bacteria cause around 700,000 deaths worldwide annually, and it is estimated that up to 10 million people will probably die from AMR each year by 2050 (**Figure 1.1**) [4]. While the large number of antibiotics in clinical, veterinary and agricultural areas relevant to human livelihoods have accumulated overloads of antibiotic pressure on bacteria, resulting in an increased occurrence of antibiotic resistance (AR). In consequence, the infections caused by multidrug-resistant (MDR) bacteria have been reported to cause 23,000 annual deaths in the United States [5], and 25,000 annual deaths in European Union [6], respectively. Despite the absence of reliable data on the number of deaths caused by antibiotic-resistant bacteria (ARB) in Japan, 8,000 deaths were confirmed in 2017 due to two representative types of ARBs: typical gram-positive bacteria (GPB) methicillin-resistant *Staphylococcus aureus* (MSRA), gram-negative bacteria (GNB) fluoroquinolone-resistant *Escherichia coli* (FQREC) [7]. In consideration of these serious circumstances, in 2017, the World Health Organization (WHO) listed *Escherichia coli* (*E. coli*), *Shigella*, and *Salmonella* in the *Enterobacteriaceae* family as ARB that pose serious threats to human health and released survey data to warn the global population of the potential severity. *Enterobacteriaceae* are the pathogens that related to gastrointestinal infections, urinary tract infections and various other intestinal infections [8, 9], among which *E. coli* is the most important bacteria of the intestinal flora of humans and animals. Generally, most *E. coli* do not carry pathogens, but there are also *E. coli* that contain pathogens. The main cause of many foods poisoning, diarrhea and abdominal pain in our living life is the pathogen *E. coli* [10].

As a result of the inappropriate use of a large number of antibiotics in clinical, agricultural or veterinary, the bacterium may become ARB through self-adaptive mutation [11]. From a genetic point of view, when AR spreads as new generations of bacteria acquire antibiotic resistance gene (ARG), known as vertical gene transfer (VGT), and

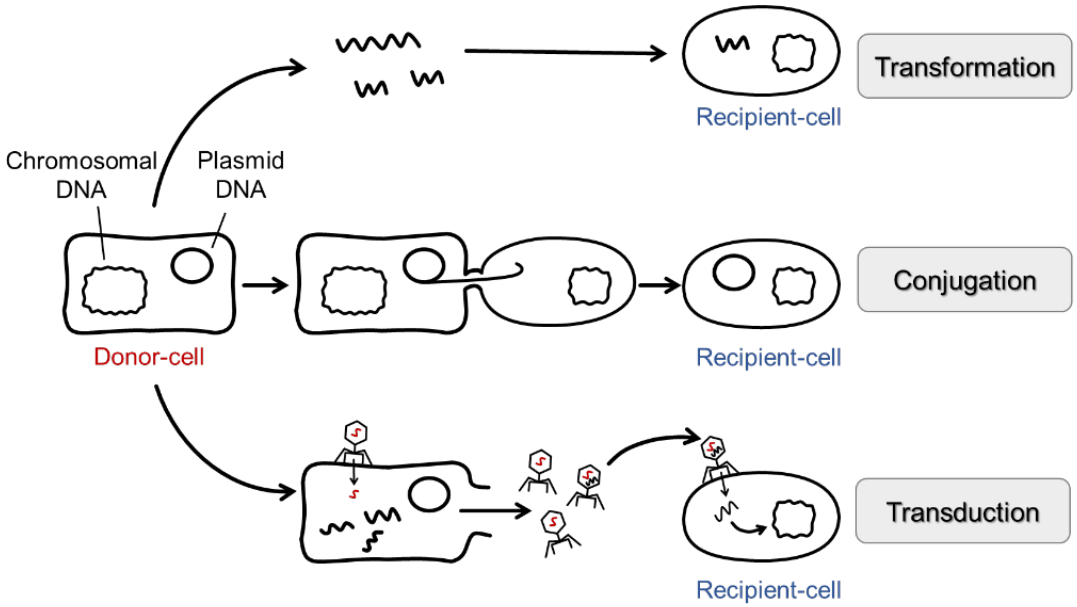

Figure 1.2 The 3 mechanisms of HGT, known as transformation, conjugation, and transduction.

when bacteria exchange genetic information with other bacteria, known as horizontal gene transfer (HGT) [12, 13]. However, the HGT, which can transfer ARGs between various species, plays a more major role in the spread of ARGs than the VGT [14]. The transfer of genetic information from one cell to another can occur by 3 mechanisms: transformation, conjugation, and transduction (**Figure 1.2**) [15]. Transformation is the second mechanism for gene transfer which is the direct uptake of naked DNA fragments into bacteria [16]. Most bacteria exchange genetic information via direct contact of the sex pili, and this process is called conjugation [17]. Transduction is the third pathway, which is the phage-mediated DNA transfer into bacteria [18].

1.1.2 The widespread antibiotic resistance in the environment

In contrast to other world concerns such as the Greenhouse Effect and the Desertification of Land, resistance in the environment is a potential risk that could lead to the next pandemic if ignored. Although antibiotics have brought great positive benefits to human health as well as to agriculture and livestock husbandry, the potential environmental risks associated with the overuse or misuse of antibiotics have received

increasing attention in the last century [19]. The *Nature* published an article in 2014 stating that the resistance of the bacteria is increasing which causes many antibiotic agents to be ineffective [20]. In Japan, the *National Action Plan on Antimicrobial Resistance (AMR) 2016-2020 and relevant activities* has been established and efforts are being made to prevent and control resistant bacteria in various fields [21]. In the healthcare and livestock sectors, testing for MDR bacteria in humans and animals (livestock, aquatic animals and pets) is published once a year to provide information to the public [22]. ARB are not only found in healthcare facilities [23], livestock farms [24] and aquaculture [25] where antibiotics are commonly used, but also in our living environment, including food [26], drinking water [27] and aquatic environments [28].

A purposed transfer pathway of ARB and ARGs which might occur in the environment is shown in **Figure 1.3**. First, the use of antibiotics allows them to enter the human gut and improper use may destroy the homeostasis of the intestinal flora [29], posing a threat to human health. Humans and animals that consume antibiotics may produce ARB in their

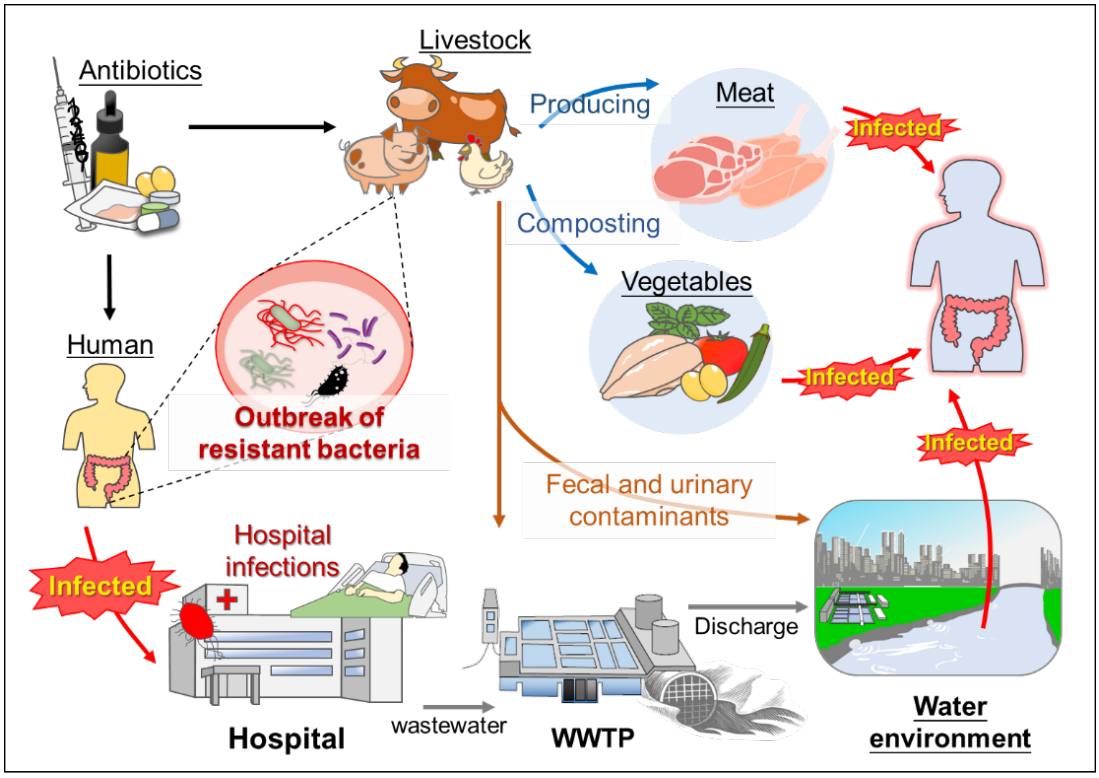

Figure 1.3 An illustration of the spread of antibiotic resistance in the environment.

intestines. These ARB have the potential to be transferred from person to person in a community or from animal to human during food consumption. However, antibiotics are difficult to metabolize in the internal tract [30], which results in a highly biologically active for a long time in the natural environment. ARB and ARGs contamination in human or animal populations may then accumulate in wastewater treatment plants (WWTPs) that receive wastewater from domestic, hospital and slaughterhouse sources. As a result of antibiotic misuse and overuse, antibiotic contamination in the receiving environment may select for surviving ARB [31]. More importantly, HGT can even overcome barriers between different bacterial species, allowing ARGs transfer from non-pathogenic ARB to pathogens [14] and thus enhancing the potential emergence of AR pathogens. In the current study, aquatic environments are one of the most abundant hotspots and reservoirs for ARGs transfer in different natural and engineered environments [32] as they are frequently affected by anthropogenic activities [33]. For example, different mixtures of antibiotics, nutrients, and other pollutants, ARB and ARGs can accumulate in the aquatic environment through treated and untreated domestic wastewater [34], hospital wastewater [35], aquaculture discharges and agricultural runoff [36, 37]. In particular, WWTPs, as reservoirs and hotspots, represent an important site for AR dissemination [38]. This is because the WWTPs environment is an ideal environment for the growth and transmission of ARGs, which includes high levels of organics [39], selection pressure from toxic compounds [40] and high bacterial diversities [41]. Thus, these favorable conditions can increase the frequency of HGT and opportunities for microbial interactions by transmitting ARB and ARGs to other organisms via HGT [14, 15]. Consequently, WWTPs are hotspot for the occurrence and transfer of ARB and ARGs in urban water systems, and possibly even in the broader environment [33].

1.1.3 Significant bacteria in the water environment

Water quality environment standards have been established for more than half a century and have improved the water environment tremendously in many countries.

There are various standards and regulations related to water environment that have been established by different organizations and governments around the world. Some examples the WHO's Guidelines for Drinking-water Quality [42], and the International Organization for Standardization's ISO 14001 Environmental Management System standard [43]. These standards and regulations aim to protect and improve the quality of water resources and the surrounding environment, and they often cover a range of issues such as water quality, wastewater treatment, water usage, and environmental management. *Escherichia coli* (*E. coli*), is a type of bacteria that is commonly used as an indicator of water quality because it is found in the intestines of warm-blooded animals, including humans. Its presence in water indicates that fecal contamination may be present, which could lead to the transmission of harmful pathogens and diseases [44]. It is used internationally as a discharge standard to test the effluent standard of WWTPs. The presence of *E. coli* in treated wastewater can indicate the presence of other harmful pathogens and fecal material that may be present in the water. In many countries, the discharge standard for WWTPs is based on the concentration of *E. coli* in the treated wastewater.

While most strains of *E. coli* are harmless and play a beneficial role in the gut microbiome, certain strains can cause illness and even be life-threatening [45]. Treated wastewater is a potential source of *E. coli* contamination in water environments. While wastewater treatment plants are designed to remove harmful bacteria and pollutants from wastewater, *E. coli* and other coliforms can survive the treatment process and spread through the effluent into rivers and other aquatic environments. In recent years, strains of *E. coli* resistant to innovative and effective antimicrobial agents, such as third and fourth-generation cephalosporins and carbapenems, have been detected in the natural environment [46, 47, 48]. In Europe, the detection rate of strains producing ESBLs that are resistant to third-generation cephalosporins has steadily increased [49]. Multidrug-resistant and ESBL-producing *E. coli* have been detected at relatively high concentrations in both treated and untreated wastewater samples [50, 51]. Furthermore, antibiotic-resistant *E. coli* has been reported in rivers and other aquatic environments [52, 53, 54]. Since *E. coli* can survive in wastewater treatment systems, it is critical to track the source of antibiotic-

resistant strains in aquatic environments, especially multidrug-resistant and ESBL-producing bacteria, which pose a grave threat to public health [55, 56, 57, 58]. In addition, one of the best-known pathogenic strains is *E. coli* O157:H7, a Shiga toxin-producing *E. coli* (STEC). This strain can cause serious, even life-threatening complications such as hemolytic uremic syndrome (HUS) [59, 60], which can lead to kidney failure and other serious health problems. *E. coli* O157:H7 and other STEC strains are commonly associated with foodborne illness outbreaks [61], but they can also be spread through contaminated water sources. In water environments, *E. coli* can enter the water from a variety of sources, including sewage overflows [62], agricultural runoff [63], and stormwater runoff [64]. The ability of *E. coli* and coliforms to develop resistance to antimicrobial agents, including ESBLs, is a significant public health concern and highlights the importance of effective monitoring and control measures to prevent the spread of these harmful bacteria in water environments.

1.1.4 Characteristic of antibiotic agents used in this thesis

- Ampicillin (AMP, ABPC)

Ampicillin is a class of penicillin β-lactam antibacterial antibiotic agent classified as broad-spectrum semisynthetic penicillins. It reacts with cross-linking enzymes present in the cell membrane to catalyze the linkage of pentapeptides and pentaglycans in the cell wall, thereby inhibiting the synthesis of solid cell walls. Its antibacterial spectrum against gram-positive bacteria is similar to that of penicillin G, but it has strong antibacterial activity against enterococci and listeria. Ampicillin also adds an amino group to penicillin G that allows it to penetrate the outer membrane of gram-negative bacteria, thus extending its antibacterial activity to gram-negative bacteria, such as *E. coli* and *Salmonella*. However, it should be noted that it is not effective against bacteria that produce penicillinase, which degrades penicillin [65].

- Amoxicillin (AMO)

Amoxicillin is a penicillin β-lactam antibiotic agent and a semisynthetic modifier of ampicillin. They exert their bactericidal action by inhibiting the biosynthesis of bacterial cell wall peptidoglycans. It has a broad antibacterial spectrum and therefore exhibits antibacterial activity against gram-negative and gram-positive bacteria. It also has superior antibacterial activity against *Salmonella* compared to ampicillin, which has superior activity against *Shigella dysenteriae* [65].

- Gentamicin (GEN)

Gentamicin is an aminoglycoside antimicrobial antibiotic agent, the composition of which is an amino sugar. Among the components of the aminoglycoside antimicrobial agents produced by the actinomycetes *Micromonospora purpurea* and *Micromonospora echinospora*, substances in group C of gentamicin are antimicrobial agents and show excellent antimicrobial activity against bacteria. Gentamicin binds irreversibly to the 30S ribosomal subunit of the cell, inhibiting protein synthesis and exhibiting bactericidal action. It is an effective antibacterial agent against gram-negative bacteria such as *Pseudomonas aeruginosa*, *Serratia marcescens* and *Aspergillus*. It is also effective against streptomycin-resistant bacteria, as it shows higher antibacterial activity than streptomycin, which belongs to the same family [65].

- Cefazolin (CFZ)

Cefazolin is a cephalosporin antibiotic agent and is classified as a first generation cephalosporin. It is classified as a β-lactam antibacterial antibiotic in the same class as penicillin and other drugs. They inhibit the biosynthesis of peptidoglycan in the bacterial cell wall. They exhibit antibacterial activity against gram-positive bacteria other than *Staphylococcus aureus* and *Enterococcus*, as well as gram-positive bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* [65].

- Cefotaxime (CTX)

Cefotaxime is a cephalosporin antibiotic agent and is classified as a third generation cephalosporin. It is classified as a β-lactam antibacterial antibiotic in the same category as penicillin and other antibiotic. They strongly inhibit the formation of peptidoglycan crosslinks in bacterial cell walls. Due to their good outer membrane passage, they are resistant to β-lactamases and therefore exhibit bactericidal activity against bacteria. It is an antimicrobial agent with a broad antibacterial spectrum, showing antibacterial activity against gram-positive and gram-positive bacteria, but not against *Pseudomonas aeruginosa* [65].

- Ceftazidime (CAZ)

Cefotaxime is a cephalosporin antibiotic agent and is classified as a third generation cephalosporin. It is classified as a β-lactam antibacterial antibiotic in the same category as penicillin and other antibiotics. They inhibit the synthesis of bacterial cell wall peptidoglycan and have a bactericidal effect on bacteria. They are also β-lactamase stabilized antibacterial agents. It is an antibacterial agent with a broad antibacterial spectrum, with antibacterial activity against *Staphylococcus aureus*, *Serratia marcescens* and even *Pseudomonas aeruginosa*. It is very effective in the cephalosporin class of antibiotics and shows better activity against *Pseudomonas aeruginosa* than the aminoglycoside antibiotics [65].

- Tetracycline (TC, TET)

Tetracycline is a class of tetracycline antibacterial antibiotic agent, produced by several species of actinomycetes. They bind to subunits on the bacterial 30S ribosome and interfere with protein synthesis. As a result, they control bacterial growth (bacteriostatic). It is also selectively toxic as it does not act on animal ribosomes. It has an extremely broad antibacterial spectrum, with antibacterial activity against gram-negative bacteria,

gram-positive bacteria, *Mycoplasma*, *Chlamydia*, *Rickettsia* and some *Protozoa*. However, their effectiveness is considerably reduced in modern times when tetracycline-resistant bacteria have proliferated [65].

- Imipenem (IPM)

Imipenem is a carbapenem β-lactam antibiotic agent with a configuration in which sulfur, normally present in β-lactam antibiotics, is replaced by carbon. They specifically inhibit the synthesis of peptidoglycans in bacterial cell walls and exhibit bactericidal activity and are very stable antimicrobial agents against β-lactamases, the enzymes that hydrolyze β-lactam antibiotics. It is also the antibacterial agent with the broadest antibacterial spectrum, exhibiting antibacterial activity against gram-positive, gram-negative and anaerobic bacteria [65].

- Ciprofloxacin (CIP)

Ciprofloxacin is a new class of quinolone antibacterial antibiotics agent. They are a group of synthetic and developed drugs based on a quinolone structure with a fluorinated chemical structure. They adsorb to the DNA gyrase of gram-negative bacteria and to the DNA topoisomerase IV complex of gram-positive bacteria, inhibiting DNA replication. It is an antibacterial agent with a broad antibacterial spectrum (exhibiting antibacterial activity against both gram-positive and gram-negative bacteria) and particularly good activity against gram-negative bacteria. It is also very effective against third generation cephalosporins and carbapenems in cases where these are ineffective [65].

- Cefepime (CPM)

Cefepime is a cephem antibiotic agent and is classified as a fourth-generation cephalosporin. It is classified as a β-lactam antibacterial antibiotic on a par with penicillin and others. They inhibit the synthesis of bacterial cell wall peptidoglycans and exert

bactericidal action on bacteria. They are also β-lactamase stable antimicrobial agents. It is an antibacterial agent with a broad antibacterial spectrum, exhibiting antibacterial activity against *Staphylococci*, *Serratia* and even gram-negative bacteria, including *Pseudomonas aeruginosa* and *Enterobacteriaceae*. They are less likely to induce resistance in gram-negative bacteria and exhibit antibacterial activity against other antibiotic-resistant bacteria [65].

- Chloramphenicol (CHL)

Cefepime is a cephem antibiotic agent and is classified as a fourth-generation cephalosporin. It is classified as a β-lactam antibacterial drug on a par with penicillin and others. They inhibit the synthesis of bacterial cell wall peptidoglycans and exert bactericidal action on bacteria. They are also β-lactamase stable antimicrobial agents. It is an antibacterial agent with a broad antibacterial spectrum, exhibiting antibacterial activity against *Staphylococci*, *Serratia* and even gram-negative bacteria, including *Pseudomonas aeruginosa* and *Enterobacteriaceae*. They are less likely to induce resistance in gram-negative bacteria and exhibit antibacterial activity against other antibiotic-resistant bacteria [65].

- Clavulanic acid (CVA)

Clavulanic acid is a β-lactamase inhibitor (clavulanic acid, sulbactam, tazobactam) that irreversibly inhibits β-lactamases and can therefore be used in combination with β-lactam antibiotics to enhance their antibacterial activity. Ceftazidime and ceftazidime have been tested by the CLSI against ESBL-producing *Escherichia coli* and suspected ESBL-producing *Klebsiella pneumoniae* with clavulanic acid against suspected ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* [65].

- Meropenem (MEP)

Meropenem is a carbapenem β-lactam antibiotic agent with a strong affinity for penicillin-binding proteins (PBPs) and a specific inhibition of bacterial cell wall peptidoglycan synthesis. Lactamase, the enzyme that hydrolyses β-lactam antibiotics. It is also the antimicrobial agent with the broadest antibacterial spectrum, with antibacterial activity against gram-positive, gram-negative and anaerobic bacteria [65].

- Nalidixic acid (NA)

Nalidixic acid is an ancient quinolone, chemically synthesized antibacterial antibiotic agent. Nalidixic acid inhibits the activity of bacterial DNA gyrase (topoisomerase I), thereby blocking DNA replication. As a result, bacteria are unable to synthesize proteins. It remains highly sensitive to gram-negative rods and shows antibacterial activity against gram-positive bacteria such as *Escherichia coli* and *Shigella dysenteria*e as well as typical bacteria such as Vibrio parahaemolyticus [65].

- Rifampicin (RFP)

Rifampicin is a semi-synthetic antibacterial agent derived from rifamycin produced by the actinomycete Streptomyces Mediterranean. It binds to the DNA-dependent RNA polymerase of bacteria, inhibits mRNA transcription and exhibits antibacterial activity against bacteria. On the other hand, it does not inhibit RNA polymerase in animal cells. It is an important anti-tubercular agent, showing antibacterial activity against *Schistosomiasis* and *Schistosomiasis tuberculosis*. It also shows potent antibacterial activity against *Neisseria*, *Haemophilus* and *Legionella* [65].

1.1.5 Characteristic of reference strain used in this thesis

- *E. coli* strain ATCC 25922

The *E. coli* strain ATCC 25922 is a gram-negative bacterium. This strain has a fully sequenced genome and nucleotide sequence accession number is available in GenBank under accession No. CP009072 [66]. It belongs to serotype O6 and single biotype. ATCC 25922 is susceptible to most antibiotics commonly used in clinical practice, including β-lactams, (such as penicillin, cephalosporins, and carbapenems), aminoglycosides (such as gentamicin), fluoroquinolones (such as ciprofloxacin), tetracyclines, and trimethoprim-sulfamethoxazole. And it doesn't carry any clinically relevant antibiotic resistance genes.

1.2 Thesis objectives

E. coli must develop AR in the water environment in which wastewater is discharged by which transmission mechanism and the current research must clarify the "WWTPs where bacteria accumulate in the living environment". There is little information on the types of bacteria that survive in the water discharged from WWTPs and their AR, and the survival of the released resistant bacteria in the environment is unclear. It is also assumed that ARGs that cannot be removed during the treatment process are released into the environment so that non-resistant bacteria present in the environment acquire resistance genes to make AR. Therefore, we verified the persistence of ARB in WWTPs by simulating aerobic treatment processes and by detecting the resistance in the whole wastewater treatment process with a small possibility of effluent inflow into rivers. In addition, we constructed an experimental system if natural-source *E. coli* develops AR in the river into which treated wastewater flows and establish a pathway for AR expression in antibiotic-sensitive *E. coli* and antibiotics transmission. Then in practical applications, verify the effectiveness of the clarified paths and transmission mechanisms.

1.3 Thesis organization

This thesis is consisted of five chapters.

Chapter 1 provided a general introduction to the background, objectives and organization of the thesis.

Chapter 2 discussed the survival of *E. coli* in wastewater and changes to the relationships between each phylogroup and AR profiles of *E. coli* isolates from wastewater were investigated under aerobic conditions for 14 days via batch experiments.

Chapter 3 discussed the prevalence of ESBL-producing *E. coli* and coliforms during the wastewater treatment process (influent wastewater, biologically-treated wastewater which before chlorination), and efferent which after chlorination) in an urban WWTP (oxidation ditch system) during different seasons.

Chapter 4 discussed the possibility that the ESBL bacteria that remain in the effluent water after chlorine disinfection may transfer genetic factors to sensitive STEC bacteria in the receiving river.

Chapter 5 provided a conclusion that resistance is enhanced in wastewater plants as the bacterial count decreases, especially as ESBL-producing bacteria remain in the effluent water after chlorine disinfection then are released into the receiving rivers. When the ARB and ARGs associate with the bacteria in the river, which increase the AR of the bacteria in the river.

The graphical organization of this thesis is shown in **Figure 1.4**.

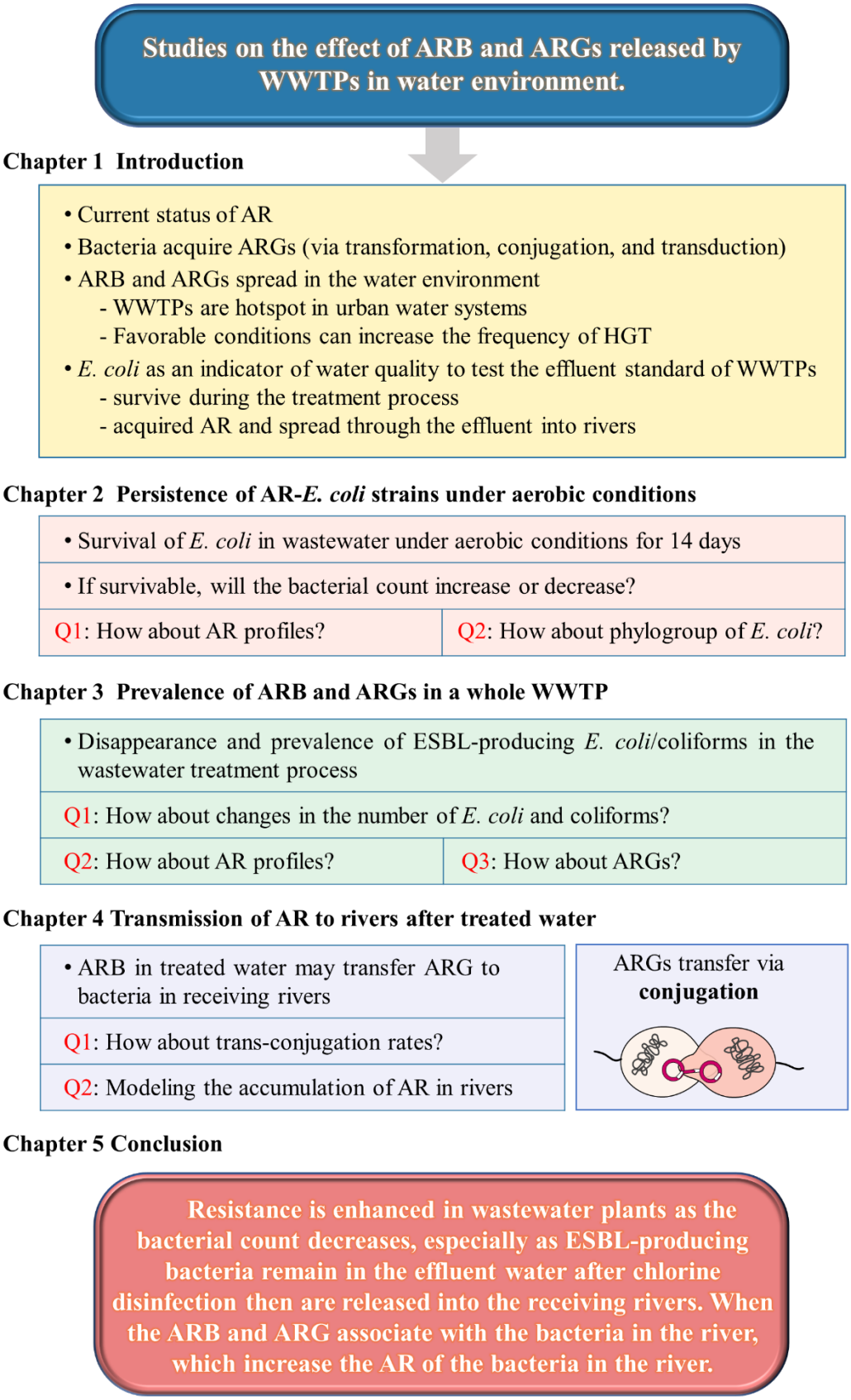

Figure 1.4 The organization structure of this thesis.

Reference

1. Powers J. H. (2004). Antimicrobial drug development--the past, the present, and the future. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 10 Suppl 4, 23–31. https://doi.org/10.1111/j.1465-0691.2004.1007.x
2. Purssell, E. (2020). Antimicrobials. In: Hood, P., Khan, E. (eds) Understanding Pharmacology in Nursing Practice . Springer, Cham. https://doi.org/10.1007/978-3-030-32004-1_6
3. Levinson, W. (2014). Antimicrobial Drugs: Resistance. In Review of Medical Microbiology and Immunology, 13th edition, McGraw-Hill Education: New York. https://doctorlib.info/immunology/microbiology/index.html
4. O'Neill, J. (2016). Review on antimicrobial resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, 1-20. https://wellcomecollection.org/works/rdpck35v.
5. CDC (Centers for Disease Control and Prevention). (2013). Antibiotic resistance threats in the United States. GA: Centers for Disease Control and Prevention. https://stacks.cdc.gov/view/cdc/20705
6. European Centre for Disease Prevention and Control. Antimicrobial resistance surveillance in Europe 2013. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC; 2014. https://www.ecdc.europa.eu/en/publications-data/antimicrobial-resistance-surveillance-europe-2013
7. Tsuzuki, S., Matsunaga, N., Yahara, K., Gu, Y., Hayakawa, K., Hirabayashi, A., Kajihara, T., Sugai, M., Shibayama, K., & Ohmagari, N. (2020). National trend of blood-stream infection attributable deaths caused by *Staphylococcus aureus* and *Escherichia coli* in Japan. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy, 26(4), 367–371. https://doi.org/10.1016/j.jiac.2019.10.017
8. Parshionikar, S. U., Willian-True, S., Fout, G. S., Robbins, D. E., Seys, S. A., Cassady,

J. D., & Harris, R. (2003). Waterborne outbreak of gastroenteritis associated with a norovirus. Applied and environmental microbiology, 69(9), 5263–5268. https://doi.org/10.1128/AEM.69.9.5263-5268.2003

9. Walters, S. P., Thebo, A. L., & Boehm, A. B. (2011). Impact of urbanization and agriculture on the occurrence of bacterial pathogens and stx genes in coastal waterbodies of central California. Water research, 45(4), 1752–1762. https://doi.org/10.1016/j.watres.2010.11.032
10. Chan, Y. M., Thoe, W., Lee, J. H. W. (2015). Field and laboratory studies of *Escherichia coli* decay rate in subtropical coastal water. Journal of Hydro-environment Research, 9, 1-14. https://doi.org/10.1016/j.jher.2014.08.002
11. Barbosa, T. M., & Levy, S. B. (2000). The impact of antibiotic use on resistance development and persistence. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy, 3(5), 303–311. https://doi.org/10.1054/drup.2000.0167
12. Ochman, H., Lawrence, J. & Groisman, E. (2000). Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299–304. https://doi.org/10.1038/35012500
13. Dunning Hotopp J. C. (2011). Horizontal gene transfer between bacteria and animals. Trends in genetics : TIG, 27(4), 157–163. https://doi.org/10.1016/j.tig.2011.01.005
14. Thomas, C. M., & Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nature reviews. Microbiology, 3(9), 711–721. https://doi.org/10.1038/nrmicro1234
15. Krishnapillai, V. (1996).Horizontal gene transfer. Journal of Genetics, 75, 219–232. https://doi.org/10.1007/BF02931763
16. Krüger, N. J., & Stingl, K. (2011). Two steps away from novelty--principles of bacterial DNA uptake. Molecular microbiology, 80(4), 860–867. https://doi.org/10.1111/j.1365-2958.2011.07647.x
17. Frost L. S. (1992). Bacterial conjugation: everybody's doin' it. Canadian journal of microbiology, 38(11), 1091–1096. https://doi.org/10.1139/m92-179
18. Griffiths A.J.F et al (2000). An introduction to genetic analysis. 7th edition. New York: W.H. Freeman.

19. Neu H. C. (1992). The crisis in antibiotic resistance. Science (New York, N.Y.), 257(5073), 1064–1073. https://doi.org/10.1126/science.257.5073.1064
20. Woolhouse, M., & Farrar, J. (2014). Policy: An intergovernmental panel on antimicrobial resistance. Nature, 509(7502), 555–557. https://doi.org/10.1038/509555a
21. Ohmagari N. (2019). National Action Plan on Antimicrobial Resistance (AMR) 2016-2020 and relevant activities in Japan. Global health & medicine, 1(2), 71–77. https://doi.org/10.35772/ghm.2019.01017
22. The AMR One Health Surveillance Committee. Nippon AMR One Health Report (NAOR) 2021.Tokyo: Infectious Diseases Control Division, Health Service Bureau, Ministry of Health, Labour and Welfare; 20
23. Donkor, G. Y., Dontoh, E., & Owusu-Ofori, A. (2019). A cross-sectional study on the prevalence of antibiotic use prior to laboratory tests at two Ghanaian hospitals. PloS one, 14(1), e0210716. https://doi.org/10.1371/journal.pone.0210716
24. Beukers, A. G., Zaheer, R., Cook, S. R., Stanford, K., Chaves, A. V., Ward, M. P., & McAllister, T. A. (2015). Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in *Enterococci* isolated from feedlot steers. Frontiers in microbiology, 6, 483. https://doi.org/10.3389/fmicb.2015.00483
25. Su, H., Liu, S., Hu, X., Xu, X., Xu, W., Xu, Y., Li, Z., Wen, G., Liu, Y., & Cao, Y. (2017). Occurrence and temporal variation of antibiotic resistance genes (ARGs) in shrimp aquaculture: ARGs dissemination from farming source to reared organisms. The Science of the total environment, 607-608, 357–366. https://doi.org/10.1016/j.scitotenv.2017.07.040
26. Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., de Schaetzen, M. A., Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J., & Herman, L. (2013). Antimicrobial resistance in the food chain: a review. International journal of environmental research and public health, 10(7), 2643–2669. https://doi.org/10.3390/ijerph10072643
27. O'Flaherty, E., Borrego, C. M., Balcázar, J. L., & Cummins, E. (2018). Human

exposure assessment to antibiotic-resistant *Escherichia coli* through drinking water. The Science of the total environment, 616-617, 1356–1364. https://doi.org/10.1016/j.scitotenv.2017.10.180

28. Müller, A., Stephan, R., & Nüesch-Inderbinen, M. (2016). Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated from the environment, livestock, food and humans. The Science of the total environment, 541, 667–672. https://doi.org/10.1016/j.scitotenv.2015.09.135
29. Neuman, H., Forsythe, P., Uzan, A., Avni, O., & Koren, O. (2018). Antibiotics in early life: dysbiosis and the damage done. FEMS microbiology reviews, 42(4), 489–499. https://doi.org/10.1093/femsre/fuy018
30. Gao, Y., Bi, W., Wu, X., Zhu, X., & Luo, Y. (2018). Chinese Journal of Biotechnology, 34(8), 1259–1269. https://doi.org/10.13345/j.cjb.180123
31. Martínez J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. Science (New York, N.Y.), 321(5887), 365–367. https://doi.org/10.1126/science.1159483
32. Biyela, P. T., Lin, J., & Bezuidenhout, C. C. (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. Water science and technology : a journal of the International Association on Water Pollution Research, 50(1), 45–50. https://doi.org/10.2166/wst.2004.0014
33. Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., Michael, I., & Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. The Science of the total environment, 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032
34. 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. https://doi.org/10.1016/j.watres.2017.07.002
35. 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. https://doi.org/10.1016/j.watres.2014.11.021

36. Biyela, P. T., Lin, J., & Bezuidenhout, C. C. (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. Water science and technology : a journal of the International Association on Water Pollution Research, 50(1), 45–50. https://doi.org/10.2166/wst.2004.0014
37. Marti, E., Variatza, E., & Balcazar, J. L. (2014). The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends in microbiology, 22(1), 36–41. https://doi.org/10.1016/j.tim.2013.11.001
38. Kümmerer K. (2009). Antibiotics in the aquatic environment--a review--part I. Chemosphere, 75(4), 417–434. https://doi.org/10.1016/j.chemosphere.2008.11.086
39. Moura, A., Henriques, I., Smalla, K., & Correia, A. (2010). Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. Research in microbiology, 161(1), 58–66. https://doi.org/10.1016/j.resmic.2009.11.004
40. Szulc, J., Okrasa, M., Majchrzycka, K., Sulyok, M., Nowak, A., Ruman, T., Nizioł, J., Szponar, B., & Gutarowska, B. (2021). Microbiological and toxicological hazards in sewage treatment plant bioaerosol and dust. Toxins, 13(10), 691. https://doi.org/10.3390/toxins13100691
41. Ahn, Y.; Choi, J. (2016). Bacterial communities and antibiotic resistance communities in a full-scale hospital wastewater treatment plant by high-throughput pyrosequencing. Water, 8, 580. https://doi.org/10.3390/w8120580
42. World Health Organization. (2017). Guidelines for drinking-water quality: fourth edition incorporating first addendum, 4th ed + 1st add. World Health Organization. https://apps.who.int/iris/handle/10665/254637.
43. Whitelaw, Ken. (2004). ISO 14001 Environmental Systems Handbook. London: Butterworth-Heinemann. https://doi.org/10.4324/9780080478807
44. Cabral J. P. (2010). Water microbiology. Bacterial pathogens and water. International

journal of environmental research and public health, 7(10), 3657–3703. https://doi.org/10.3390/ijerph7103657

45. Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. BMJ (Clinical research ed.), 361, k2179. https://doi.org/10.1136/bmj.k2179
46. Daniel, D. S., Lee, S. M., Gan, H. M., Dykes, G. A., & Rahman, S. (2017). Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia. Journal of infection and public health, 10(5), 617–623. https://doi.org/10.1016/j.jiph.2017.02.006
47. Rosenberg Goldstein, R. E., Micallef, S. A., Gibbs, S. G., George, A., Claye, E., Sapkota, A., Joseph, S. W., & Sapkota, A. R. (2014). Detection of vancomycin-resistant *Enterococci* (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. The Science of the total environment, 466-467, 404–411. https://doi.org/10.1016/j.scitotenv.2013.07.039
48. Diwan, V., Hanna, N., Purohit, M., Chandran, S., Riggi, E., Parashar, V., Tamhankar, A. J., & Stålsby Lundborg, C. (2018). Seasonal variations in water-quality, antibiotic residues, resistant bacteria and antibiotic resistance genes of *Escherichia coli* isolates from water and sediments of the Kshipra River in central India. International journal of environmental research and public health, 15(6), 1281. https://doi.org/10.3390/ijerph15061281
49. ECDC/EMEA Joint Technical Report: The bacterial challenge: time to react. A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents. European Centre for Disease Prevention and Control (ECDPC) 2009, 1-54. https://www.ecdc.europa.eu/en/publications-data/ecdcemea-joint-technical-report-bacterial-challenge-time-react
50. Aristizábal-Hoyos, A. M., Rodríguez, E. A., Arias, L., & Jiménez, J. N. (2019). High clonal diversity of multidrug-resistant and extended spectrum beta-lactamase-producing *Escherichia coli* in a wastewater treatment plant. Journal of environmental management, 245, 37–47.

https://doi.org/10.1016/j.jenvman.2019.05.073

51. Adefisoye, M. A., & Okoh, A. I. (2016). Identification and antimicrobial resistance prevalence of pathogenic *Escherichia coli* strains from treated wastewater effluents in Eastern Cape, South Africa. MicrobiologyOpen, 5(1), 143–151. https://doi.org/10.1002/mbo3.319
52. Kümmerer K. (2009). Antibiotics in the aquatic environment--a review--part II. Chemosphere, 75(4), 435–441. https://doi.org/10.1016/j.chemosphere.2008.12.006
53. Müller, A., Stephan, R., & Nüesch-Inderbinen, M. (2016). Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated from the environment, livestock, food and humans. The Science of the total environment, 541, 667–672. https://doi.org/10.1016/j.scitotenv.2015.09.135
54. Abia, A. L., Ubomba-Jaswa, E., & Momba, M. N. (2016). Occurrence of diarrhoeagenic *Escherichia coli* virulence genes in water and bed sediments of a river used by communities in Gauteng, South Africa. Environmental science and pollution research international, 23(15), 15665–15674. https://doi.org/10.1007/s11356-016-6762-6
55. Centers for Disease Control and Prevention (CDC). (2019). National Center for Emerging Zoonotic and Infectious Diseases (U.S.); Division of Healthcare Quality Promotion; Antibiotic Resistance Coordination and Strategy Unit. Antibiotic resistance threats in the United States, 1-148, http://dx.doi.org/10.15620/cdc:82532.
56. Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., Kretzschmar, M. E., Devleesschauwer, B., Cecchini, M., Ouakrim, D. A., Oliveira, T. C., Struelens, M. J., Suetens, C., Monnet, D. L., & Burden of AMR Collaborative Group (2019). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. The Lancet. Infectious diseases, 19(1), 56–66. https://doi.org/10.1016/S1473-3099(18)30605-4
57. Woerther, P. L., Burdet, C., Chachaty, E., & Andremont, A. (2013). Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. Clinical microbiology reviews, 26(4), 744–758.

https://doi.org/10.1128/CMR.00023-13

58. Manges, A. R., Johnson, J. R., Foxman, B., O'Bryan, T. T., Fullerton, K. E., & Riley, L. W. (2001). Widespread distribution of urinary tract infections caused by a multidrug-resistant *Escherichia coli* clonal group. The New England journal of medicine, 345(14), 1007–1013. https://doi.org/10.1056/NEJMoa011265

59. George, J. N., & Nester, C. M. (2014). Syndromes of thrombotic microangiopathy. The New England journal of medicine, 371(7), 654–666. https://doi.org/10.1056/NEJMra1312353

60. Joseph, A., Cointe, A., Mariani Kurkdjian, P., Rafat, C., and Hertig, A. (2020) Shiga toxin-associated hemolytic uremic syndrome: a narrative review. Toxins, 12(2), 67. https://doi.org/10.3390/toxins12020067

61. Swaminathan, B., Gerner-Smidt, P., Ng, L. K., Lukinmaa, S., Kam, K. M., Rolando, S., Gutiérrez, E. P., & Binsztein, N. (2006). Building PulseNet International: an interconnected system of laboratory networks to facilitate timely public health recognition and response to foodborne disease outbreaks and emerging foodborne diseases. Foodborne pathogens and disease, 3(1), 36–50. https://doi.org/10.1089/fpd.2006.3.36

62. Locatelli, L., Russo, B., Acero Oliete, A., Sánchez Catalán, J. C., Martínez-Gomariz, E., and Martínez, M.(2020). Modeling of *E. coli* distribution for hazard assessment of bathing waters affected by combined sewer overflows. Natural Hazards and Earth System Sciences, 20, 1219–1232. https://doi.org/10.5194/nhess-20-1219-2020

63. Topalcengiz, Z., Strawn, L. K., & Danyluk, M. D. (2017). Microbial quality of agricultural water in Central Florida. PloS one, 12(4), e0174889. https://doi.org/10.1371/journal.pone.0174889

64. Ahmed, W., Hamilton, K., Toze, S., Cook, S., & Page, D. (2019). A review on microbial contaminants in stormwater runoff and outfalls: Potential health risks and mitigation strategies. The Science of the total environment, 692, 1304–1321. https://doi.org/10.1016/j.scitotenv.2019.07.055

65. Nishi, K. (2017). Basic knowledge of antibiotics, Journal of the Japanese Society for

Clinical Anesthesia, 37(5), 687-694. https://doi.org/10.2199/jjsca.37.687

66. Minogue, T. D., Daligault, H. A., Davenport, K. W., Bishop-Lilly, K. A., Broomall, S. M., Bruce, D. C., Chain, P. S., Chertkov, O., Coyne, S. R., Freitas, T., Frey, K. G., Gibbons, H. S., Jaissle, J., Redden, C. L., Rosenzweig, C. N., Xu, Y., & Johnson, S. L. (2014) Complete Genome Assembly of *Escherichia coli* ATCC 25922, a Serotype O6 Reference Strain. Genome announcements, 2: e00969-14. https://doi.org/10.1128/genomeA.00969-14

Chapter 2. Persistence of antibiotic resistant *E. coli* strains under aerobic conditions

2.1 Introduction

E. coli is a GNB that is endemic in the intestinal tracts of thermostatic animals, including humans [1]. It is therefore ubiquitous in aquatic environments where wastewater, treated wastewater and wild animal feces enter the waterways, and is used as an indicator bacterium for fecal contamination to assess water quality in aquatic environments [2]. In the past, the presence of AR-*E. coli* was primarily limited to specific facilities such as medical institutions [3] and livestock farms [4]. The overuse and misuse of antibiotics in these facilities have led to the development of ARB, including *E. coli*. Moreover, these bacteria are now also being detected in the environment, such as soil and aquatic environments [5]. MDR- and ESBL-producing *E. coli* have been detected at relatively high concentrations in both treated and untreated wastewater samples [6, 7]. Furthermore, antibiotic-resistant *E. coli* has been reported in rivers and other aquatic environments [8-10]. Since *E. coli* can survive in wastewater treatment systems, it is critical to track the source of antibiotic-resistant strains in aquatic environments, especially multidrug-resistant and ESBL-producing *E. coli*, which pose a grave threat to public health [11-14].

Based on gene structure and sequence data, *E. coli* is commonly classified into four major phylogenetic groups (A, B1, B2, and D), which exhibit differences in ecological specificity [15]. Since host and environmental factors impact the diversity and abundance of *E. coli*, the source of the host and the pathogenicity of *E. coli* strains can be distinguished by gene sequencing technology [16-19]. In general, *E. coli* strains of phylogroups A and B1 are most likely to inhabit the intestinal tracts of humans and animals. Symbiotic strains isolated from humans mainly belong to phylogroup A [17], while most strains isolated from animals belong to phylogroup B1 [20] and strains isolated from wastewater systems mainly belong to phylogroups B2 and D [21]. However, there is a little information about the relationship between phylogroups and antibiotic-resistant *E. coli* in municipal wastewater [22], especially for processing of wastewater. Hence, the aim of the

present study was to investigate changes to the relationship between each phylogroup and antibiotic-resistant profiles of *E. coli* isolates from municipal wastewater under aerobic mixing conditions for 14 days via batch experiments.

2.2 Materials and Methods

2.2.1 Sampling and water quality analysis

Wastewater samples were collected from WWTPs A and B. Plant A treats sewage from a population of approximately 10,000 people with an average daily flow volume of 6,600 m^3. Plant B treats sewage from a population of approximately 163,000 people with an average daily flow volume of 92,500 m^3. Samples were collected from plants A and B on January 24, 2018, and January 15, 2019, respectively, stored in sterile 5-L polyethylene bottles, and immediately transported for microbial and water quality analyses, which were conducted within 4 h.

The pH value, electrical conductivity, and turbidity were determined using a pH meter (HM-30G; DKK-TOA Corporation, Tokyo, Japan), conductivity meter (CM30S; DKK-TOA Corporation), and turbidity meter (SEP-PT-706D; Mitsubishi Chemical Corporation, Tokyo, Japan), respectively. In addition, the concentrations of total organic carbon (TOC) in the samples were determined as the state of aerobic decomposition of organic substances using a TOC analyzer (TOC Analyzer Wet Oxidation/Non-Dispersive Infra-Red Method Model; Shimadzu Corporation, Kyoto, Japan).

2.2.2 Batch mixing experiment and isolation of *E. coli*

Under batch aerobic conditions (in the dark at 20°C), the wastewater was stirred and mixed (200 rpm) for two weeks and samples were collected on days 0, 7, and 14. In case of high concentrations of *E. coli* in the inflowing water, the samples were diluted with sterilized physiological saline. All samples were filtered through a sterile cellulose ester membrane (pore, 0.45 μm; diameter, 47 mm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and

incubated on CHROMagar™ ECC plates (CHROMagar, Paris, France) at 37°C for 24 h. Afterward, mean numbers of colony-forming units (CFUs) of coliform bacteria and *E. coli* in each sample were determined from three replicates. Bacterial counts are expressed as CFU/100 mL. On CHROMagar™ ECC plates, colonies of *E. coli* and coliform bacteria are blue and mauve, respectively. One hundred *E. coli* isolates were collected from each sample on days 0, 7, and 14, thus 300 samples were collected from plant A and 300 from plant B. For confirmation, all *E. coli* isolates were streaked on brain heart infusion (BHI) agar plates (1.5% agar; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37°C for 24 h.

2.2.3 Identification of *E. coli* by MALDI-TOF MS

The *E. coli*-positive isolates were pre-incubated on nutrient liquid BHI agar medium at 37°C for 18 h. The species were identified by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) [23]. An aliquot (1.0 mL) of the template was spotted directly into the wells of a 384-well stainless-steel target plate (MTP 384; Bruker Daltonics, Billerica, MA, USA), air-dried for 10 min, and then overlaid with 1.0 mL of matrix solution. All samples were analyzed using an autoflex® III TOF/TOF system (Bruker Daltonics) operated in the linear positive mode within a mass range of 2,000–20,000 Da, according to the manufacturer's instructions. For database construction and validation, measurements were obtained in the auto-execute mode using flexControl 3.0 software (Bruker Daltonics) with the follow parameters: linear positive, 3–20 kDa; detector gain, 1,900 V; laser shots, 500–1,000; and laser power, 30%. The instrument was calibrated using a Bruker bacterial test standard (part no. 8255343; Bruker Daltonics).

Recorded mass spectra were processed with the MALDI Biotyper Compass microbial identification system (Bruker Daltonics) using standard settings. The MALDI Biotyper output is a log score value in the range of 0.000 to 3.000, representing the probability of correct identification of the isolate computed by a comparison of the peak list for an

unknown isolate with the reference spectrum in the database. *E. coli* was identified by a log score value greater than 2.000.

2.2.4 Classification of phylogroups of *E. coli* by multiplex PCR

DNA samples were extracted from *E. coli* isolates using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA). The phylogroups (A, B1, B2, and D) were determined according to the multiplex PCR method reported by Clermont et al. [24], which is widely adopted as a simple but reliable *E. coli* phylotyping method [25].

The PCR reaction was conducted using the Kapa Taq extra PCR kit (Kapa Biosystems), primers for detection of three genes (arpA, chuA, and yjaA), and one DNA fragment (TspE4.C2), as presented in **Table 2.1**. Each 20-μL reaction consisted of 4.3 μL of sterilized distilled water, 4.0 μL of 5 × KAPA Extra Buffer, 0.1 μL of Taq polymerase, 0.4 μL of deoxynucleoside triphosphates (dNTPs), 1 μL of each primer, and 2 μL of the DNA template. The PCR reaction was performed using a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the following reaction conditions: denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 5 s and 59°C for 20 s, and a final extension step at 72°C for 5 min. After the PCR reaction, 5 μL of the PCR product and 1 μL of 6 × loading buffer (Takara Bio, Inc., Shiga, Japan) were mixed, loaded into the wells of 2% agarose gels, and separated with the Mupid®-One electrophoresis system (Nippon Genetics Co., Ltd., Tokyo, Japan) at 100 V for 40 min. After electrophoresis, the agarose gel was stained with ethidium bromide solution (0.05

Table 2.1 Design of primers sequence for detection of phylogroups of *E. coli* isolates.

Primer name	Target	Primer sequences	PCR product (bp)
chuA.1b chuA.2	*chuA*	F: 5'-ATGGTACCGGACGAACCAAC-3' R: 5'-TGCCGCCAGTACCAAAGACA-3'	288
yjaA.1b yjaA.2b	*yjaA*	F: 5'-CAAACGTGAAGTGTCAGGAG-3' R: 5'-AATGCGTTCCTCAACCTGTG-3'	211
TspE4C2.1b TspE4C2.2b	TspE4.C2	F: 5'-CACTATTCGTAAGGTCATCC-3' R: 5'-AGTTTATCGCTGCGGGTCGC-3'	152
AceK.f ArpA1.r	*arpA*	F: 5'-AACGCTATTCGCCAGCTTGC-3' R: 5'-TCTCCCCATACCGTACGCTA-3'	400

μL/mL) for 10 min and then shaken in distilled water for 10 min to confirm the PCR amplification products. This method is based on the classification of amplification patterns of four primers (arpA, chuA, yjaA, and TspE4.C2) into each phylogroup. *E. coli* ATCC 25922 was used as a positive control for the PCR reaction.

2.2.5 Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of each antimicrobial agent was determined on Mueller–Hinton (MH) agar using the agar dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [26]. *E. coli* isolates were cultured at 37°C for 18 h in MH broth (Becton, Dickinson and Company, Sparks, MD, USA) and then diluted to a final concentration corresponding to the 0.5 McFarland turbidity standard with fresh MH broth. The *E. coli* isolates were then inoculated on the surface of 1.5% MH agar containing graded concentrations of each antimicrobial in the wells of a microplate (Sakuma Co., Tokyo, Japan). Following incubation of the plates at 37°C for 18 h, the MICs were determined. MIC breakpoints for resistance were based on the CLSI criteria.

The antimicrobials used in the current study included ampicillin (AMP; graded concentrations of 4–64 μg/mL), gentamicin (GEN; 2–32 μg/mL), cefazolin (CFZ; 1–16 μg/mL), cefotaxime (CTX; 0.5–8 μg/mL), ceftazidime (CAZ; 2–32 μg/mL), tetracycline (TET; 2–32 μg/mL), imipenem (IMP), ciprofloxacin (CIP; 0.5–8 μg/mL), cefepime (CPM; 4–64 μg/mL) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and chloramphenicol (CHL; 4–64 μg/mL) (Sigma-Aldrich Corporation, St. Louis, MO, USA). Each of the tested agents was dissolved in distilled water or other appropriate solvents in accordance with the CLSI recommendations. The *E. coli* reference strain ATCC 25922 was used for quality control.

2.2.6 ESBL genotypes of *E. coli* by multiplex PCR

Table 2.2 Design of primers sequence for detection of ESBL-producing genotypes of *E. coli* isolates.

β-Lactamase(s) genotypes	Primer sequences	PCR product (bp)
TEM-type	F: 5'-CCGTGTCGCCCTTATTCC-3' R: 5'-AGGCACCTATCTCAGCGA-3'	824
SHV-type	F: 5'-ATTTGTCGCTTCTTTACTCGC-3' R: 5'-TTTATGGCGTTACCTTTGACC-3'	1051
CTX-M-1-type	F: 5'-GCTGTTGTTAGGAAGTGTGC-3' R: 5'- CCATTGCCCGAGGTGAAG-3'	516
CTX-M-2-type	F: 5'-ACGCTACCCCTGCTATTT-3' R: 5'- GCTTTCCGCCTTCTGCTC-3'	779 or 780
CTX-M-9-type	F: 5'- GCAGATAATACGCAGGTG-3' R: 5'-CGGCGTGGTGGTGTCTCT-3'	393

DNA was extracted by the same method as described above. The ESBL genotypes (TEM, SHV, CTX-M-1, CTX-M-2, and CTX-M-9) of the *E. coli* strains were determined by multiplex PCR as described elsewhere [27, 28]. Five primer of ESBL genotypes were shown in **Table 2.2**.

The primers for all the genes. Each 50-µl reaction consisted of 29.75 µl of sterilized distilled water, 0.25 µl of Takara Taq HS (Takara Bio Inc., Shiga, Japan), 5 µl of 10 × KAPA Extra Buffer (Takara Bio Inc., Shiga, Japan), 5 µl of deoxynucleoside triphosphate Mix (dNTP Mix) (Takara Bio Inc., Shig a, Japan), 1 µl of each primer, and 1 µl of the DNA template. The PCR reaction was performed using a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the following reaction conditions: denaturation at 94°C for 2 minutes, followed by 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 90 seconds, and a final extension step at 72°C for 5 minutes. After the PCR reaction, 5 µl of the PCR product and 1 µl of 6 × loading buffer (Takara Bio, Inc., Shiga, Japan) were mixed, loaded into the wells of 2% agarose gels, and separated using the Mupid®-One electrophoresis system (Nippon Genetics Co., Ltd., Tokyo, Japan) at 100 V for 40 minutes. After electrophoresis, the agarose gel was stained with ethidium bromide solution (0.05 µl/ml) for 10 minutes and then shaken in distilled water for 10 minutes to confirm the PCR amplification products.

2.3 Results and Discussion

2.3.1 Changes to TOC concentrations and number of *E. coli* colonies under aerobic conditions

Changes to the TOC concentrations and number of *E. coli* colonies in wastewater under aerobic conditions are shown in **Figure. 2.1-(a)**. The other parameters of water quality and the number of bacteria is presented in **Table 2.3**. The TOC concentration gradually decreased after 14 days under aerobic conditions. The TOC concentrations of wastewater samples collected from plants A and B decreased from 55.4 to 11.0 mg/L and 53.5 to 12.7 mg/L after 14 days, respectively, while turbidity decreased from 102.4 to 59.1 and 114.8 to 53.2. These findings confirmed the decomposition of organic matter in wastewater under aerobic conditions for 14 days.

The number of *E. coli* CFUs in the wastewater samples collected from plants A and B decreased from 6.9×10^6 and 7.3×10^6/100 mL on day 0 to 2.6×10^4 and 9.3×10^4/100 mL on day 14, respectively, under aerobic conditions (**Figure. 2.1-b**). Under aerobic mixing conditions, the removal efficiency of *E. coli* from the samples collected from plants A and B was 99.6% and 98.7%, respectively. The decreases in *E. coli* content confirmed successful biological treatment of the wastewater under aerobic conditions.

Table 2.3 The parameters of water quality and the number of bacteria in each sample from plant A and B.

Sample	Mixed batch time	pH	EC	Turbidity	TOC	Bacteria (CFU/100 mL)		
		(-)	(μs/cm)	(ppm)	(mg·C/L)	Total coliforms	*Escherichia coli*	Enterococci
Plant A	0 day	8.036	0.487	102.40	55.36	1.2×10^7	6.9×10^6	4.2×10^6
	7 days	6.649	0.589	74.14	20.21	1.4×10^5	5.5×10^4	3.7×10^4
	14 days	5.483	0.480	59.10	10.99	1.2×10^5	2.6×10^4	1.9×10^4
Plant B	0 day	7.286	1.640	144.76	53.50	1.2×10^7	7.3×10^6	2.1×10^6
	7 days	6.891	1.723	50.60	16.29	9.0×10^5	5.0×10^5	2.5×10^4
	14 days	5.752	1.675	60.30	12.66	4.0×10^4	9.3×10^4	1.0×10^2

EC, Electrical conductivity; TOC, Total Organic Carbon; N, no data

2.3.2 Identification of *E. coli*

In the batch mixing experiment, MALDI-TOF MS was used to identify *E. coli* in all 600 wastewater samples collected from plants A and B (days 0, 7, and 14). Of the 300

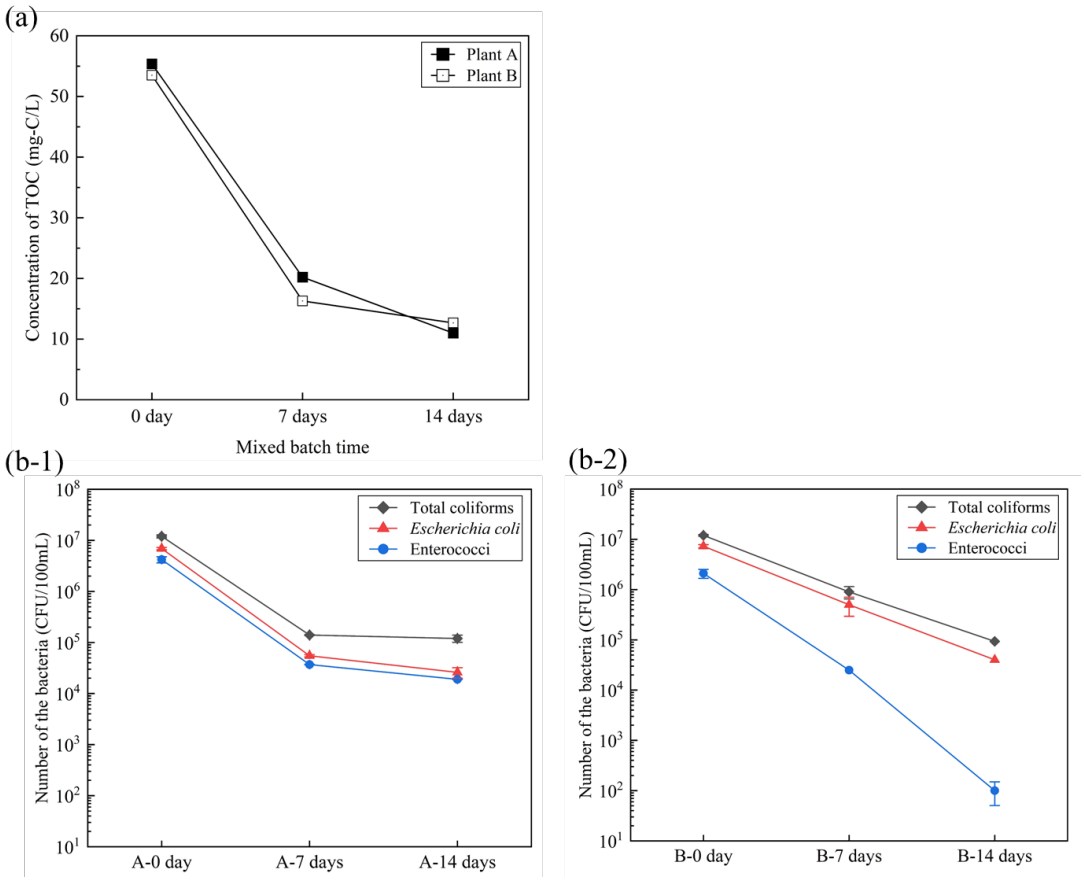

Figure 2.1 Changes to the TOC concentrations (a) and number of *E. coli* colonies in plant A wastewater (b-1) and plant B wastewater (b-2) under aerobic conditions.

samples collected from plant A, 296 (98.7%) were positive for *E. coli*, while three were positive for *Enterobacter cloacae* and one for *Citrobacter braakii*. Meanwhile, all 300 samples collected from plant B were positive for *E. coli*. These results confirm that *E. coli*, but not pseudo-positive strains, were prevalent in wastewater collected from plants A and B.

2.3.3 Changes to the composition of *E. coli* phylogroups

Changes to the composition of *E. coli* phylogroups occurred over time in municipal wastewater samples collected from plants A and B under aerobic conditions (**Figure. 2.2**). In the samples collected from plants A and B, 94.3% (279/296) and 82.3% (247/300) of isolates, respectively, were identified as the major phylogroups (A, B1, B2, and D).

Before aerobic treatment, the most dominant phylogroup was B2 in samples collected from both plants. Interestingly, after aerobic treatment, although the abundances of the major phylogroups other than B2 were considerably decreased in both plant samples, the abundance of phylogroup B2 was constant in the samples collected from plant B and had increased in those from plants A (**Figure. 2.2**). These results indicate that the survival rate of strains belonging to phylogroup B2 was much higher than that of strains belonging to the other major phylogroups in municipal wastewater samples under aerobic conditions.

Among various pathotypes of pathogenic *E. coli*, extraintestinal pathogenic *E. coli* (ExPEC) causes various human infections, including life-threatening sepsis and neonatal meningitis, thereby posing a significant public health concern [29]. ExPEC strains mostly belonged to phylogroup B2 [30, 31]. In this study, phylogroups B2 strains were the most predominant in the wastewater samples collected from both treatment plants at different areas within the same city and exhibited the greatest tolerance among all *E. coli* strains to aerobic treatment of wastewater. From a public health point of view, survival of phylogroup B2 strains in treated wastewater poses serious public health concerns. *E. coli* phylogroups D and B2 are dominant in WWTPs in subtropical regions [21, 32]. However, since the population structure of *E. coli* strains in wastewater is greatly affected by various

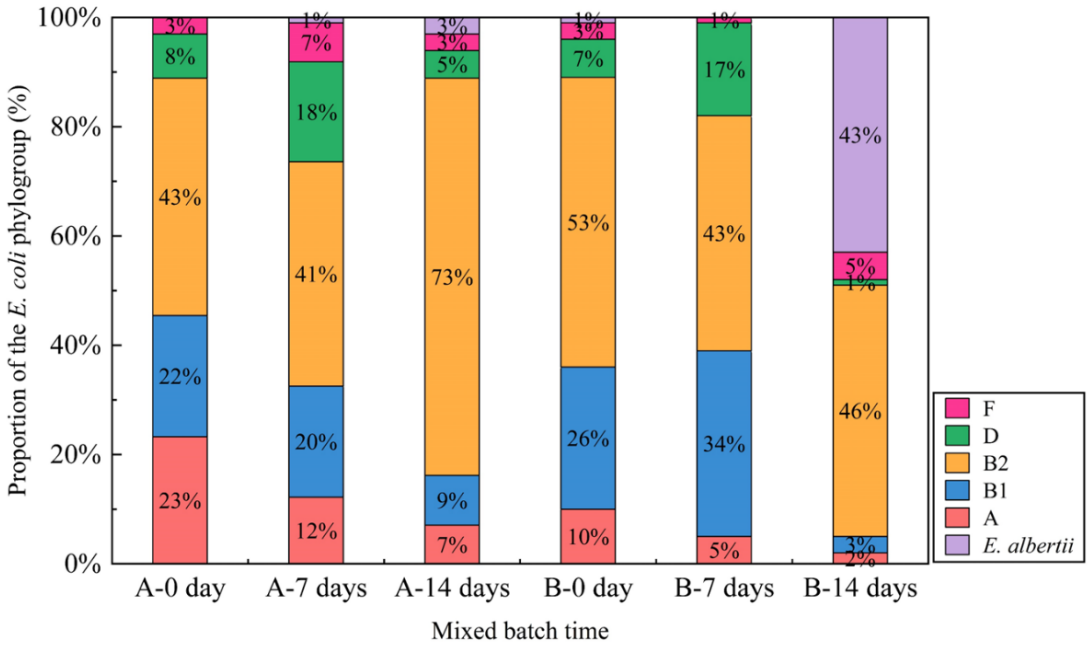

Figure 2.2 A bar chart of the cumulative percentage wastewater from plants A and B in phylogenetic groups A, B1, B2, and D.

factors, such as temperature, treatment processes, and sampling regions, further research in this field is required to support our findings.

In this study, among the strains identified as *E. coli* by MALDI-TOF MS, 48 were actually *E. albertii*, as determined by the phylotyping method developed by [24]. Although *E. albertii* strains are often misidentified as *E. coli* by routine phenotyping methods and even MALDI-TOF MS [33], several studies have supported the classification of *E. albertii* as a distinct species in the genus Escherichia [34-37]. More importantly, *E. albertii* was recently recognized as an emerging human pathogen, which encodes a type III secretion system and to a lesser extent, Shiga toxin, and causes outbreaks of gastroenteritis as well as sporadic infections [38]. In the wastewater samples, especially those collected from plant B, the abundance of *E. albertii* was extremely elevated after aerobic treatment (**Figure. 2.2**), suggesting that *E. albertii* can survive in municipal wastewater. Hence, further studies are warranted to investigate the release of *E. albertii* from wastewater plants into aquatic environments.

2.3.4 Antibiotic susceptibility of *E. coli*

All 596 *E. coli* isolates were tested for susceptibility to 11 antibiotics to assess changes to the prevalence of antibiotic-resistant *E. coli* in wastewater samples collected from plants A and B over a 2-week period under aerobic conditions. Here, prevalence was defined as the percentage of isolates from each sample that were resistant to one or more antibiotics. Although the abundance of *E. coli* was relatively lower in the samples collected from plant A, (**Figure. 2.1-b**), the prevalence of antibiotic-resistant *E. coli* isolates increased from 14% (14/99) on day 0 to 44% (44/99) on day 14 (**Figure. 2.3**). In contrast, the prevalence of antibiotic-resistant *E. coli* isolates in the samples collected from plant B increased from 69% (69/100) on day 0 to 79% (79/100) on day 7 and then decreased to 48% (48/100) on day 14. In the wastewater samples from plant B, the abundance of *E. albertii* had increased under aerobic conditions by 16% from day 0 to

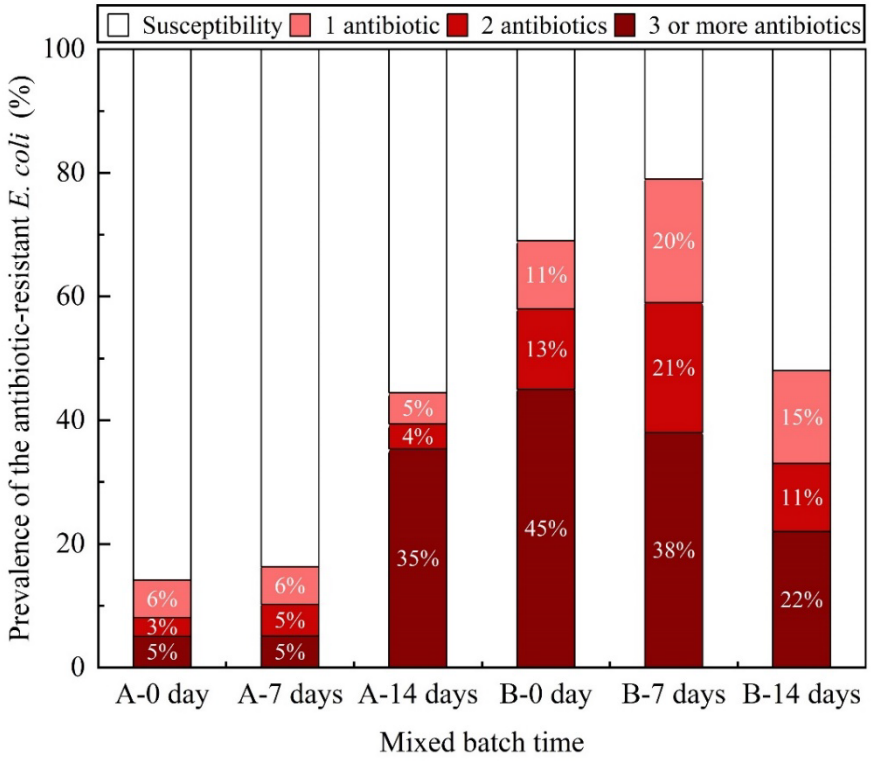

Figure 2.3 A bar chart of the cumulative percentage of *E. coli* isolates from plants A and B that were resistant to 1, 2, and 3 or more antibiotics.

day 14. Notably, 14% and 69% of the isolates in wastewater samples collected from plants A and B, respectively, were resistant to at least one of the 11 antibiotics. In contrast, 79% and 48% of the isolates in wastewater samples collected from plant B were resistant to at least one antibiotic on day 7 and 14, respectively. In a previous study, 44% of *E. coli* isolates in urban wastewater were resistant to at least one of the nine tested antibiotics [39].

Changes to the prevalence of isolates resistant to each tested antibiotic over the 14-day period are shown in **Figure. 2.4**. The prevalence of isolates from plant A resistant to any of the 11 antibiotics had increased with time, especially those resistant to AMP, CFZ, CIP, and TET had increased by more than 30% on day 14. The significant increase in the prevalence of antibiotic-resistant isolates was responsible for the overall increase in **Figure. 2.4**. In contrast, the prevalence of isolates in wastewater samples collected from plant B resistant to AMP, CFZ, and CTX was relatively high on day 0 and then significantly decreased on day 14. The rapid increase in the abundance of TET-resistant isolates on day 7 was responsible for the overall increase in prevalence in **Figure. 2.4**.

The *E. coli* isolates in two wastewater samples collected from plants A and B were susceptible to IMP.

The *E. coli* isolates in wastewater samples collected from plants A and B were highly resistant to AMP, CFZ, CTX, TET, and CIP. However, there was no significant increase in the prevalence of resistant isolates in wastewater samples collected from plant B over the 14-day experimental period. According to the 2018 "National Action Plan on Antimicrobial Resistance" [40], the prevalence of *E. coli* isolates resistant to the penicillin antibacterial, AMP and piperacillin had increased from 2011 to 2017 and remained greater than 40% for 6 consecutive years. In addition, approximately 20% of *E. coli* isolates are resistant to cephalosporins. The antibiotic susceptibility results of *E. coli* in this study were consistent with the trends described in the Nippon Antimicrobial Resistance One Health Report.

Information on multidrug-resistant bacteria is extremely important from a public health perspective. The prevalence of multidrug-resistant strains in wastewater samples collected from plants A and B is shown in **Figure. 2.4**. By day 14, the prevalence of multidrug-resistant *E. coli* strains increased to 35% (35/99) in the plant A samples but decreased to 22% (22/100) in the plant B samples. *E. coli* strains resistant to five antibiotics were also detected in wastewater samples collected from both plants A and B, but the prevalence was six-fold greater in samples from plant B as compared to those

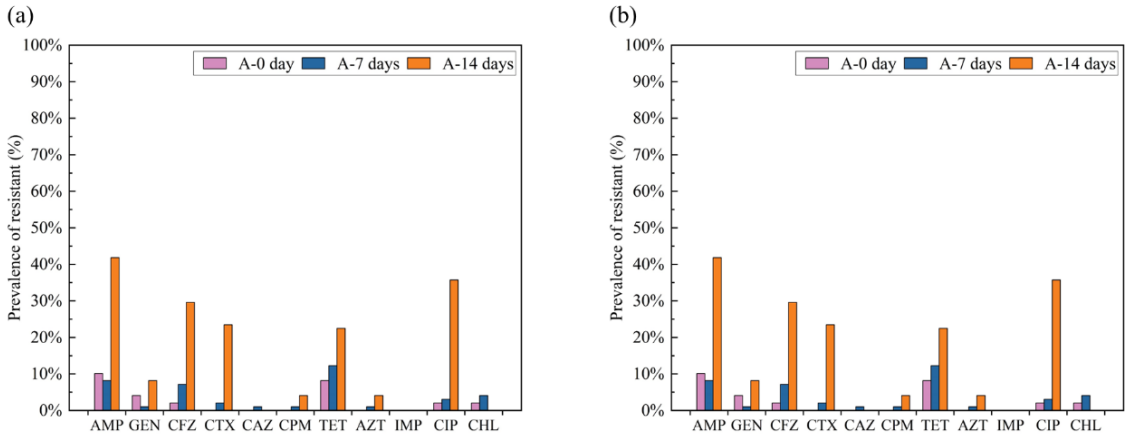

Figure 2.4 Changes to the prevalence of isolates resistant to each tested antibiotic over the 14-day period.

from plant A. The prevalence of multidrug-resistant isolates in wastewater samples collected from plant A had increased under aerobic conditions, while more than 10% of the isolates from plant B were resistant to five or more antibiotics. These findings suggest that the prevalence of antibiotic-resistant *E. coli* isolates differs among wastewater samples and is increased under aerobic conditions.

2.3.5 Relationship between phylogroups and ESBL-producing *E. coli* isolates

The relationship between phylogroups and AR of *E. coli* was evaluated based on phylogroup analysis and antibiotic-resistance profiling (**Figure. 2.5**). The prevalence of AR was highest in phylogroup B2. Furthermore, the prevalence of AR increased with time from 8% on day 0 to 39% on day 14 among the isolates in wastewater samples collected from plant A. Similarly, among the wastewater sample collected from plant B, AR was highest among isolates classified as phylogroup B2. In the plant B samples, although there was no increasing trend in the resistance rate, isolates classified as phylogroup B2 were the most prevalent on day 14 under aerobic conditions. Further evaluation of the relationship between phylogroups and AR found that *E. coli* isolates classified as phylogroup B2 were most capable to survive under aerobic conditions for 14 days. Previous studies have reported that members of phylogroup B2 are more pathogenic than members of the other phylogroups [41, 42]. Based on gene structure and sequencing data, *E. coli* strains of phylogroup B2 differ from other strains and are more likely to cause parenteral infections [41, 43, 44]. Following biological treatment of municipal wastewater, although the abundance of *E. coli* had decreased by about 2log, the prevalence of AR increased due to the survival of members of phylogroup B2, which were the most resistant to the tested antibiotics.

Testing for ESBL-producing genes in multidrug-resistant bacteria showed that 25 (8.4%) of 296 and 51 (17%) of 300 isolates in wastewater samples collected from plants A and B, respectively, were ESBL-producing *E. coli*. The relationship between phylogroups and ESBL-producing *E. coli* was also evaluated based on the phylogroup

analysis and ESBL-production profiles (**Figure. 2.5**; **Table 2.5**). While *E. coli* counts gradually decreased with time under aerobic conditions, the prevalence of ESBL-producing *E. coli* accounted for 23% of surviving *E. coli* isolates in wastewater samples collected from plant A on day 14. The prevalence of ESBL-producing *E. coli* had increased in the wastewater samples collected from plant A, but not plant B, from day 0 to day 14. Most of the ESBL-producing isolates were classified as phylogroup B2, while one was classified as phylogroup B1. The CTX-M-9 type was dominant among the isolates in wastewater samples collected from plant A. In contrast, the prevalence of ESBL-producing *E. coli* under aerobic conditions for 14 days did not increase in the samples from plant B, of which the SHV and CTX-M-9 types (29 and 22 isolates, respectively) were predominant, as determined by PCR analysis. All five phylogroups were identified in samples collected from plant B and were almost evenly divided among phylogroups B2, B1, A, and D, and to a lesser extent, in phylogroup F (24, 10, 7, 7, and 3 isolates, respectively). The phylogroups of isolates from plant B were more complex and diverse as compared to those from plant A. The survival rate of phylogroup B2 isolates carrying beta-lactamase genes was relatively high under aerobic conditions.

A previous study reported a significant increase in the abundance of CTX-M-producing *E. coli*, which is also a potential pandemic genotype [45]. The presence of clonal strains from patient isolates of phylogenetic group B2 that can produce CTX-M may be responsible for the spread of ESBL resistance [46]. As a collection and storage site for pathogenic bacteria and pathogenic genes, wastewater plants provide an invaluable venue for the growth and spread of ARB, which is undoubtably increased by the constant agitation and flow of wastewater. Although bacteria gradually weaken and die during the wastewater treatment process, antibiotic-resistance genes can be acquired by bacteria with stronger survival capabilities. Municipal WWTPs can promote the spread of multiple ARB that are potentially harmful to human health and the environment. Hence, continued monitoring of ARB in WWTPs and sewage systems is needed.

2.4 Conclusions

Batch-type agitation and mixing experiments were conducted to monitor changes to *E. coli* strains and antibiotic-resistant profiles in wastewater samples under aerobic conditions. The survival rate of *E. coli* in phylogroup B2, accounting for approximately 73% of the isolates, was the highest in aerobically treated wastewater in plant A after 14 days. In addition, B2 was the dominant phylogroup of antibiotic-resistant *E. coli* isolates at the end of the 14-day experimental period. Under aerobic conditions, the survival of bacteria in wastewater samples significantly differed among strains and the prevalence antibiotic-resistant *E. coli* had increased. Hence, the behavior of members of phylogroup B2, which is the major phylogroup of *E. coli*, should be closely monitored.

Table 2.4 The detection of phylogroups and ESBL-producing *E. coli* isolates.
* Y (yes) or N (no) to indicate whether it is an ESBL genotype.

Isolates from plant A	ESBL types by the following PCR type					Phylogroup
	TEM	SHV	CTX-M-1	CTX-M-2	CTX-M-9	
A-7 days-91	N	N	N	N	Y	B2
A-7 days-100	N	N	N	Y	Y	B2
A-14 days-5	N	N	Y	N	N	B2
A-14 days-7	N	N	N	Y	N	B2
A-14 days-8	N	N	N	N	Y	B2
A-14 days-9	N	N	N	N	Y	B2
A-14 days-21	N	N	N	Y	Y	B2
A-14 days-23	N	N	N	N	Y	B2
A-14 days-42	N	N	N	N	Y	B2
A-14 days-54	N	N	N	N	Y	B2
A-14 days-59	N	N	N	N	Y	B2
A-14 days-62	N	N	N	N	Y	B2
A-14 days-71	N	N	N	N	Y	B2
A-14 days-73	N	N	N	N	Y	B2
A-14 days-80	N	Y	N	N	N	B2
A-14 days-82	N	Y	N	N	N	B2
A-14 days-83	N	N	Y	Y	N	B2
A-14 days-84	N	N	Y	Y	N	B2
A-14 days-85	N	N	Y	N	N	B2
A-14 days-86	N	N	N	N	Y	B2
A-14 days-87	N	N	N	N	Y	B2
A-14 days-88	N	N	N	N	Y	B2
A-14 days-92	N	N	N	N	Y	B2
A-14 days-94	N	N	N	N	Y	B2
A-14 days-100	N	Y	N	N	N	B1
Strains (%)	0 (0)	3 (12%)	4 (16%)	5 (20%)	17 (68%)	total: 25 strains

Isolates from plant B	ESBL types by the following PCR type					Phylogroup
	TEM	SHV	CTX-M-1	CTX-M-2	CTX-M-9	
B-0 day-1	N	N	N	N	Y	B2
B-0 day-2	N	N	N	N	Y	D
B-0 day-3	N	N	N	Y	N	B2
B-0 day-4	N	Y	N	N	N	B2
B-0 day-12	N	N	N	N	Y	D
B-0 day-13	N	Y	N	N	N	D
B-0 day-14	N	N	N	N	Y	F
B-0 day-23	N	N	N	N	Y	B2
B-0 day-24	N	N	Y	N	N	B2
B-0 day-25	N	N	N	N	Y	B2
B-0 day-26	N	Y	N	N	N	A
B-0 day-38	N	N	N	Y	Y	B2
B-0 day-39	N	Y	N	N	N	B1
B-0 day-43	N	Y	N	N	N	A
B-0 day-60	N	N	N	Y	Y	B2
B-0 day-62	N	Y	N	N	N	B2
B-0 day-64	N	Y	N	N	N	B2
B-0 day-65	N	Y	N	N	N	A
B-0 day-66	N	Y	N	N	N	A
B-0 day-85	N	N	N	N	Y	D
B-0 day-86	N	Y	N	N	N	B1
B-0 day-87	N	N	N	N	Y	B2
B-0 day-88	N	Y	N	N	N	F
B-0 day-89	N	Y	N	N	N	B2
B-7 days-17	N	N	N	Y	N	B2
B-7 days-18	N	Y	N	N	N	B1
B-7 days-19	N	N	Y	N	Y	B1
B-7 days-20	N	Y	N	N	Y	F
B-7 days-26	N	Y	N	N	Y	D
B-7 days-40	N	Y	Y	N	N	B2
B-7 days-45	N	Y	N	N	Y	B2
B-7 days-46	N	Y	N	N	Y	B2
B-7 days-47	Y	Y	N	N	N	A
B-7 days-48	N	Y	N	N	N	D
B-7 days-49	N	N	N	Y	N	B2
B-7 days-50	N	N	N	N	Y	A
B-7 days-51	N	Y	Y	N	N	A
B-7 days-53	N	Y	N	N	N	B1
B-7 days-58	N	N	N	N	Y	D
B-7 days-67	N	N	N	N	Y	B2
B-7 days-68	N	N	N	N	Y	B2
B-7 days-69	N	N	N	Y	Y	B1
B-7 days-70	N	Y	Y	N	N	B1
B-7 days-71	N	N	N	N	Y	B1
B-7 days-84	N	Y	N	N	N	B2
B-7 days-88	N	Y	N	N	N	B2
B-7 days-91	Y	Y	N	N	Y	B2
B-14 days-27	N	Y	N	N	N	B2
B-14 days-28	N	N	N	Y	N	B1
B-14 days-30	N	Y	N	N	N	B1
B-14 days-37	N	N	N	Y	Y	B2
Strains (%)	2 (4%)	28 (55%)	5 (10%)	8 (16%)	23 (45%)	total: 51 strains

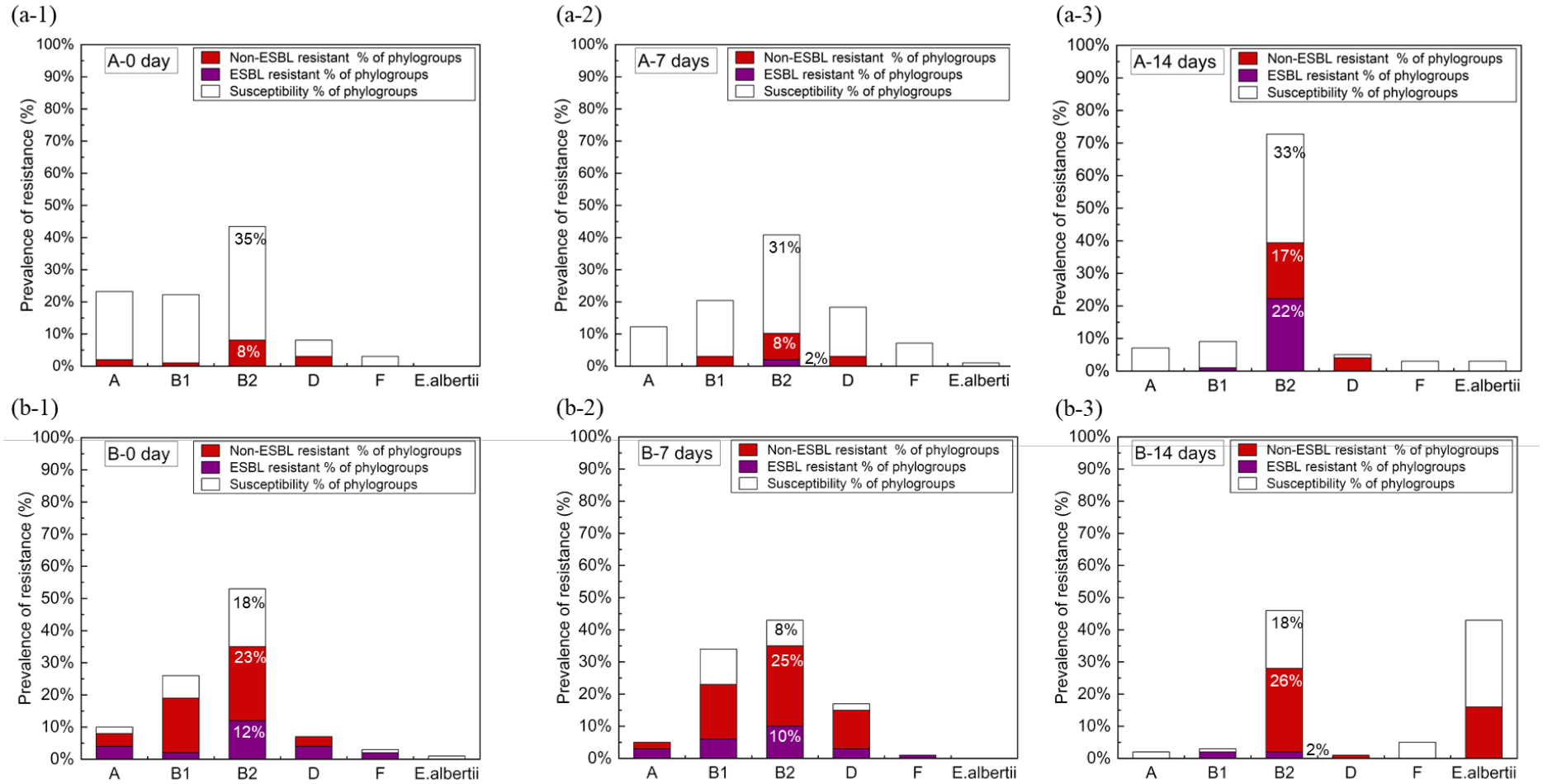

Figure 2.5 The relationship between phylogroups and AR (ESBL and non-ESBL) of *E. coli* isolates in wastewater samples collected from plants A and B. *E. coli* isolates obtained from plant A samples on days 0 (a-1), 7 (a-2), and 14 (a-3). *E. coli* isolates obtained from plant B on days 0 (b-1), 7 (b-2), and 14 (b-3).

Reference

1. Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. BMJ (Clinical research ed.), 361, k2179. https://doi.org/10.1136/bmj.k2179
2. Cabral J. P. (2010). Water microbiology. Bacterial pathogens and water. International journal of environmental research and public health, 7(10), 3657–3703. https://doi.org/10.3390/ijerph7103657
3. Nagarjuna, D., Mittal, G., Dhanda, R. S., Verma, P. K., Gaind, R., & Yadav, M. (2015). Faecal *Escherichia coli* isolates show potential to cause endogenous infection in patients admitted to the ICU in a tertiary care hospital. New microbes and new infections, 7, 57–66. https://doi.org/10.1016/j.nmni.2015.05.006
4. Zhang, X. S., Chase-Topping, M. E., McKendrick, I. J., Savill, N. J., & Woolhouse, M. E. (2010). Spread of *E. coli* O157 infection among Scottish cattle farms: stochastic models and model selection. Epidemics, 2(1), 11–20. https://doi.org/10.1016/j.epidem.2010.02.001
5. van Elsas, J., Semenov, A., Costa, R., & Trevors T. J. (2011). Survival of *Escherichia coli* in the environment: fundamental and public health aspects, 5, 173–183. https://doi.org/10.1038/ismej.2010.80
6. Aristizábal-Hoyos, A. M., Rodríguez, E. A., Arias, L., & Jiménez, J. N. (2019). High clonal diversity of multidrug-resistant and extended spectrum beta-lactamase-producing *Escherichia coli* in a wastewater treatment plant. Journal of environmental management, 245, 37–47. https://doi.org/10.1016/j.jenvman.2019.05.073
7. Adefisoye, M. A., & Okoh, A. I. (2016). Identification and antimicrobial resistance prevalence of pathogenic *Escherichia coli* strains from treated wastewater effluents in Eastern Cape, South Africa. MicrobiologyOpen, 5(1), 143–151. https://doi.org/10.1002/mbo3.319
8. Kümmerer K. (2009). Antibiotics in the aquatic environment--a review--part II. Chemosphere, 75(4), 435–441. https://doi.org/10.1016/j.chemosphere.2008.12.006

9. Müller, A., Stephan, R., & Nüesch-Inderbinen, M. (2016). Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated from the environment, livestock, food and humans. The Science of the total environment, 541, 667–672. https://doi.org/10.1016/j.scitotenv.2015.09.135
10. Abia, A. L., Ubomba-Jaswa, E., & Momba, M. N. (2016). Occurrence of diarrhoeagenic *Escherichia coli* virulence genes in water and bed sediments of a river used by communities in Gauteng, South Africa. Environmental science and pollution research international, 23(15), 15665–15674. https://doi.org/10.1007/s11356-016-6762-6
11. Centers for Disease Control and Prevention (CDC). (2019). National Center for Emerging Zoonotic and Infectious Diseases (U.S.); Division of Healthcare Quality Promotion; Antibiotic Resistance Coordination and Strategy Unit. Antibiotic resistance threats in the United States, 1-148. http://dx.doi.org/10.15620/cdc:82532.
12. Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., Kretzschmar, M. E., Devleesschauwer, B., Cecchini, M., Ouakrim, D. A., Oliveira, T. C., Struelens, M. J., Suetens, C., Monnet, D. L., & Burden of AMR Collaborative Group (2019). Attributable deaths and disability-adjusted life-years caused by infections with ARB in the EU and the European Economic Area in 2015: a population-level modelling analysis. The Lancet. Infectious diseases, 19(1), 56–66. https://doi.org/10.1016/S1473-3099(18)30605-4
13. Woerther, P. L., Burdet, C., Chachaty, E., & Andremont, A. (2013). Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. Clinical microbiology reviews, 26(4), 744–758. https://doi.org/10.1128/CMR.00023-13
14. Manges, A. R., Johnson, J. R., Foxman, B., O'Bryan, T. T., Fullerton, K. E., & Riley, L. W. (2001). Widespread distribution of urinary tract infections caused by a multidrug-resistant *Escherichia coli* clonal group. The New England journal of medicine, 345(14), 1007–1013. https://doi.org/10.1056/NEJMoa011265
15. Chaudhuri, R. R., & Henderson, I. R. (2012). The evolution of the *Escherichia coli* phylogeny. Infection, genetics and evolution : journal of molecular epidemiology and

evolutionary genetics in infectious diseases, 12(2), 214–226. https://doi.org/10.1016/j.meegid.2012.01.005

16. Duriez, P., Clermont, O., Bonacorsi, S., Bingen, E., Chaventré, A., Elion, J., Picard, B., & Denamur, E. (2001). Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. Microbiology (Reading, England), 147(Pt 6), 1671–1676. https://doi.org/10.1099/00221287-147-6-1671
17. Escobar-Páramo, P., Le Menac'h, A., Le Gall, T., Amorin, C., Gouriou, S., Picard, B., Skurnik, D., & Denamur, E. (2006). Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. Environmental microbiology, 8(11), 1975–1984. https://doi.org/10.1111/j.1462-2920.2006.01077.x
18. Orsi, R.H., Stoppe, N.C., Sato, M.I.Z., Gomes, T.A.T., Prado, P.I., Manfio, G.P., Ottoboni, L.M.M. (2007). Genetic variability and pathogenicity potential of *Escherichia coli* isolated from recreational water reservoirs. Research in Microbiology, 158, 420-427. https://doi.org/10.1016/j.resmic.2007.02.009
19. Gordon, D. M., Clermont, O., Tolley, H., & Denamur, E. (2008). Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. Environmental microbiology, 10(10), 2484–2496. https://doi.org/10.1111/j.1462-2920.2008.01669.x
20. Higgins, J., Hohn, C., Hornor, S., Frana, M., Denver, M., & Joerger, R. (2007). Genotyping of *Escherichia coli* from environmental and animal samples. Journal of microbiological methods, 70(2), 227–235. https://doi.org/10.1016/j.mimet.2007.04.009
21. Anastasi, E. M., Matthews, B., Gundogdu, A., Vollmerhausen, T. L., Ramos, N. L., Stratton, H., Ahmed, W., & Katouli, M. (2010). Prevalence and persistence of *Escherichia coli* strains with uropathogenic virulence characteristics in sewage treatment plants. Applied and environmental microbiology, 76(17), 5882–5886. https://doi.org/10.1128/AEM.00141-10
22. Bojar, B., Sheridan, J., Beattie, R., Cahak, C., Liedhegner, E., Munoz-Price, L. S.,

Hristova, K. R., & Skwor, T. (2021). Antibiotic resistance patterns of *Escherichia coli* isolates from the clinic through the wastewater pathway. International journal of hygiene and environmental health, 238, 113863. https://doi.org/10.1016/j.ijheh.2021.113863

23. Suzuki, Y., Niina, K., Matsuwaki, T., Nukazawa, K., & Iguchi, A. (2018). Bacterial flora analysis of coliforms in sewage, river water, and ground water using MALDI-TOF mass spectrometry. Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering, 53(2), 160–173. https://doi.org/10.1080/10934529.2017.1383128
24. Clermont, O., Christenson, J. K., Denamur, E., & Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environmental microbiology reports, 5(1), 58–65. https://doi.org/10.1111/1758-2229.12019
25. Cabrolier, N., Sauget, M., Bertrand, X., & Hocquet, D. (2015). Matrix-assisted laser desorption ionization-time of flight mass spectrometry identifies Pseudomonas aeruginosa high-risk clones. Journal of clinical microbiology, 53(4), 1395–1398. https://doi.org/10.1128/JCM.00210-15
26. Clinical and Laboratory Standards Institute (CLSI) (2017). Performance standards for antimicrobial susceptibility testing. M100-27th ed. CLSI supplement M100.Wayne, PA: Clinical and Laboratory Standards Institute, 1-282, ISBN 1-56238-804-5. https://clsi.org/standards/.
27. Shibata, N., Kurokawa, H., Doi, Y., Yagi, T., Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Ishikawa, S., Kato, H., Ozawa, Y., Shibayama, K., Kai, K., Konda, T., & Arakawa, Y. (2006). PCR classification of CTX-M-type beta-lactamase genes identified in clinically isolated gram-negative bacilli in Japan. Antimicrobial agents and chemotherapy, 50(2), 791–795. https://doi.org/10.1128/AAC.50.2.791-795.2006
28. Yagi, T., Kurokawa, H., Shibata, N., Shibayama, K., & Arakawa, Y. (2000). A preliminary survey of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS microbiology letters, 184(1), 53–56.

https://doi.org/10.1111/j.1574-6968.2000.tb08989.x

29. Biran, D., & Ron, E. Z. (2018). Extraintestinal Pathogenic *Escherichia coli*. Current topics in microbiology and immunology, 416, 149–161. https://doi.org/10.1007/82_2018_108
30. Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G., & Choroszy-Krol, I. (2019). Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. Gut pathogens, 11, 10. https://doi.org/10.1186/s13099-019-0290-0
31. Johnson, J. R., Kuskowski, M. A., O'Bryan, T. T., & Maslow, J. N. (2002). Epidemiological correlates of virulence genotype and phylogenetic background among *Escherichia coli* blood isolates from adults with diverse-source bacteremia. The Journal of infectious diseases, 185(10), 1439–1447. https://doi.org/10.1086/340506
32. Mokracka, J., Koczura, R., Jabłońska, L., & Kaznowski, A. (2011). Phylogenetic groups, virulence genes and quinolone resistance of integron-bearing *Escherichia coli* strains isolated from a wastewater treatment plant. Antonie van Leeuwenhoek, 99(4), 817–824. https://doi.org/10.1007/s10482-011-9555-4
33. Inglis, T.J.J., Merritt, A.J., Bzdyl, N., Lansley, S., Urosevic, M.N. (2015) First bacteraemic human infection with *Escherichia albertii*. New Microbes and New Infections, 8, 171-173, https://doi.org/10.1016/j.nmni.2015.07.003
34. Hyma, K. E., Lacher, D. W., Nelson, A. M., Bumbaugh, A. C., Janda, J. M., Strockbine, N. A., Young, V. B., & Whittam, T. S. (2005). Evolutionary genetics of a new pathogenic Escherichia species: *Escherichia albertii* and related *Shigella boydii* strains. Journal of bacteriology, 187(2), 619–628. https://doi.org/10.1128/JB.187.2.619-628.2005
35. Ooka, T., Seto, K., Kawano, K., Kobayashi, H., Etoh, Y., Ichihara, S., Kaneko, A., Isobe, J., Yamaguchi, K., Horikawa, K., Gomes, T. A., Linden, A., Bardiau, M., Mainil, J. G., Beutin, L., Ogura, Y., & Hayashi, T. (2012). Clinical significance of *Escherichia albertii*. Emerging infectious diseases, 18(3), 488–492.

https://doi.org/10.3201/eid1803.111401

36. Walk, S. T., Alm, E. W., Gordon, D. M., Ram, J. L., Toranzos, G. A., Tiedje, J. M., & Whittam, T. S. (2009). Cryptic lineages of the genus *Escherichia*. Applied and environmental microbiology, 75(20), 6534–6544. https://doi.org/10.1128/AEM.01262-09
37. Abbott, S. L., O'Connor, J., Robin, T., Zimmer, B. L., & Janda, J. M. (2003). Biochemical properties of a newly described *Escherichia* species, *Escherichia albertii*. Journal of clinical microbiology, 41(10), 4852–4854. https://doi.org/10.1128/JCM.41.10.4852-4854.2003
38. Gomes, T. A. T., Ooka, T., Hernandes, R. T., Yamamoto, D., & Hayashi, T. (2020). *Escherichia albertii* Pathogenesis. EcoSal Plus, 9(1), 10.1128/ecosalplus.ESP-0015-2019. https://doi.org/10.1128/ecosalplus.ESP-0015-2019
39. Paulshus, E., Kühn, I., Möllby, R., Colque, P., O'Sullivan, K., Midtvedt, T., Lingaas, E., Holmstad, R., & Sørum, H. (2019). Diversity and antibiotic resistance among *Escherichia coli* populations in hospital and community wastewater compared to wastewater at the receiving urban treatment plant. Water research, 161, 232–241. https://doi.org/10.1016/j.watres.2019.05.102
40. Nippon, A.M.R. (2019) One health report (NAOR). A.M.R. One Health Surveillance Committee. http://resistancecontrol.info/2019-1/the-one-health-approach-and-public-education-and-awareness-raising-activities-in-japan/.
41. Bingen, E., Picard, B., Brahimi, N., Mathy, S., Desjardins, P., Elion, J., & Denamur, E. (1998). Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. The Journal of infectious diseases, 177(3), 642–650. https://doi.org/10.1086/514217
42. Desjardins, P., Picard, B., Kaltenböck, B., Elion, J., & Denamur, E. (1995). Sex in *Escherichia coli* does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism. Journal of molecular evolution, 41(4), 440–448. https://doi.org/10.1007/BF00160315
43. Picard, B., Garcia, J. S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J., &

Denamur, E. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infection and immunity, 67(2), 546–553. https://doi.org/10.1128/IAI.67.2.546-553.1999

44. Le Gall, T., Clermont, O., Gouriou, S., Picard, B., Nassif, X., Denamur, E., & Tenaillon, O. (2007). Extraintestinal virulence is a coincidental by-product of commensalism in B2 phylogenetic group *Escherichia coli* strains. Molecular biology and evolution, 24(11), 2373–2384. https://doi.org/10.1093/molbev/msm172

45. Mugnaioli, C., Luzzaro, F., De Luca, F., Brigante, G., Perilli, M., Amicosante, G., Stefani, S., Toniolo, A., & Rossolini, G. M. (2006). CTX-M-type extended-spectrum beta-lactamases in Italy: molecular epidemiology of an emerging countrywide problem. Antimicrobial agents and chemotherapy, 50(8), 2700–2706. https://doi.org/10.1128/AAC.00068-06

46. Nicolas-Chanoine, M. H., Blanco, J., Leflon-Guibout, V., Demarty, R., Alonso, M. P., Caniça, M. M., Park, Y. J., Lavigne, J. P., Pitout, J., & Johnson, J. R. (2008). Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. The Journal of antimicrobial chemotherapy, 61(2), 273–281. https://doi.org/10.1093/jac/dkm464

Chapter 3. Prevalence of antibiotic resistant bacteria and antibiotic resistant genes in a whole wastewater treatment plant

3.1 Introduction

Antibiotics have been widely utilized as the most popular form of chemotherapy due to their effectiveness in killing bacteria and inhibiting bacterial proliferation [1]. With the increasing use of antibiotics, the emergence of MDR bacteria has been observed worldwide [2-4]. Nosocomial infections caused by ARB with deaths due to the lack of therapeutic options for affected individuals have been reported worldwide, and they pose the most serious human health risk worldwide [5, 6].

Identified ARBs have been reported to be resistant to not only conventional effective antibiotics but also to newer antibiotics such as fourth-generation cephalosporins, ampicillin, carbapenems, and vancomycin, which is another problem [7-9]. In particular, ESBL-producing bacteria, which are regarded as major contributors to nosocomial infections, have become the most critical challenge. ESBLs have been identified as β-lactamase-producing genes (TEM-, SHV-, CTX-M-types, etc.) encoded on transmissible plasmids (R-plasmids) carried by bacteria such as *E. coli* and coliforms, as well as β-lactamase-producing genes that also degrade third-generation cephalosporins [10-12]. Nowadays, much information and findings on the prevalence, transmission, and dissemination of ESBL-producing *E. coli* and coliforms have been reported, covering human living environments and diverse activity areas, including hospital facilities, livestock, and aquaculture farms [13-15].

WWTPs are the infrastructure facilities of utmost priority for the efficient collection and purification of human excreta. WWTPs can be a hot spot for bacteria survival because they provide an ideal environment for the bacteria to grow and thrive. WWTPs receive huge quantities of raw wastewater containing *E. coli* and coliforms, which are removed via various treatment processes and discharged into rivers or coastal public water bodies after being disinfected. Therefore, several research studies have investigated the prevalence of ARB (including *E. coli* and coliforms) contamination throughout WWTPs

in various countries [16-19]. It has already been reported that ESBL-producing *E. coli* and coliforms are released from human excreta through WWTPs into rivers, possibly increasing the prevalence of ARBs contamination [20, 21]. However, there is insufficient information on changes in the disappearance and prevalence of ESBL-producing *E. coli* and coliforms passing the treatment process of WWTPs. There is little information on the changes in the microbiota of ESBL-producing coliforms and their survival in each treatment process. However, the presence of bacteria in the influent (i.e., the raw wastewater that enters the WWTP) can make it difficult to remove the bacteria completely. Furthermore, WWTPs can also be a source of ARB. This is because antibiotics are often used to treat bacterial infections in humans and animals, and these antibiotics can end up in the wastewater. The presence of antibiotics in the wastewater can create a selection pressure for ARB to survive and thrive, including *E. coli*.

Therefore, in this study, we investigated the changes in bacterial disappearance and the prevalence of ESBL-producing bacteria in each process in the WWTP. In addition, changes in the microbiota of ESBL-producing coliforms were followed. Furthermore, we identified the types of antibiotic-resistance genes harbored by ESBL-producing *E. coli* and coliforms.

3.2 Materials and Methods

3.2.1 Sampling and water quality analysis

Influent wastewater, biologically-treated wastewater (before chlorination), and efferent (after chlorination) samples were collected from an urban WWTP (oxidation ditch system) that treats wastewater from a town with a population of approximately 14,000 people. The average daily flow through the plant is 6,300 m^3. The survey was conducted over a total of three sampling dates, between June, September, and November of 2021. There was no rainfall on the day before and on the day of sampling. Water samples were collected from the surface layer into polyethylene bottles using a ladle.

These samples were brought back to the laboratory immediately after collection, and the bacterial counts of *E. coli* and coliforms, as well as general water quality parameters, were measured. A benchtop pH/water quality analyzer (LAQUA, Horiba) was used to measure the pH and electrical conductivity (EC). Turbidity (TU; based on the kaolin standard) was determined using a turbidity meter (PT-200, NittoSeiko Analytech, Japan). The amount of residual chlorine was determined via the diethyl-p-phenylenediamine method (DR2800, HACH). The concentrations of total organic carbon (TOC) were determined using a TOC analyzer (TOC-V Model, Shimadzu, Japan).

3.2.2 Enumeration and isolation of bacteria

E. coli and coliforms were enumerated using CHROMagar ECC (CHROMagar, France). In brief, 10^{-5} to 100 ml of water samples were filtered through a sterile 0.45-μm-pore mixed cellulose membrane filter (47 mm diameter, Advantec, Japan). The membrane filters were incubated on CHROMagar ECC agar plates at 37 ± 0.5 °C for 24 hours. Afterward, mean numbers of CFUs of *E. coli* and coliforms in each sample were determined from three replicates. Bacterial counts are expressed as CFU/100 mL. On CHROMagar™ ECC plates, blue colonies were counted as *E. coli* while mauve colonies were counted as other coliforms. For isolation and preservation of bacteria, all *E. coli* and coliforms isolates were streaked on BHI agar plates (1.5% agar; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37 ± 0.5 °C for 24 h.

3.2.3 Screening for ampicillin-resistant bacteria

In order to identify potential ESBL-producing *E.coli*/coliforms in this study, pre-selection was carried out with ampicillin (ABPC) antibiotics in the target WWTP. Filters passing through the samples were attached to ABPC-screening ECC agar plates (ABPC screening plates) supplemented with ABPC at 32 μg/ml [22] and incubated at 37 ± 0.5°C for 24 hours. Positive colonies of *E. coli* and coliforms formed on ABPC screening plates

were counted. The ABPC resistance rate was calculated as the ratio (%) of the number of bacteria on ABPC screening plates to the number of bacteria on regular ECC agar plates. After counting, a total of 60 positive colonies of *E. coli* and coliforms on each of the three ABPC screening plates were randomly isolated. The isolated colonies were applied to BHI agar plates and incubated at 37 ± 0.5°C for 24 hours. After incubation, single colonies were collected as ABPC-resistant strains.

3.2.4 Bacterial identification by MALDI-TOF MS

The method is the same as the one used in previous publications [23, 24]. Positive isolates of *E. coli* and coliforms were incubated at 37 ± 0.5 °C for 18 hours. Samples were spread directly into 384-well stainless-steel target plates (MTP 384; Bruker Daltonics, Billerica, MA, USA) and then covered with 1.0 ml of matrix solution. All samples were analyzed for the probability of correct isolates using the autoflex® III TOF/TOF system (Bruker Daltonics) and the MALDI Biotyper Compass microbial identification system (Bruker Daltonics, version 4.1.60.2) per the manufacturer's instructions. *E. coli* and coliforms were identified by a logarithmic score of >1.700.

3.2.5 Screening for ESBL-producing bacteria

ABPC-resistant isolates identified from each species of *E. coli* and coliforms were further screened for ESBL-producing strains using the CHROMagar-ESBL-selective agar medium (Kanto Chemical, Tokyo, Japan). Pre-culture was performed on the BHI agar medium (37 ± 0.5°C for 18 hours); thereafter, growing colonies were suspended in 3 ml of sterile saline to achieve McFarland 0.5 bacterial concentration. The suspension of each strain was inoculated onto a CHROMagar-ESBL-selective agar plates (ESBL-selective plates) for the ESBL-producing bacteria screening test. After incubation at 37 ± 0.5°C for 24 hours, ESBL-producing bacteria were identified based on their color (red colonies for *E. coli* and blue colonies for other coliforms), and all positive colonies were isolated and

stored.

3.2.6 Confirmation test of ESBL-producing bacteria

Confirmation tests were performed on positive isolates after screening for ESBL-producing bacteria. The confirmation test was determined by the inhibition circle for ceftazidime (CAZ) (30 mg, Becton Dickinson, Tokyo, Japan), ceftazidime/clavulanic acid (CAZ/CVA) (30 mg/10 mg, Eiken Chemical, Tokyo, Japan), cefotaxime (CTX) (30 mg, Becton Dickinson, Tokyo, Japan), and cefotaxime/clavulanic acid (CTX/CVA) (30 mg/10 mg, Eiken Chemical, Tokyo, Japan) per the disk diffusion method (CLSI, 2017). The positive strains screened with ESBL-selective plates were pre-cultured on the BHI agar medium, and the bacterial concentration was adjusted to the Macfarlane 0.5 standard as described above. Then, the adjusted bacterial solution was applied to Mueller Hinton (Becton Dickinson, Tokyo, Japan) agar medium within 15 minutes. Discs of four antibiotics (CAZ, CAZ/CVA, CTX, and CTX/CVA) were stamped on an agar medium using a Sensi-Disc dispenser (Becton Dickinson, Tokyo, Japan). After incubation at 37 ± 0.5°C for 18–24 hours, ESBL-producing bacteria were identified by an inhibition circle with a minimum diameter of 5 mm in CAZ and CAZ/CVA or CTX and CTX/CVA.

3.2.7 ESBL genotypes of *E. coli* and coliforms by multiplex PCR analysis

DNA samples were extracted from ABPC-resistant *E. coli* and ABPC-resistant coliforms isolates using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA). The five ESBL genotypes (TEM, SHV, CTX-M-1, CTX-M-2, CTX-M-9) were determined according to the multiplex PCR technique [25].

The primers for all the genes. Each 50-µl reaction consisted of 29.75 µl of sterilized distilled water, 0.25 µl of Takara Taq HS (Takara Bio Inc., Shiga, Japan), 5 µl of 10 × KAPA Extra Buffer (Takara Bio Inc., Shiga, Japan), 5 µl of deoxynucleoside triphosphate Mix (dNTP Mix) (Takara Bio Inc., Shiga, Japan), 1 µl of each primer, and 1 µl of the DNA template. The PCR reaction was performed using a SimpliAmp™ Thermal Cycler

(Thermo Fisher Scientific, Waltham, MA, USA) with the following reaction conditions: denaturation at 94°C for 2 minutes, followed by 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 90 seconds, and a final extension step at 72°C for 5 minutes. After the PCR reaction, 5 μl of the PCR product and 1 μl of 6 × loading buffer (Takara Bio, Inc., Shiga, Japan) were mixed, loaded into the wells of 2% agarose gels, and separated using the Mupid®-One electrophoresis system (Nippon Genetics Co., Ltd., Tokyo, Japan) at 100 V for 40 minutes. After electrophoresis, the agarose gel was stained with ethidium bromide solution (0.05 μl/ml) for 10 minutes and then shaken in distilled water for 10 minutes to confirm the PCR amplification products. The *E. coli* strain, ATCC 25922, was used as a positive control for the PCR reaction.

3.2.8 Statistical analysis

Continuous data are presented as mean values and their respective standard deviations. The means of the ARBs prevalence data for each sampling site were statistically compared using the independent samples t-test to determine significant differences ($p < 0.05$). The chi-square test was also used to check the independence of ARB prevalence in each sample with a statistical significance threshold of $p<0.05$ to determine whether there was a significant association between the two categorical variables. Based on the proportions of coliforms species, the Bray-Curtis distances between ABPC-resistant coliforms species were determined for each sampling period. Arbitrary distance matrices were visualized for the bacterial component species by Non-metric Multidimensional Scaling (nMDS) using the vegan package (R version 3.6.3) metaMDS function. The individual ESBL-generating genes from the wastewater samples were normalized to the data using the Z score in the originlab program, and then hierarchical clustering heatmaps were performed using the heatmap package.

3.3 Results and discussion

3.3.1 Quality of water samples

The determination of general water quality parameters is shown in **Table 3.1**. The pH varied within a narrow neutral range of 6.69–7.39 at each point from influent to effluent. No decrease in pH was observed during the biological treatment process, suggesting that the reaction up to ammonia nitrification was not in progress. The EC decreased slightly as the treatment process progressed. The turbidity of the influent varied widely from 94.1 TU to 158.8 TU during the months of sampling. The turbidity components were removed via the biological treatment process, and the turbidity of the final effluent decreased below 2.4 TU in all sampling data. The TOC, the main objective of the WWTP, ranged from 94.2 mg-C/L to 100.4 mg-C/L in the influent during the survey, indicating that the organic load to the treatment process at the WWTP was stable. The TOC in the effluent was maintained below 20 mg-C/L, and the removal efficiency was 80.4%. Based on the results of the above general water quality parameters, the samples collected in this study were assumed to have been routinely treated at the WWTP.

Table 3.1 The determination of general water quality parameters.

Sample	Collection days (month)	pH (-)	EC (μs/cm)	Turbidity (ppm)	TOC (mg·C/L)
Influent	6	7.39	514	158.80	100.36
Influent	9	6.88	554	94.10	95.96
Influent	11	7.12	567	112.34	94.18
Biological treated water	6	6.97	401	2.23	17.60
Biological treated water	9	7.01	459	1.44	19.91
Biological treated water	11	6.69	505	2.62	20.98
Effluent	6	7.02	457	2.39	18.17
Effluent	9	7.19	465	1.36	19.63
Effluent	11	7.01	437	1.30	19.16

3.3.2 Changes in bacterial counts of *E. coli* and coliforms during the treatment process

Figure 3.1-(a) shows the changes in *E. coli* counts in each sample during the three sampling events in June, September, and November. The number of *E. coli* in the influent was $1.2 \pm 0.6 \times 10^7$ CFU/100 ml. In the biologically-treated water, the *E. coli* count was $5.3 \pm 0.1 \times 10^4$ CFU/100 ml and was removed at a high proportion of 99.7% by the biological process. In the effluent, the *E. coli* count was 5.7 ± 0.5 CFU/100 ml, confirming that through the treatment process, 6 logs (99.9999%) of *E. coli* in the influent water were removed. **Figure 3.1-(b)** shows the changes in the number of coliforms in each sample during the three sampling events in June, September, and November. The coliforms count was $2.0 \pm 0.1 \times 10^8$ CFU/100 ml in the influent, one order of magnitude higher than the *E. coli* count. In the biologically-treated water, it was $1.8 \pm 0.10 \times 10^6$ CFU/100 ml, with a 99.1% reduction obtained through the biotreatment process. The coliforms count was reduced to $2.4 \pm 0.01 \times 10^2$ CFU/100 ml in the effluent after chlorination, showing that 5 logs of coliforms were removed throughout the treatment process.

The ABPC-resistant *E. coli* count was $1.7 \pm 0.5 \times 10^6$ CFU/100 ml in the influent (**Figure. 3.1-(a)**). The number of ABPC-resistant *E. coli* was one order of magnitude

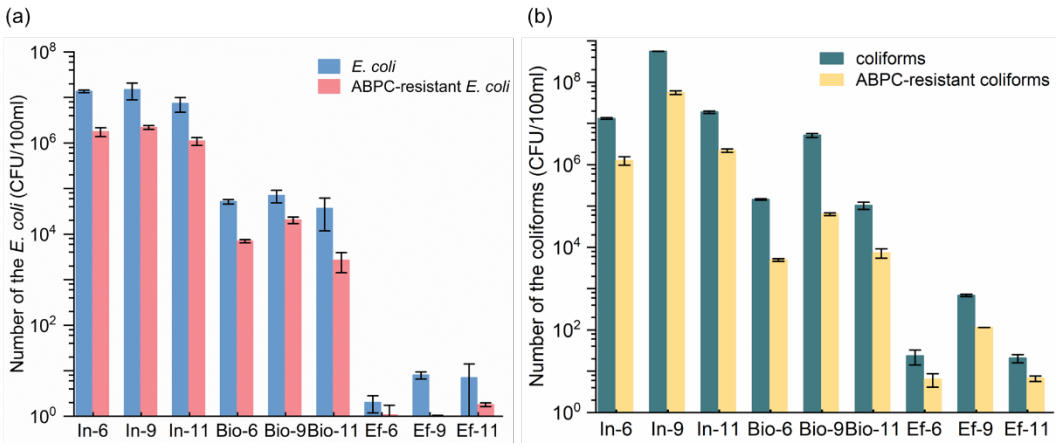

Figure 3.1 Influent, biologically-treated water, and effluent samples from WWTPs in June, September, and November with changes in the number of *E. coli* and ABPC-resistant *E. coli* (a). Changes in the number of coliforms and ABPC-resistant coliforms (b). In; influent, Bio; biologically-treated water, Ef; effluent.

lower than the total number of *E. coli* in the general population. The ABPC-resistant *E. coli* count in the influent was $1.69 \pm 0.5 \times 10^6$ CFU/100 ml, and in the biologically-treated water, it was $1.0 \pm 0.2 \times 10^4$ CFU/100 ml, with an ABPC-resistant prevalence of 16.6% for *E. coli* after biological treatment. The ABPC-resistant *E. coli* count in the effluent was 1.2 ± 0.4 CFU/100 ml and the ABPC-resistant prevalence was 30.0%. ABPC- resistant *E. coli* that entered the WWTP were found to be removed in amounts equivalent to 6 logs of *E. coli* after the treatment processes until they were discharged. However, the prevalence of ABPC tended to increase from the influent to biologically-treated water and then to the effluent. In a similar phenomenon, we have reported that the prevalence of antibiotic-resistant *E. coli* in batch biological wastewater increases after treatment [24]. It has been suggested that antibiotic-resistant *E. coli* has a high survival rate during wastewater treatment. In addition, ABPC-resistant *E. coli* was found in the chlorinated effluent, although the bacterial count remained minimal (average: 1.2 CFU/100 ml).

The number of ABPC-resistant coliforms in the influent was $2.0 \pm 0.4 \times 10^7$ CFU/100 ml, one order of magnitude lower than coliforms count. The ABPC-resistant prevalence of the coliforms present in the influent was 10.5%. The coliforms count in the biologically-treated water was $2.5 \pm 0.1 \times 10^4$ CFU/100 ml, and the ABPC resistance of the coliforms after biological treatment was 3.9%. The number of ABPC-resistant coliforms in the effluent was 42.4 ± 0.1 CFU/100 ml, and the ABPC-resistant prevalence was 25.5%. During the three monthly time series, it was observed that ABPC-resistant coliforms entering the WWTP were removed through each treatment process, with 5 logs comparable to coliforms. However, ABPC-resistant coliforms count tended to be higher in the effluent than in the influent, similar to what was found with *E. coli*. It was also observed that more than 40.0 CFU/100 ml of ABPC-resistant coliforms were released into the effluent. The ABPC-resistant coliforms were found to have a higher load of bacteria in the effluent than the ABPC-resistant *E. coli*. The results showed that in all samples, the WWTP markedly reduced the number of *E. coli* and coliforms, whereas ABPC-resistant *E. coli* and coliforms remained viable in the effluent and were discharged into the water body. It has been reported that wastewater effluent discharged into rivers

can retain trace amounts of bacteria, leading to increased bacterial concentrations and altering the microbial diversity of river water [27].

3.3.3 Species identification of ABPC-resistant positive strains of *E. coli* and coliforms

A total of 378 *E. coli* isolates from each sample were identified as *E. coli* (90.0%; 378/420 isolates). For a total of three sampling events in the influent, biologically-treated water, and the effluent, the identification rate of ABPC-resistant *E. coli* among the *E. coli*-positive strains were 93.9 ± 4.8%, 82.2 ± 8.8%, and 86.9 ± 4.0%, respectively, among the *E. coli* suspicious positive strains. The positive strains of *E. coli* on ABPC screening plates

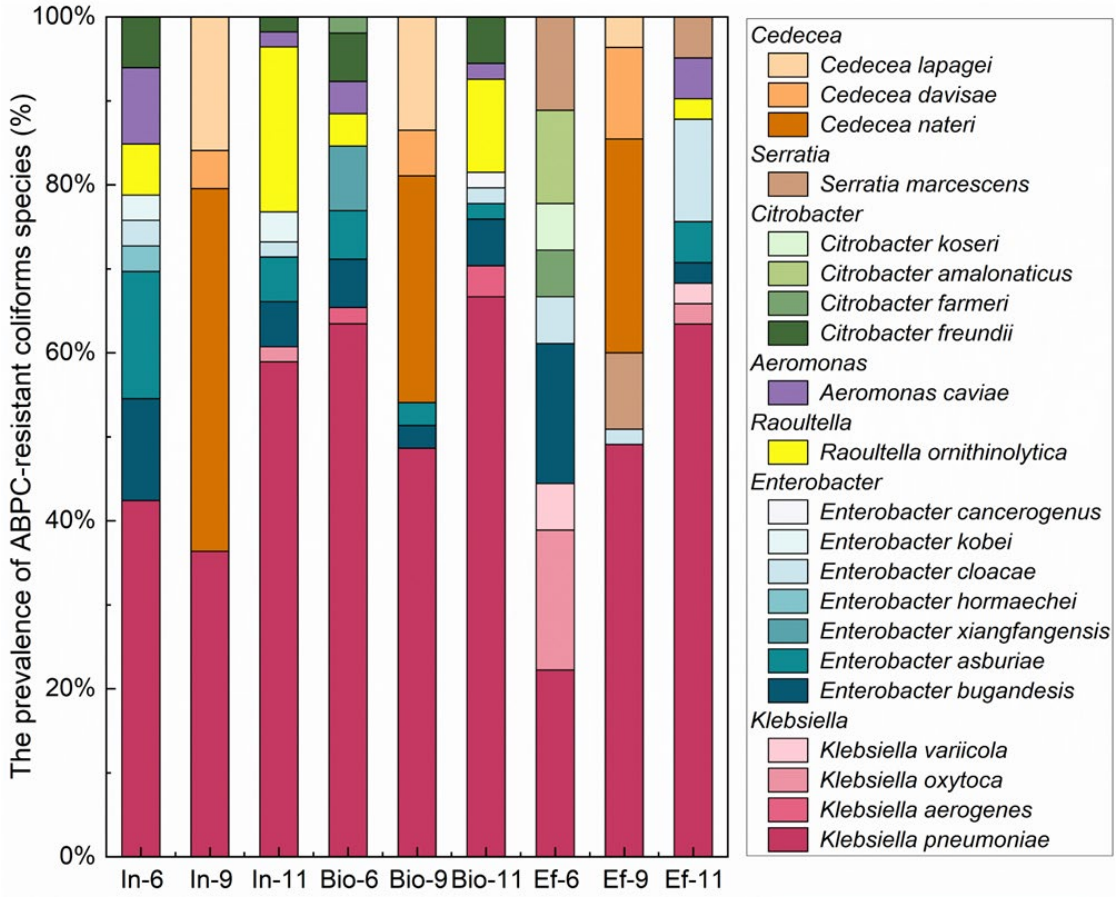

Figure 3.2 The prevalence of ABPC-resistant coliforms species from influent, biologically-treated water, and effluent samples of WWTPs in June, September, and November. In; influent, Bio; biologically-treated water, Ef; effluent.

ranged from 70.0% to 100%, confirming their high selectivity for screening for ABPC-resistant *E. coli*. However, the identification rate of *E. coli* in the effluent water after chlorination was lower than in the influent water samples.

Out of a total of 453 coliforms isolates, 86.1% (390/453 isolates) were identified as coliforms species. The identification rates of ABPC-resistant coliforms in the positive coliforms strains during three sampling events of the influent, biologically-treated water, and effluent were 87.9 ± 1.1%, 80.4 ± 13.4%, and 89.4 ± 5.4%, respectively. Changes in the microbiota of coliforms identified from each sample during the June, September, and November surveys were examined. Twenty-one species from seven bacterial genera were identified from ABPC-resistant coliforms (four species of the genera *Klebsiella*: *K. oxytoca*, *K. pneumoniae*, *K. aerogenes*, and *K. variicola*; one species of the genera *Raoultella*: *R. ornithinolytica*; seven species of the genera *Enterobacter*: *E. asburiae*, *E. bugandesis*, *E. xiangfangensis*, *E. hormaechei*, *E. cloacae*, *E. kobei*, and *E. cancerogenu*; four species of the genera *Citrobacter*: *C. freundii*, *C. farmer*, *C. koseri*, and *C. amalonaticus*; one species of the genera *Serratia*: *S. marcescens*; three species of the genera *Cedecea*: *C. nateri*, *C. lapagei*, and *C. davisae*; one species of the genera *Aeromonas*: *A. caviae*. **Figure 3.2** shows the genera -level microbiota of ABPC-resistant coliforms changes in the influent, biologically-treated water, and effluent. In common with all samples, *Klebsiella* was the most dominant genera, with its percentage prevalence ranging from 36.4% to 60.7%. *Enterobacter* was the second dominant genera in all samples in June and November. In contrast, the *Cedecea* showed a higher dominance in the community in September (38.6%–68.6%) than in the other survey months, with a similar occupancy of *Klebsiella*. However, *Cedecea* was not detected in samples from June to November. The results indicated that the community microbiota of coliforms in wastewater varied significantly over a period of six months. Based on the findings of the present study, we could infer that ABPC-resistant *Klebsiella*, *Enterobacter,* and *Serratia* are constantly discharged from WWTP into receiving rivers. The highest occupancy of *K. pneumoniae* was detected up to a maximum of 66.7%. Similar to the findings of our study, *K. pneumoniae* was also detected in wastewater at an occupancy rate of 62.2% [28]. *K.*

pneumoniae has also been one of the most dominant species in European countries [29], accounting for 11.3% of the total bacterial population. They have also been isolated from the wastewater environment [30]. *K. pneumoniae* is the most abundant species of *Enterobacteriaceae* carrying the transmissible carbapenems gene [31], which is an important contributor to the survival and spread of multidrug-resistant bacteria by being discharged into the environment from WWTPs.

The nMDS analysis was performed to evaluate the similarity of the microbiota at the species level of ABPC-resistant coliforms between each sample by month and wastewater treatment process nMDS (**Figure. 3.3**). The ABPC-resistant coliforms community in September had a smaller plot area and similar microbiota compared to the June and November data. In contrast, the plot positions of the effluent in June and November were

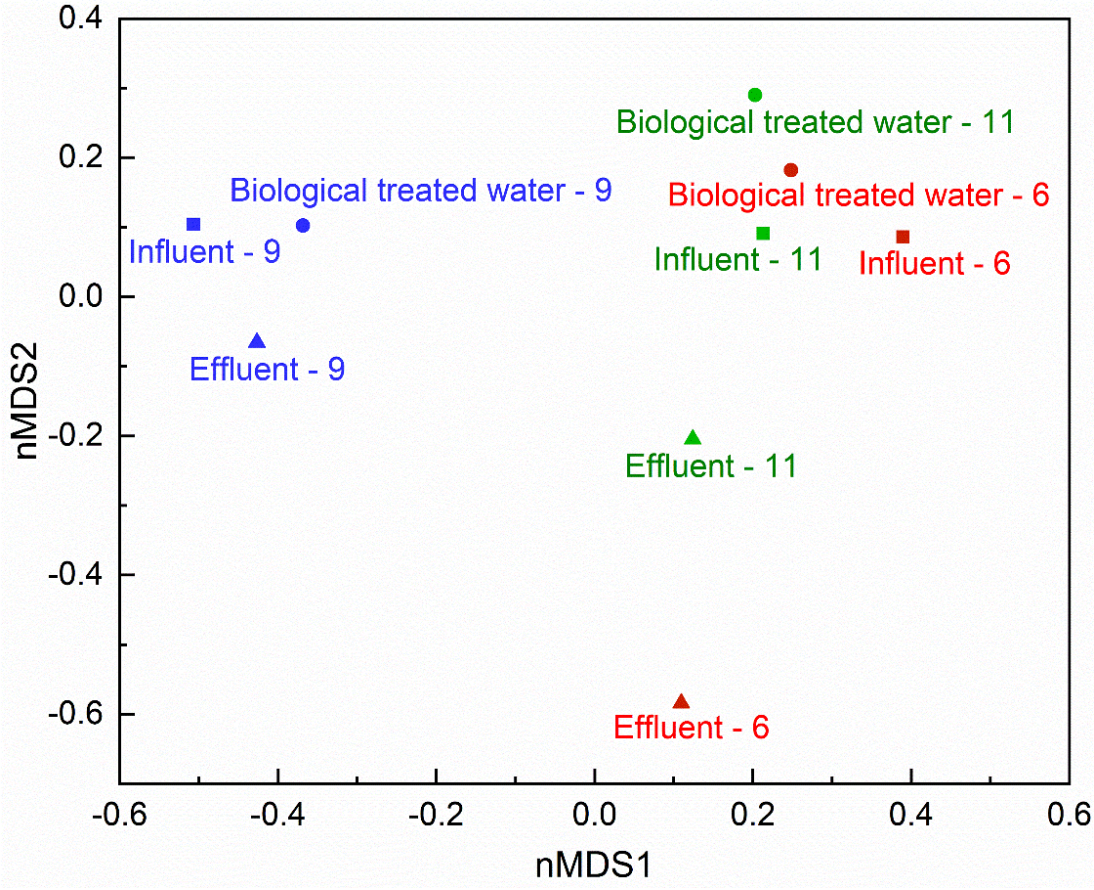

Figure 3.3 The nMDS analysis was performed to determine the similarity of the ABPC-resistant coliforms at the species level between each sample by month and the wastewater treatment process.

microbiota differed significantly from those in the other samples. In this study, *K. pneumoniae*, *K. aerogenes*, *K. oxytoca*, *E. asburiae*, *E. bugandesis*, *E. kobei*, *C. nateri*, *C. lapagei,* and *C. davisae* were the main bacterial species that remained in the effluent. Stanish et al. [32] have reported alterations in the bacterial community due to chlorination, and it is highly probable that chlorination affects the bacterial community by leaving chlorine-resistant species.

3.3.4 Change in the prevalence of ESBL-producing *E. coli*

In the 378 isolates of ABPC-resistant *E. coli*, 84 isolates were positive per the ESBL-producing bacteria screening test, and 82 isolates of them were determined to be ESBL-producing *E. coli* by a confirmation test. ESBL-producing *E. coli* isolates were effectively

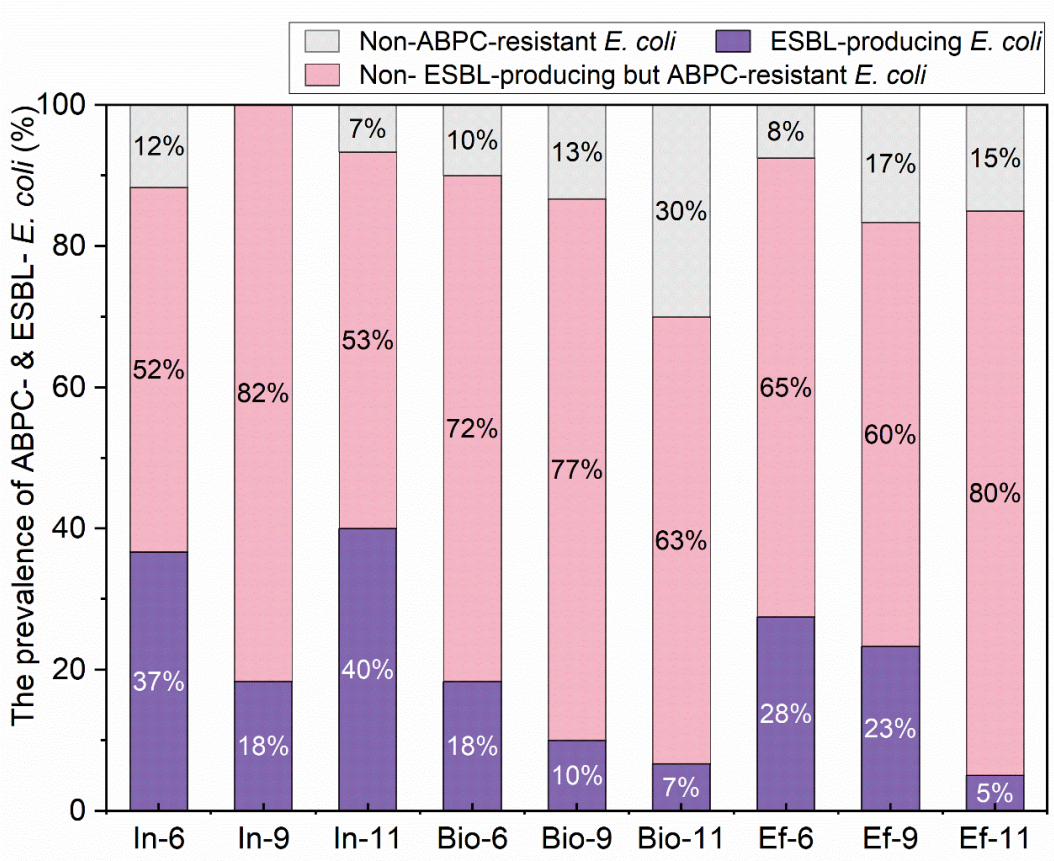

Figure 3.4 The prevalence of ABPC-resistant *E. coli* and ESBL-producing *E. coli* from influent, biologically-treated water, and effluent samples of WWTPs in June, September, and November. In; influent, Bio; biologically-treated water, Ef; effluent.

selected via screening tests of ESBL-producing bacteria using the ESBL-selective plates (97.6%; 82/84 isolates). The prevalence of ESBL-producing *E. coli* in the influent, biologically-treated water, and effluent over the three sampling periods are shown in **Figure. 3.4**. Results of the chi-square test confirmed that the prevalence of ESBL-producing *E. coli* in the influent, biologically-treated water, and effluent in the three samplings periods were independent at a significance level of 5%. The results suggest that there is no significant association between the prevalence of ESBL-producing *E. coli* in the influent, biologically-treated water, and effluent in the three sampling periods. This means that the prevalence of ESBL-producing *E. coli* is not dependent on the stage of the wastewater treatment process. In addition, the mean percentage prevalence of ESBL-producing *E. coli* in June, September, and November were 31.7 ± 9.5%, 11.7 ± 4.9%, and 18.6 ± 9.8%, respectively. A significant decrease in the prevalence of ESBL-producing *E. coli* was observed in the biological treatment process. In June, the prevalence of ESBL-producing *E. coli* decreased from 36.7% in the influent to 18.3% in biologically-treated water; however, it increased again to 27.5% in the effluent. In September, the prevalence of ESBL-producing *E. coli* in biologically-treated water and the effluent increased from 10.0% to 23.3%. Therefore, we compared the prevalence of ESBL-producing *E. coli* in biologically-treated water and the effluent in each month and found that the prevalence of ESBL-producing *E. coli* was significantly higher ($p > 0.05$, t-test) in June and September in the effluent after chlorination. This suggests that there may be seasonal variations in the prevalence of ESBL-producing *E. coli* in the WWPT, which may be due to factors such as changes in the environment, water temperature, organic matter in the water, or other variables. It has been reported that antibiotic-resistant *E. coli* bacteria are highly resistant to chlorine disinfection and survive the ensuing treatments [33].

3.3.5 Change in the prevalence and microbiota of ESBL-producing coliforms

In the ESBL screening experiment and confirmation test determination of all ABPC-resistant *E. coli* isolates, there were 390 ABPC-resistant coliforms isolates in total and

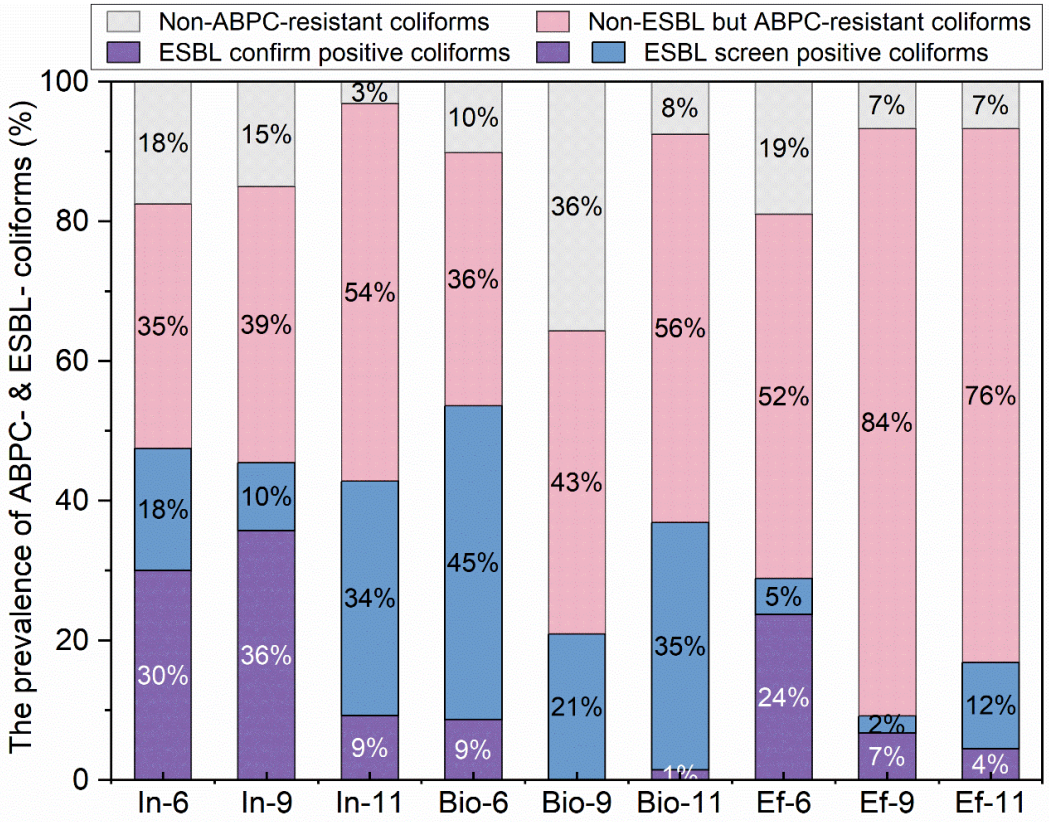

Figure 3.5 The prevalence of ABPC-resistant and ESBL-producing coliforms from influent, biologically-treated water, and effluent samples of WWTPs in June, September, and November. In; influent, Bio; biologically-treated water, Ef; effluent.

135 positive isolates per the screening test of ESBL-producing bacteria, of which 54 (40.0%; 54/135 isolates) were determined to be ESBL-producing coliforms via the confirmation test. The ESBL-producing coliforms were less selective than ESBL-producing *E. coli* per the screening test, and the results of the ESBL confirmation test meant that 60.0% of isolates that formed positive colonies on the ESBL-selective plates were not confirmed as ESBL-producing coliforms. **Figure 3.5** shows the changes in the prevalence of ABPC-resistant coliforms, the presence of positive isolates for the screening test of ESBL-producing bacteria and coliforms the presence of ESBL-producing isolates in each sample. The prevalence of ESBL-producing coliforms in the influent, biologically-treated water, and effluent during the three sampling periods was independent of the chi-square test at a significance level of 5%, means that there is no clear correlation between prevalence at each stage of the wastewater treatment process.

The average prevalence rates of ESBL-producing coliforms in the influent, biologically-treated water, and effluent were 25.6 ± 3.3%, 4.0 ± 0.8%, and 11.4 ± 1.1%, respectively. This in the biological treatment process, the prevalence of ESBL-producing coliforms, as well as that of ESBL-producing *E. coli*, was found to be decreased. However, it is concerning that the prevalence of ESBL-producing coliforms in the effluent was still over 11%. Upon comparing the prevalence of ESBL-producing coliforms in biologically-treated water and the effluent, we found that the prevalence in the effluent was significantly higher in September and November ($p < 0.05$, t-test), whereas it was lower in effluent water in the June. This suggests that there may be a seasonal variation in the prevalence of ESBL-producing coliforms in the WWPT, which may be influenced by factors such as changes in water temperature, rainfall patterns, or other environmental variables. Furthermore, the lower prevalence of ESBL-producing coliforms in the effluent

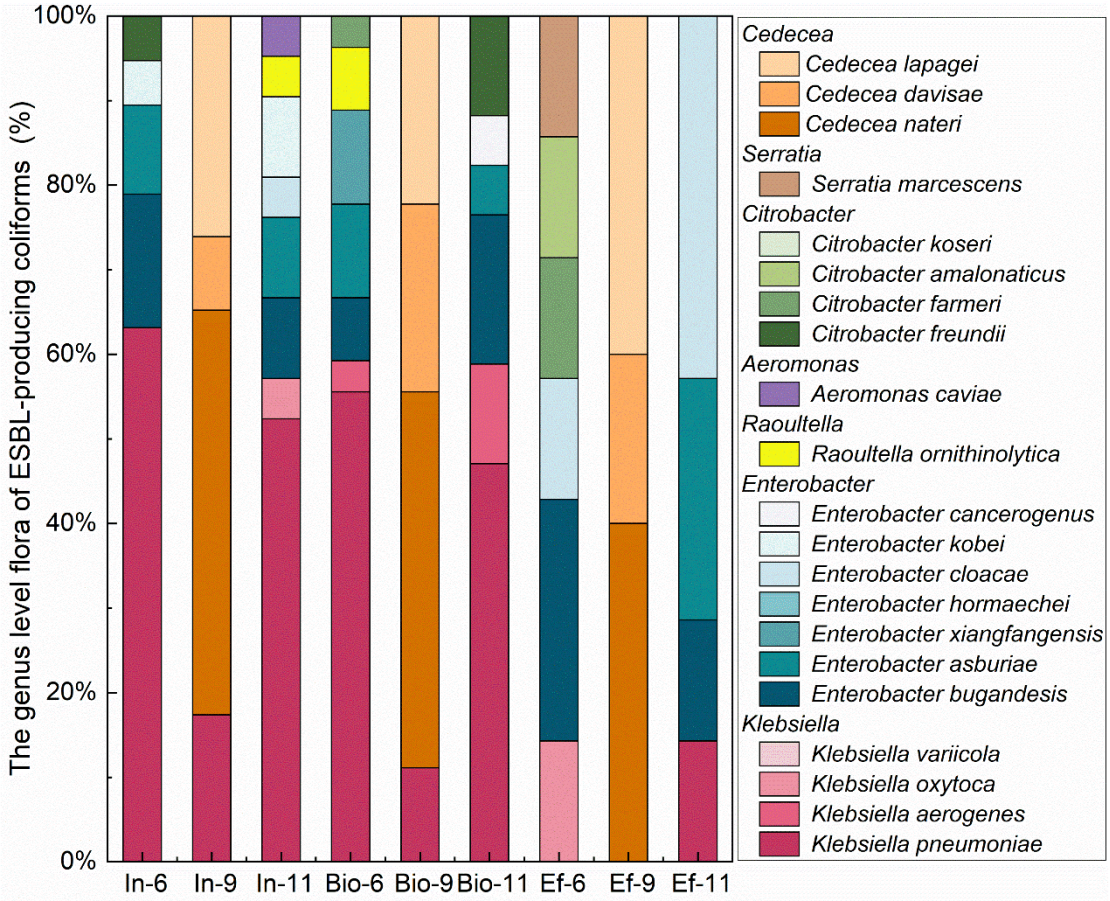

Figure 3.6 The prevalence of ESBL-resistant coliforms species from influent, biologically-treated water, and effluent samples of WWTPs in June, September, and November. In; influent, Bio; biologically-treated water, Ef; effluent

in June compared to biologically-treated water is encouraging and suggests that the wastewater treatment process may be more effective at removing these microorganisms during certain times of the year. However, more research is needed to identify the underlying reasons for this seasonal variation in prevalence rates. In addition, the fact that *Klebsiella* is highly resistant to chlorine disinfection, as previously reported [33, 34], may help explain the higher prevalence of ESBL-producing coliforms in the effluent during these months.

Figure 3.6 shows the microbiota of ESBL-producing coliforms in the influent, biologically-treated water, and effluent in June, September, and November. Seven genera and 19 species (*Klebsiella*, *Raoultella*, *Enterobacter*, *Citrobacter*, *Serratia*, *Cedecea,* and *Aeromonas*) were identified among the ESBL-producing coliforms. In common with all

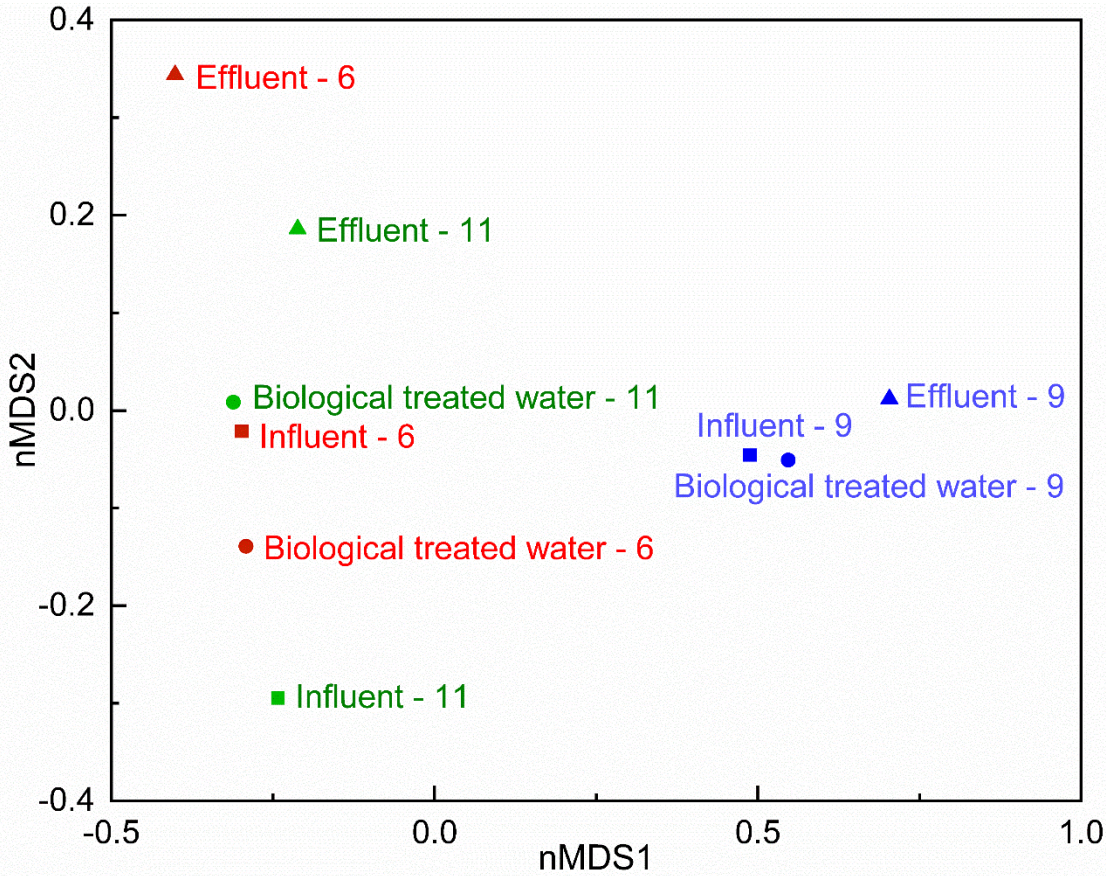

Figure 3.7 The nMDS analysis was performed to determine the similarity of the ESBL-producing coliforms at the species level between each sample by month and the wastewater treatment process.

samples from June and November, *Klebsiella* and *Enterobacter* were the dominant genera, with prevalence rates ranging from 11.1% to 63.2% and 29.4% to 85.7%, respectively. In contrast, *Cedecea* dominated the composition of the bacterial microbiota in September, unlike the other survey months, with a higher prevalence of *Cedecea* ranging from 82.6% to 100%. However, *Cedecea* was not detected in samples collected in June and November. These results suggest that the community of coliforms in wastewater varied widely over the six-month period. The nMDS analysis of the component species of the ESBL-producing coliforms showed that the plots of the effluent tended to be separated from those of the influent and biologically-treated water (**Figure. 3.7**), which is in line with the results of the nMDS analysis of the ABPC-resistant coliforms. This suggests that the coliforms community in the effluent is distinct from that of the influent and biologically-treated water and may include different species of bacteria that are more resistant to disinfection. The disinfection process does kill most of the bacteria, but as the number of bacteria decreases there is still a residue of resistant bacteria released into the receiving river. In reality, ESBL-producing coliforms such as *K. pneumoniae*, *E. asburiae*, *E. bugandesis*, *E. kobei*, *C. nateri*, *C. lapagei*, and *C. davisae* were discharged from the WWTP into the river. This is concerning because ESBL-producing bacteria are highly resistant to antibiotics and can cause serious infections in humans health. Moreover, disinfection with chlorine in WWTPs has been shown to promote the survival of ESBL-producing bacteria, as reported by Rolbiecki et al. [35]. This, coupled with the accumulation of antibiotics at the discharge of municipal wastewater, as reported in several studies [36, 37], raises the possibility that ESBL-producing genes could be transmitted to non-ESBL-producing bacteria in the environment. Therefore, it is crucial to track the behavior of ESBL-producing bacteria in the receiving water body to ensure that they do not pose a threat to public health or environmental safety.

3.3.6 Possession of ESBL-producing genes

ESBL-producing *E. coli* and coliforms were classified according to their ESBL-

Table 3.2 ESBL-producing *E. coli* and coliforms were classified according to their ESBL-producing genes.

Bacteria	Samples sites	Month	ESBL genetypes, identified strains, isolated strains (%)				
			TEM	SHV	CTX-M-1	CTX-M-2	CTX-M-9
E. coli	Influent	6	11/22 (50)	0/22 (0)	1/22 (5)	0/22 (0)	21/22 (95)
	Influent	9	4/10 (40)	6/10 (60)	3/10 (30)	0/10 (0)	7/10 (70)
	Influent	11	3/11 (27)	5/11 (45)	4/11 (36)	0/11 (0)	1/11 (9)
	Biological treated water	6	5/11 (45)	0/11 (0)	5/11 (45)	0/11 (0)	6/11 (55)
	Biological treated water	9	3/6 (50)	2/6 (33)	1/6 (17)	0/6 (0)	5/6 (83)
	Biological treated water	11	6/14 (43)	0/14 (0)	5/14 (36)	0/14 (0)	4/14 (29)
	Effluent	6	10/12 (83)	0/12 (0)	3/12 (25)	0/12 (0)	10/12 (83)
	Effluent	9	0/2 (50)	1/2 (50)	1/2 (50)	0/2 (50)	1/2 (50)
	Effluent	11	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Coliforms	Influent	6	14/19 (74)	6/19 (32)	7/19 (37)	8/19 (42)	5/19 (26)
	Influent	9	3/23 (13)	14/23 (61)	3/23 (13)	0/23 (0)	19/23 (83)
	Influent	11	4/21 (19)	1/21 (5)	7/21 (33)	0/21 (0)	19/21 (90)
	Biological treated water	6	18/27 (67)	8/27 (30)	11/27 (41)	10/27 (37)	2/27 (7)
	Biological treated water	9	0/9 (0)	8/9 (89)	0/9 (0)	0/9 (0)	9/9 (100)
	Biological treated water	11	0/17 (0)	2/17 (12)	0/17 (0)	1/17 (6)	0/17 (0)
	Effluent	6	4/7 (57)	1/7 (14)	3/7 (43)	1/7 (4)	0/27 (0)
	Effluent	9	0/5 (0)	3/5 (60)	0/5 (0)	0/5 (0)	3/5 (60)
	Effluent	11	0/7 (0)	3/7 (43)	0/7 (0)	1/7 (14)	0/7 (0)

producing genes (**Table 3.2**). The prevalence of ESBL-producing *E. coli* genotypes was as follows: TEM type: 47.2%, SHV type: 15.7%, CTX-M-1 type: 25.8%, CTX-M-2 type: 0% and CTX-M-9 type: 62.9% (Table S3-a). The combination of TEM and CTX-M-9 types was the most common. In June samples, CTX-M-9 was the most common type of *E. coli* in the influent (95.5%), followed by the TEM type (50.0%), and SHV and CTX-M-2 were not detected. In the September samples, CTX-M-9 was the most abundant, followed by SHV and TEM, and the total gene carriage also showed an increasing trend. In the November samples, ESBL gene carriage in *E. coli* decreased, and only CTX-M-9 was detected in the effluent. *E. coli* carrying multiple ESBL-producing genes, including the CTX-M-9 type, were identified in the samples. The prevalence of CTX-M-9 ESBL-producing *E. coli* in aquatic environments in Japan has been reported in previous studies [34, 38], which suggests that it is a common genotype in the country's water bodies. However, the absence of *E. coli* carrying the CTX-M-2 genotype in this study, which is frequently detected in Japan according to previous reports [39-42], suggests that the prevalence and diversity of ESBL-producing *E. coli* in aquatic environments in Japan may vary depending on several factors, including the location and time of sampling.

The frequency of occurrence of the ESBL genotype in ESBL-producing coliforms is

shown in **Figure. 8** as a heatmap. The TEM type (31.9%), SHV type (34.1%), CTX-M-1 type (23.0%), CTX-M-2 type (15.6%), and CTX-M-9 type (42.2%) constituted the genotypes of the ESBL-producing coliforms. The CTX-M-9 type was frequently detected in *Cedecea* (20.7%), *Klebsiella* (14.1%), and *Enterobacter* (6.7%) but not in *Citrobacter*. In the TEM type, *Klebsiella* (17.0%) and *Enterobacter* (10.4%) were the predominant strains, while no isolates of *Aeromonas* were detected. Of these, *K. pneumoniae* (15.6%) was the dominant species in the TEM type. In the SHV type, *Cedecea* (12.6%), *Klebsiella* (10.4%), and *Enterobacter* (6.0%) were dominant, while *Citrobacter*, *Raoultella,* and *Aeromonas* were not detected. Of these, *K. pneumoniae* (9.6%) and *C. nateri* (7.4%) were the dominant species of the SHV type. In the CTX-M-1 type, *Klebsiella* (11.1%) and *Enterobacter* (8.1%) were predominant, with *K. pneumoniae* being the most dominant in both CTX-M-1 and CTX-M-2 types. It was found that the major bacterial species that composed the coliforms in the samples corrected at the WWTPs carried important ESBL-producing genes.

3.4 Conclusion

This study aimed to investigate the prevalence of ESBL-producing *E. coli* and coliforms during the wastewater treatment process in a WWTP. The results showed that although the chlorination process reduced the number of *E. coli* and coliforms, some ESBL-producing *E. coli* and coliforms were still released into the environment through the effluent. The changes in prevalence of ESBL- producing *E. coli* compared to the total *E. coli* counts in the wastewater treatment process showed that the average prevalence was 4.5% in the influent, 1.9% in biologically-treated water, and 5.6% in the effluent. It is noteworthy that opportunistic pathogens such as *Klebsiella* and *Enterobacter* carrying ESBL-producing genes were detected in the effluent. Further monitoring of ESBL-producing bacteria in the effluent is necessary to identify their transmission pathways and prevent the spread of resistance in the *Enterobacteriaceae* family, which can pose a risk to human health.

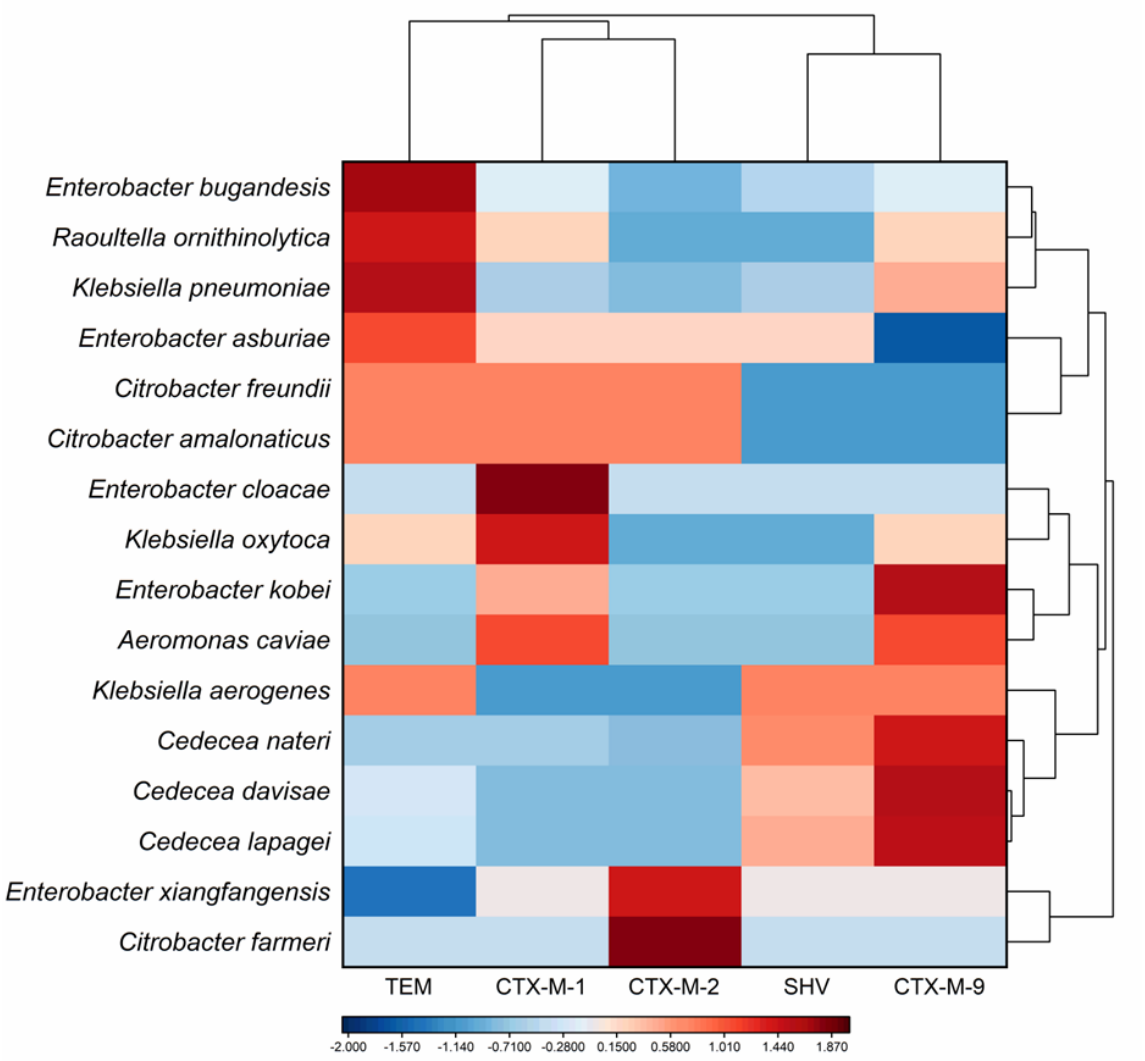

Figure 3.8 Heatmap of ESBL-producing coliforms from influent, biologically-treated water, and effluent samples showing hierarchical clustering of species and factors. Colors represent Z-scores calculated from the relative abundance of the ESBL genes above 3% in all classifications. Binary factors (ESBL-producing genes) are indicated in red for their presence (relative response of 1) or in blue for their absence (relative response of 0).

Reference

1. David AW, Timothy MC, John D. Antimicrobial chemotherapy. 5th ed. Oxford: Oxford Academic; 2010.
2. Prestinaci, F., Pezzotti, P., & Pantosti, A. (2015). Antimicrobial resistance: a global multifaceted phenomenon. Pathogens and global health, 109(7), 309–318. https://doi.org/10.1179/2047773215Y.0000000030
3. Antimicrobial Resistance Collaborators (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet (London, England), 399(10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0
4. O'Neill, J. (2016). Review on antimicrobial resistance. Antimicrobial resistance: tackling a crisis for the health and wealth of nations, 1-20. https://wellcomecollection.org/works/rdpck35v.
5. Ak, O., Batirel, A., Ozer, S., & Çolakoğlu, S. (2011). Nosocomial infections and risk factors in the intensive care unit of a teaching and research hospital: a prospective cohort study. Medical science monitor : international medical journal of experimental and clinical research, 17(5), PH29–PH34. https://doi.org/10.12659/msm.881750
6. Azargun, R., Sadeghi, M. R., Soroush Barhaghi, M. H., Samadi Kafil, H., Yeganeh, F., Ahangar Oskouee, M., & Ghotaslou, R. (2018). The prevalence of plasmid-mediated quinolone resistance and ESBL-production in *Enterobacteriaceae* isolated from urinary tract infections. Infection and drug resistance, 11, 1007–1014. https://doi.org/10.2147/IDR.S160720
7. Meyer, E., Schwab, F., Schroeren-Boersch, B., & Gastmeier, P. (2010). Dramatic increase of third-generation cephalosporin-resistant *E. coli* in German intensive care units: secular trends in antibiotic drug use and bacterial resistance, 2001 to 2008. Critical care (London, England), 14(3), R113. https://doi.org/10.1186/cc9062
8. Dancer S. J. (2001). The problem with cephalosporins. The Journal of antimicrobial chemotherapy, 48(4), 463–478. https://doi.org/10.1093/jac/48.4.463
9. Rosenberg Goldstein, R. E., Micallef, S. A., Gibbs, S. G., George, A., Claye, E.,

Sapkota, A., Joseph, S. W., & Sapkota, A. R. (2014). Detection of vancomycin-resistant enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. The Science of the total environment, 466-467, 404–411. https://doi.org/10.1016/j.scitotenv.2013.07.039

10. Bonnet R. (2004). Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrobial agents and chemotherapy, 48(1), 1–14. https://doi.org/10.1128/AAC.48.1.1-14.2004
11. Rossolini, G. M., D'Andrea, M. M., & Mugnaioli, C. (2008). The spread of CTX-M-type extended-spectrum beta-lactamases. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 14 Suppl 1, 33–41. https://doi.org/10.1111/j.1469-0691.2007.01867.x
12. Cantón, R., González-Alba, J. M., & Galán, J. C. (2012). CTX-M Enzymes: Origin and Diffusion. Frontiers in microbiology, 3, 110. https://doi.org/10.3389/fmicb.2012.00110
13. Wei R, Ge F, Huang S, Chen M, Wang R (2011) Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Jiangsu Province, China. Chemosphere l82,1408-1414. https://doi.org/10.1016/j.chemosphere.2010.11.067
14. Marti, R., Scott, A., Tien, Y. C., Murray, R., Sabourin, L., Zhang, Y., & Topp, E. (2013). Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Applied and environmental microbiology, 79(18), 5701–5709. https://doi.org/10.1128/AEM.01682-13
15. Börjesson, S., Ny, S., Egervärn, M., Bergström, J., Rosengren, Å., Englund, S., Löfmark, S., & Byfors, S. (2016). Limited Dissemination of extended-spectrum β-lactamase- and plasmid-encoded AmpC-producing *Escherichia coli* from food and farm animals, Sweden. Emerging infectious diseases, 22(4), 634–640. https://doi.org/10.3201/eid2204.151142
16. Baba, H., Nishiyama, M., Watanabe, T., & Kanamori, H. (2022). Review of antimicrobial resistance in wastewater in Japan: current challenges and future

perspectives. Antibiotics (Basel, Switzerland), 11(7), 849. https://doi.org/10.3390/antibiotics11070849

17. Qiao, M., Ying, G. G., Singer, A. C., & Zhu, Y. G. (2018). Review of antibiotic resistance in China and its environment. Environment international, 110, 160–172. https://doi.org/10.1016/j.envint.2017.10.016
18. Mukherjee, M., Laird, E., Gentry, T. J., Brooks, J. P., & Karthikeyan, R. (2021). Increased antimicrobial and multidrug resistance downstream of wastewater treatment plants in an urban watershed. Frontiers in microbiology, 12, 657353. https://doi.org/10.3389/fmicb.2021.657353
19. Barancheshme F, Munir M (2019) Development of antibiotic resistance in wastewater treatment plants. In: Kumar Y (ed) antimicrobial resistance-a global threat. IntechOpen, London. https://doi.org/10.5772/intechopen.81538
20. Kutilova, I., Medvecky, M., Leekitcharoenphon, P., Munk, P., Masarikova, M., Davidova-Gerzova, L., Jamborova, I., Bortolaia, V., Pamp, S. J., & Dolejska, M. (2021). Extended-spectrum beta-lactamase-producing *Escherichia coli* and antimicrobial resistance in municipal and hospital wastewaters in Czech Republic: Culture-based and metagenomic approaches. Environmental research, 193, 110487. https://doi.org/10.1016/j.envres.2020.110487
21. Gomi, R., Matsuda, T., Matsumura, Y., Yamamoto, M., Tanaka, M., Ichiyama, S., & Yoneda, M. (2017). Occurrence of clinically important lineages, including the sequence type 131 C1-M27 subclone, among extended-spectrum-β-lactamase-producing *Escherichia coli* in wastewater. Antimicrobial agents and chemotherapy, 61(9), e00564-17. https://doi.org/10.1128/AAC.00564-17
22. Clinical and Laboratory Standards Institute (CLSI) (2017) Performance standards for antimicrobial susceptibility testing. M100-27th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute. https://clsi.org/standards/products/microbiology/documents/m100/
23. Suzuki, Y., Niina, K., Matsuwaki, T., Nukazawa, K., & Iguchi, A. (2018). Bacterial flora analysis of coliforms in sewage, river water, and ground water using MALDI-TOF mass spectrometry. Journal of environmental science and health. Part A,

Toxic/hazardous substances & environmental engineering, 53(2), 160–173. https://doi.org/10.1080/10934529.2017.1383128

24. Xie, H., Ogura, Y., & Suzuki, Y. (2022). Persistence of Antibiotic-Resistant *Escherichia coli* Strains Belonging to the B2 Phylogroup in Municipal Wastewater under Aerobic Conditions. Antibiotics (Basel, Switzerland), 11(2), 202. https://doi.org/10.3390/antibiotics11020202
25. Shibata, N., Kurokawa, H., Doi, Y., Yagi, T., Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Ishikawa, S., Kato, H., Ozawa, Y., Shibayama, K., Kai, K., Konda, T., & Arakawa, Y. (2006). PCR classification of CTX-M-type beta-lactamase genes identified in clinically isolated gram-negative bacilli in Japan. Antimicrobial agents and chemotherapy, 50(2), 791–795. https://doi.org/10.1128/AAC.50.2.791-795.2006
26. Minogue, T. D., Daligault, H. A., Davenport, K. W., Bishop-Lilly, K. A., Broomall, S. M., Bruce, D. C., Chain, P. S., Chertkov, O., Coyne, S. R., Freitas, T., Frey, K. G., Gibbons, H. S., Jaissle, J., Redden, C. L., Rosenzweig, C. N., Xu, Y., & Johnson, S. L. (2014) Complete genome assembly of *Escherichia coli* ATCC 25922, a Serotype O6 reference strain. Genome announcements, 2: e00969-14. https://doi.org/10.1128/genomeA.00969-14
27. Proia, L., von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C. M., Rodríguez-Mozaz, S., & Balcázar, J. L. (2016). Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. Environmental pollution (Barking, Essex : 1987), 210, 121–128. https://doi.org/10.1016/j.envpol.2015.11.035
28. Rolbiecki, D., Harnisz, M., Korzeniewska, E., Buta, M., Hubeny, J., & Zieliński, W. (2021). Detection of carbapenemase-producing, hypervirulent *Klebsiella spp.* in wastewater and their potential transmission to river water and WWTP employees. International journal of hygiene and environmental health, 237, 113831. https://doi.org/10.1016/j.ijheh.2021.113831
29. European Centre for Disease Prevention and Control (2019) Carbapenem-resistant *Enterobacteriaceae,* Second Update. ECDC: Stockholm.

https://www.ecdc.europa.eu/en/publications-data/carbapenem-resistant-enterobacteriaceae-second-update

30. Savin, M., Bierbaum, G., Mutters, N. T., Schmithausen, R. M., Kreyenschmidt, J., García-Meniño, I., Schmoger, S., Käsbohrer, A., & Hammerl, J. A. (2022). Genetic characterization of carbapenem-resistant *klebsiella spp.* from municipal and slaughterhouse wastewater. Antibiotics (Basel, Switzerland), 11(4), 435. https://doi.org/10.3390/antibiotics11040435
31. Logan, L. K., & Weinstein, R. A. (2017). The epidemiology of carbapenem-resistant *Enterobacteriaceae*: the impact and evolution of a global menace. The Journal of infectious diseases, 215(suppl_1), S28–S36. https://doi.org/10.1093/infdis/jiw282
32. Stanish, L. F., Hull, N. M., Robertson, C. E., Harris, J. K., Stevens, M. J., Spear, J. R., & Pace, N. R. (2016). Factors influencing bacterial diversity and community composition in municipal drinking waters in the Ohio River basin, USA. PloS one, 11(6), e0157966. https://doi.org/10.1371/journal.pone.0157966
33. Yu D, Ryu K, Otto SJG, Stothard P, Banting G, Ruecker N, Neumann FN, Zhi S (2022) Differential survival of potentially pathogenic, septicemia- and meningitis-causing *E. coli* across the wastewater treatment train. npj Clean Water 5:3. https://doi.org/10.1038/s41545-022-00177-y
34. Popa, L. I., Gheorghe, I., Barbu, I. C., Surleac, M., Paraschiv, S., Măruţescu, L., Popa, M., Pîrcălăbioru, G. G., Talapan, D., Niţă, M., Streinu-Cercel, A., Streinu-Cercel, A., Oţelea, D., & Chifiriuc, M. C. (2021). Multidrug resistant *Klebsiella pneumoniae* st101 clone survival chain from inpatients to hospital effluent after chlorine treatment. frontiers in microbiology, 11, 610296. https://doi.org/10.3389/fmicb.2020.610296
35. Rolbiecki, D., Korzeniewska, E., Czatzkowska, M., & Harnisz, M. (2022). The impact of chlorine disinfection of hospital wastewater on clonal similarity and ESBL-production in selected bacteria of the family *Enterobacteriaceae*. International journal of environmental research and public health, 19(21), 13868. https://doi.org/10.3390/ijerph192113868
36. Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Cahill, J. D., Zaugg, S. D., Werner,

S. L., Meyer, M. T., & Kryak, D. D. (2005). Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. Environmental science & technology, 39(14), 5157–5169. https://doi.org/10.1021/es048120k

37. Metcalfe, C. D., Chu, S., Judt, C., Li, H., Oakes, K. D., Servos, M. R., & Andrews, D. M. (2010). Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environmental toxicology and chemistry, 29(1), 79–89. https://doi.org/10.1002/etc.27
38. Azuma, T., Uchiyama, T., Zhang, D., Usui, M., & Hayashi, T. (2022). Distribution and characteristics of carbapenem-resistant and extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* in hospital effluents, sewage treatment plants, and river water in an urban area of Japan. The Science of the total environment, 839, 156232. https://doi.org/10.1016/j.scitotenv.2022.156232
39. Kojima, A., Ishii, Y., Ishihara, K., Esaki, H., Asai, T., Oda, C., Tamura, Y., Takahashi, T., & Yamaguchi, K. (2005). Extended-spectrum-beta-lactamase-producing *Escherichia coli* strains isolated from farm animals from 1999 to 2002: report from the Japanese Veterinary Antimicrobial Resistance Monitoring Program. Antimicrobial agents and chemotherapy, 49(8), 3533–3537. https://doi.org/10.1128/AAC.49.8.3533-3537.2005
40. Shiraki, Y., Shibata, N., Doi, Y., & Arakawa, Y. (2004). *Escherichia coli* producing CTX-M-2 beta-lactamase in cattle, Japan. Emerging infectious diseases, 10(1), 69–75. https://doi.org/10.3201/eid1001.030219
41. Yagi, T., Kurokawa, H., Shibata, N., Shibayama, K., & Arakawa, Y. (2000). A preliminary survey of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS microbiology letters, 184(1), 53–56. https://doi.org/10.1111/j.1574-6968.2000.tb08989.x
42. Gomi, R., Yamamoto, M., Tanaka, M., & Matsumura, Y. (2022). Chromosomal integration of blaCTX-M genes in diverse *Escherichia coli* isolates recovered from river water in Japan. Current research in microbial sciences, 3, 100144. https://doi.org/10.1016/j.crmicr.2022.100144

Chapter 4. Transmission of antibiotic resistance genes from ESBL-producing *E. coli* to Shiga toxin-producing *E. coli*

4.1 Introduction

While most strains of *E. coli* are harmless, certain pathogenic strains can cause symptoms such as diarrhea in humans [1]. These strains are highly infectious and toxic, and there have been reports of food poisoning and waterborne infections caused by them, not only in developing countries but also in developed countries [2, 3]. Therefore, the WHO has listed pathogenic *E. coli* as one of the major causes of water-borne infections [4]. Pathogenic *E. coli*, also known as diarrheagenic *E. coli*, can be classified into several types, including Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), Enteroaggregative *E. coli* (EAEC), and Diffusely Adherent *E. coli* (DAEC) [5, 6, 7]. Among these categories, STEC is particularly dangerous, as it can cause enteritis accompanied by bleeding and hemolytic uremic syndrome (HUS) [1, 3, 8]. Infections caused by STEC have become a significant public health concern worldwide, with the WHO estimating that over 1 million people contract STEC each year, resulting in over 100 deaths [8]. Pathogenic *E. coli* strains can spread through rivers and drainage canals, contaminating water sources that are used for recreation, drinking, and irrigation. In developing countries, there have been reports of STEC present in river water that is still used as a source of drinking water [9]. This poses an urgent public health issue that needs to be addressed. It has been reported that *E. coli* strains with acquired resistance to antibiotics, which are commonly used to treat bacterial infections, are spreading in rivers [10, 11]. This is a serious issue as it indicates the presence of drug-resistant pathogenic *E. coli* strains such as STEC. Therefore, the existence of STEC that has acquired AR cannot be denied.

As introduced in the previous chapters, ESBL-producing bacteria, which are regarded as major contributors to nosocomial infections, have become the most critical challenge. According to the conclusions in Chapter 3, although the chlorination process reduced the number of *E. coli*, some ESBL-producing *E. coli* were still released into the environment

through the effluent. This raise concerns that the ARGs carried by these bacteria may be transferred to other pathogenic strains, such as STEC, which can be present in the river water. Horizontal gene transfer, particularly via conjugation, is a well-documented method for the spread of ARGs, including those encoding ESBLs [12]. The success of conjugation is dependent on several factors, including the compatibility of the mating pair and the presence of restriction/ modification systems in the recipient cell [13].

This study aims to investigate the spread of drug resistance from ESBL-producing *E. coli*, which has been isolated from treated wastewater, to STEC in rivers. Specifically, the study will analyze the acquisition and transmission of resistance by *E. coli* in the river water environment and quantify the rate of water-borne transmission of AR.

4.2 Materials and Methods

4.2.1 Sampling and isolation of strain

Chapter 3 outlines the procedure used to isolate *E. coli* from chlorinated effluent obtained from a WWTP. And the STEC positive strains were collected from river water. River samples were collected and processed using the flocculation/foam concentration method and selective agar media. This enabled the recovery of STEC, even at very low concentrations in the river water. After foam concentration, the water was centrifuged for 1 min at 4,000 rpm using a desktop centrifuge (H-36, KOKUSAN). The supernatant was then removed, and the precipitate was suspended in 1 mL of sterilized physiological saline. The suspended mixture was spread onto CHROMagar STEC (CHROMagar, France) medium, a selective medium for STEC, using a conlarge stick (SFC-1000, AS-ONE) to ensure even spreading. The plates were then incubated at 37±0.5°C for 24 hours. The purple-red positive colonies formed on the medium were subsequently counted. The STEC isolates were enumerated using. Formed colonies were isolated as STEC-positive strains by streaking on BHI agar medium (agar 1.5%, BD). A total of 10 strains of *E. coli* and 30 strains of STEC were isolated, respectively, from the treated wastewater and river

water samples.

4.2.2 Bacterial identification by MALDI-TOF MS

The method is the same as the one used in previous publications [14, 15]. Positive isolates of bacteria were incubated at 37 ± 0.5 °C for 18 hours. Samples were spread directly into 384-well stainless-steel target plates (MTP 384; Bruker Daltonics, Billerica, MA, USA) and then covered with 1.0 ml of matrix solution. All samples were analyzed for the probability of correct isolates using the autoflex® III TOF/TOF system (Bruker Daltonics) and the MALDI Biotyper Compass microbial identification system (Bruker Daltonics, version 4.1.60.2) per the manufacturer's instructions. *E. coli* and coliforms were identified by a logarithmic score of >1.700.

4.2.3 Antimicrobial susceptibility testing

The identified strains were subjected to the agar plate dilution method to determine their MIC against 13 antibiotics in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The isolates were cultured at 37°C for 18 h in MH broth (Becton, Dickinson and Company, Sparks, MD, USA) and then diluted to a final concentration corresponding to the 0.5 McFarland turbidity standard with fresh MH broth. The isolates were then inoculated on the surface of 1.5% MH agar containing graded concentrations of each antimicrobial in the wells of a microplate (Sakuma Co., Tokyo, Japan). Following incubation of the plates at 37°C for 18 h, the MICs were determined. MIC breakpoints for resistance were based on the CLSI criteria.

The antimicrobials used in the current study included ampicillin (ABPC; graded concentrations of 4–64 μg/mL), gentamicin (GEN; 2–32 μg/mL), cefazolin (CFZ; 1–16 μg/mL), cefotaxime (CTX; 0.5–8 μg/mL), ceftazidime (CAZ; 2–32 μg/mL), tetracycline (TC; 2–32 μg/mL), imipenem (IMP), ciprofloxacin (CIP; 0.5–8 μg/mL), cefepime (CPM; 4–64 μg/mL) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), Amoxicillin (AMO; 4–64 μg/mL), Meropenem (MEP; 0.5–8 μg/mL), Nalidixic acid (NA; 4–64 μg/mL),

(RFP) and chloramphenicol (CHL; 4–64 µg/mL) (Sigma-Aldrich Corporation, St. Louis, MO, USA). Each of the tested agents was dissolved in distilled water or other appropriate solvents in accordance with the CLSI recommendations. The *E. coli* reference strain ATCC 25922 was used for quality control.

4.2.4 ESBL confirmation test

Confirmation tests were performed on positive isolates after screening for ESBL-producing bacteria. The confirmation test was determined by the inhibition circle for ceftazidime (CAZ) (30 mg, Becton Dickinson, Tokyo, Japan), ceftazidime/clavulanic acid (CAZ/CVA) (30 mg/10 mg, Eiken Chemical, Tokyo, Japan), cefotaxime (CTX) (30 mg, Becton Dickinson, Tokyo, Japan), and cefotaxime/clavulanic acid (CTX/CVA) (30 mg/10 mg, Eiken Chemical, Tokyo, Japan) per the disk diffusion method (CLSI, 2017). The positive strains screened with ESBL-selective plates were pre-cultured on the BHI agar medium, and the bacterial concentration was adjusted to the Macfarlane 0.5 standard as described above. Then, the adjusted bacterial solution was applied to Mueller Hinton (Becton Dickinson, Tokyo, Japan) agar medium within 15 minutes. Discs of four antibiotics (CAZ, CAZ/CVA, CTX, and CTX/CVA) were stamped on an agar medium using a Sensi-Disc dispenser (Becton Dickinson, Tokyo, Japan). After incubation at 37 ± 0.5°C for 18–24 hours, ESBL-producing bacteria were identified by an inhibition circle with a minimum diameter of 5 mm in CAZ and CAZ/CVA or CTX and CTX/CVA.

4.2.5 ESBL genotypes of *E. coli* by multiplex PCR analysis

DNA samples were extracted from ABPC-resistant *E. coli* and ABPC-resistant coliforms isolates using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA). The five ESBL genotypes (TEM, SHV, CTX-M-1, CTX-M-2, CTX-M-9) were determined according to the multiplex PCR technique [17].

The primers for all the genes. Each 50-µl reaction consisted of 29.75 µl of sterilized

distilled water, 0.25 µl of Takara Taq HS (Takara Bio Inc., Shiga, Japan), 5 µl of 10 × KAPA Extra Buffer (Takara Bio Inc., Shiga, Japan), 5 µl of deoxynucleoside triphosphate Mix (dNTP Mix) (Takara Bio Inc., Shiga, Japan), 1 µl of each primer, and 1 µl of the DNA template. The PCR reaction was performed using a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the following reaction conditions: denaturation at 94°C for 2 minutes, followed by 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 90 seconds, and a final extension step at 72°C for 5 minutes. After the PCR reaction, 5 µl of the PCR product and 1 µl of 6 × loading buffer (Takara Bio, Inc., Shiga, Japan) were mixed, loaded into the wells of 2% agarose gels, and separated using the Mupid®-One electrophoresis system (Nippon Genetics Co., Ltd., Tokyo, Japan) at 100 V for 40 minutes. After electrophoresis, the agarose gel was stained with ethidium bromide solution (0.05 µl/ml) for 10 minutes and then shaken in distilled water for 10 minutes to confirm the PCR amplification products. The *E. coli* strain, ATCC 25922, was used as a positive control for the PCR reaction.

4.2.6 STEC genotypes of *E. coli* by multiplex PCR analysis

The virulence genes targeted in STEC are *stx1* and *stx2*, which are associated with the production of Shiga toxin, and *eae*, which is involved in the production of intimin [18]. Intimin is deposited on the intestinal surface and is known to cause severe symptoms. The analysis targets three types of virulence genes. DNA samples were extracted from STEC isolates using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA). The primers used for the analysis of *stx1* and *stx2* genes were prepared as per Cebula et al.[19],

Table 4.1 Design of primers sequence for detection of STEC genotypes.

STEC genotypes	Primer sequences	PCR product (bp)
stx1	F: 5'-CAGTTAATGTGGTGGCGAAGG-3' R: 5'-CACCAGACAATGTAACCGCTG-3'	348
stx2	F: 5'-ATCCTATTCCCGGGAGTTTACG-3' R: 5'-GCGTCATCGTATACACAGGAGC-3'	584
eae	F: 5'-CCCGAATTCGGCACAAGCATAAGC-3' R: 5'-CCCGGATCCGTCTCGCCAGTATTCG-3'	881

while the *eae* gene primers were prepared according to Oswald et al.[20]. The primers for all the genes shown in **Table 4.1**.

Each 30-µl reaction consisted of grade water, 6 µl of 5 ×KAPATaq EXtra Buffer (Mg2+ Free) (Takara Bio Inc., Shiga, Japan), 3 µl of $MgCl_2$ (25 mM) (Takara Bio Inc., Shiga, Japan), 0.9 µl of KAPA dNTP Mix (10 mM) (Takara Bio Inc., Shiga, Japan), 0.16 µl of KAPATaq EXtra DNA polymerase (5 U/µL), primer (Forward and Reverse), and 2 µl of the DNA template. Primers were adjusted to final concentrations of 0.13 µM for *stx1* and *stx2* genes, and 0.27 µM for *eae* and *uidA* genes, respectively. The PCR reaction was performed using a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the following reaction conditions: denaturation at 94°C for 1 minutes, followed by 25 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, and a final extension step at 72°C for 2 minutes. After the PCR reaction, 5 µl of the PCR product and 1 µl of 6 × loading buffer (Takara Bio, Inc., Shiga, Japan) were mixed, loaded into the wells of 2% agarose gels, and separated using the Mupid®-One electrophoresis system (Nippon Genetics Co., Ltd., Tokyo, Japan) at 100 V for 40 minutes.

4.2.7 Conjugation experiment by filter matching

ESBL-producing *E. coli* was confirmed based on ESBL confirmation test and the presence of ESBL-related genes. Six strains of ESBL-producing *E. coli* from the treated wastewater were chosen as donor strains. The STEC-specific genes were identified in STEC-positive strains isolated from river water using the *Enterohemorrhagic E. coli test and Diagnosis Manual* from the National Institute of Infectious Diseases [21]. Furthermore, STEC strains were selected and applied to MacConkey medium containing 50 µg/ml rifampicin (RFP). The STEC strains that became resistant to RFP were classified as RFP-resistant STEC strains, and three strains (STEC 63, STEC 4, and STEC 9) were recovered. These three RFP-resistant STEC strains were then used as recipient strains.

The mating process involved combining 100 µl of the donor and recipient strains, which had been cultured in Luria-Bertani (LB) liquid medium, with 10 ml of

physiological saline. The mixture was then filtered through a membrane filter using suction to separate the cells from the growth medium. The filtered cells were applied onto LB medium and incubated at 37±0.5°C for 24 hours to allow for mating to occur. After 24 hours of incubation, the resulting filters were suspended in 30 ml of physiological saline and screened on MacConkey medium containing both 32 µg/ml ABPC and 25 µg/ml RFP. The use of both antibiotics was to select for cells that had acquired the plasmid containing the resistance genes for both antibiotics. The number of colonies that grew on the MacConkey medium was used to determine the level of AR expressed. The donor and recipient strains were enumerated prior to propagation onto MacConkey medium containing 32 µg/ml ABPC and 25 µg/ml RFP, respectively. The growth of colonies on the MacConkey medium indicated successful conjugation of plasmids carrying ARGs from the donor to the recipient strain. The conjugation frequency refers to the ratio of transconjugant bacteria, which are the recipient bacteria that have acquired the plasmid containing the resistance genes from the donor strain to the total number of recipient bacteria. Mathematically, it is calculated as the number of transconjugant bacteria divided by the number of recipient bacteria.

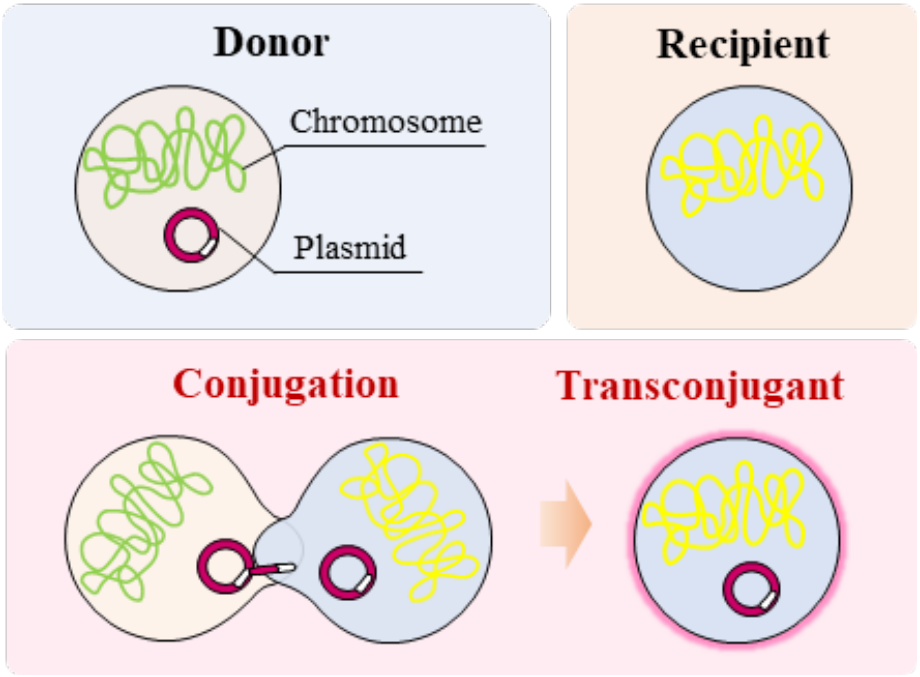

Figure 4.1 Conjugation process occurs in two bacteria strain.

4.3 Results and discussion

4.3.1 Resistant profile of donor strains and recipient strains

The six ESBL-producing *E. coli* bacteria were used as donor bacteria in this study. The resistance of these bacteria to different antibiotics was indicated in **Table 4.2**. It was observed that all six donor bacteria were resistant to ABPC, CFZ, CTX, CIP, CPM, AMO, and NA antibiotics. Furthermore, all of them carried the CTX-M-9 ESBL-producing gene, which is one of the most common genes associated with ESBL production in *Enterobacteriaceae* [22, 23]. In addition to the CTX-M-9 gene, the second *E. coli* donor bacterium was found to carry an extra TEM ESBL-producing gene. The TEM gene is another common type of ESBL gene, which is often found in combination with CTX-M genes in ESBL-producing bacteria [24].

Table 4.2 Resistant profile of donor strains and recipient strains.

NO.	Strain	ABPC	GM	CFZ	CTX	CAZ	TC	IPM	CIP	CHL	CPM	AMO	MEP	NA	RFP	Genotypes
1	ESBL- *E. coli*	R	S	R	R	S	S	S	R	S	R	R	S	R	S	***CTX-M-9***
2	ESBL- *E. coli*	R	S	R	R	S	S	S	R	S	R	R	S	R	S	***CTX-M-9 TEM***
3	ESBL- *E. coli*	R	S	R	R	S	S	S	R	S	R	R	S	R	S	***CTX-M-9***
4	ESBL- *E. coli*	R	S	R	R	I	S	S	R	S	R	R	S	R	S	***CTX-M-9***
5	ESBL- *E. coli*	R	S	R	R	S	S	S	R	S	R	R	S	R	S	***CTX-M-9***
6	ESBL- *E. coli*	R	S	R	R	S	S	S	R	S	R	R	S	R	S	***CTX-M-9***

NO.	Strain	ABPC	GM	CFZ	CTX	CAZ	TC	IPM	CIP	CHL	CPM	AMO	MEP	NA	RFP	Genotypes
11	STEC- 63	S	S	S	S	S	S	S	S	S	S	S	S	S	R	***stx2***
12	STEC- 4	S	S	S	S	S	S	S	S	S	S	S	S	S	R	***stx2***
22	STEC- 9	S	S	S	S	S	R	S	S	S	S	S	S	S	R	***stx2***

4.3.2 The rate of transmission

In this study, the rate of transmission of AR on the filter was measured using a filter mating method. The bacterial conjugation experiments carried out on supplemented media containing both ABPC and RFP revealed the presence of positive colonies of *E. coli*. This suggests that the transfer of ABPC resistance from the ESBL-producing *E. coli*

Table 4.3 Transmission rates of resistance from donor strains to recipient strains.

Donor strains	Recipient strains					
	STEC 63		STEC 4		STEC 9	
	Trans-/ Donor	Trans-/ Recipient	Trans-/ Donor	Trans-/ Recipient	Trans-/ Donor	Trans-/ Recipient
ESBL 1	2.15×10^{-6}	9.77×10^{-7}	2.67×10^{-6}	4.71×10^{-7}	6.06×10^{-7}	3.23×10^{-7}
ESBL 2	1.02×10^{-5}	1.18×10^{-5}	4.23×10^{-3}	1.10×10^{-3}	5.00×10^{-4}	4.76×10^{-4}
ESBL 3	1.76×10^{-6}	2.05×10^{-6}	1.43×10^{-6}	3.70×10^{-7}	3.00×10^{-6}	2.86×10^{-6}
ESBL 4	1.08×10^{-6}	9.14×10^{-7}	2.23×10^{-5}	3.63×10^{-7}	2.59×10^{-7}	4.96×10^{-7}
ESBL 5	8.40×10^{-7}	6.00×10^{-7}	3.12×10^{-5}	1.16×10^{-6}	4.84×10^{-7}	1.91×10^{-6}
ESBL 6	8.90×10^{-6}	6.07×10^{-6}	8.57×10^{-5}	4.08×10^{-6}	4.29×10^{-6}	1.96×10^{-5}

donor strain to the STEC recipient strain is possible. **Table 4.3** displays the transmission rates of ABPC resistance from ESBL-producing *E. coli* donor strains to STEC recipient strains. The rate of transmission on the filter was found to range from 1.10×10^{-3} to 9.77×10^{-7}. The transmission of AR through plasmids can occur at a relatively low frequency. Interestingly, the same donor strain exhibited different resistance transmission rates when tested against different recipient strains. However, it is noteworthy that there were slight variations in transmission rates even when the same combination of strains was tested between the donor and recipient strains. This indicates the need for further investigation and monitoring of transmission dynamics between different bacterial strains. The results showed that the rate of transmission varied depending on the combination of donor and recipient strains. After screening trans-conjugation bacteria that showed five of the six donor bacteria will successfully transfer the ESBL-associated gene CTX-M-9 into recipient after filter mating experiment (**Table 4.4**). The results of the conjugation experiment showed that the donor strain ESBL 2 was resistant to both ABPC and RIP antibiotics but failed to transfer the ESBL genes to recipient strains. Interestingly, the donor strains ESBL 1 and 3 were able to successfully transmit the CTX-M-9 gene to some recipients, but not to others, indicating that the conjugation process is influenced by many factors. The plasmid carrying the antibiotic resistance gene may have been lost during bacterial isolation or not successfully integrated into the recipient bacteria during the conjugation process, either due to exposure to external stresses or internal degradation

mechanisms. Alternatively, the recipient bacteria may have been unable to take up the plasmid due to a lack of competence or a barrier to uptake. The success rate of plasmid transfer can depend on various factors, such as the genetic relatedness between the donor and recipient strains, the availability of suitable receptors on the recipient's surface, and the presence of restriction-modification systems in either strain that could prevent the transfer of foreign DNA. The observed differences in transmission rates highlight the complexity of AR transmission and the need for a better understanding of the mechanisms underlying this process. Several studies have reported that ESBL-producing *E. coli* carrying the CTX-M gene can transfer their resistance to other bacteria through horizontal gene transfer mechanisms, such as conjugation [25-28]. The transmission of AR through plasmids is a major factor contributing to the spread of AR in bacteria [29]. HGT is the main mechanism for the production and spread of ARGs and ARB in the environment. Plasmids conferring multidrug resistance (MDR) are often conjugative, meaning that they are capable of initiating their own transfer from one bacterial cell to another through direct cell-to-cell contact [30]. In addition to their own transfer, these plasmids can also promote the transfer of other plasmids, further increasing the potential for horizontal transfer of ARGs. Further research is required to investigate the factors that influence the transmission of AR, including the role of plasmids in conjugation.

Table 4.4 ESBL-producing gene detected from recipient strains.

Donor strains	Recipient strains		
	STEC 63	STEC 4	STEC 9
ESBL 1	CTX-M-9	CTX-M-9	×
ESBL 2	×	×	×
ESBL 3	×	CTX-M-9	CTX-M-9
ESBL 4	CTX-M-9	CTX-M-9	CTX-M-9
ESBL 5	CTX-M-9	CTX-M-9	CTX-M-9
ESBL 6	CTX-M-9	CTX-M-9	CTX-M-9

4.4 Conclusion

The filter-mating conjugation experiment to assess the transmission of ESBL-producing *E. coli* from treated wastewater and STEC from rivers. This experiment confirms that ESBL-producing *E. coli* that persist in treated wastewater can transmit AR and ARG to STEC in rivers. The difference in the rate of AR transmission depending on the specific donor and recipient bacterial strains involved in the process was observed. These finding highlights the potential risk posed by the release of effluent from WWTPs, which may contain ARB and ARGs contribute to the spread of resistance in the environment.

Reference

1. Croxen, M. A., & Finlay, B. B. (2010). Molecular mechanisms of *Escherichia coli* pathogenicity. Nature reviews. Microbiology, 8(1), 26–38. https://doi.org/10.1038/nrmicro2265
2. Huang, S. W., Hsu, B. M., Su, Y. J., Ji, D. D., Lin, W. C., Chen, J. L., Shih, F. C., Kao, P. M., & Chiu, Y. C. (2011). Occurrence of diarrheagenic *Escherichia coli* genes in raw water of water treatment plants. Environmental science and pollution research international, 19(7), 2776–2783. https://doi.org/10.1007/s11356-012-0777-4
3. Boisen, N., Melton-Celsa, A. R., Scheutz, F., O'Brien, A. D., & Nataro, J. P. (2015). Shiga toxin 2a and Enteroaggregative *Escherichia coli*--a deadly combination. Gut microbes, 6(4), 272–278. https://doi.org/10.1080/19490976.2015.1054591
4. World Health Organization (WHO) : Guidelines for drinking-water quality, fourth edition, pp. 294-305, 2011.
5. Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. Clinical microbiology reviews, 11(1), 142–201. https://doi.org/10.1128/CMR.11.1.142
6. Kubomura, A., Misaki, T., Homma, S., Matsuo, C., & Okabe, N. (2017). Phenotypic and molecular characterization of Enteroaggregative *Escherichia coli* isolated in Kawasaki, Japan. Japanese journal of infectious diseases, 70 5, 507-512.

https://doi.org/10.7883/yoken.JJID.2016.387

7. Farajzadeh-Sheikh, A., Savari, M., Ahmadi, K., Hosseini Nave, H., Shahin, M., & Afzali, M. (2020). Distribution of genes encoding virulence factors and the genetic diversity of Enteroinvasive *Escherichia coli* (EIEC) isolates from patients with diarrhea in Ahvaz, Iran. Infection and drug resistance, 13, 119–127. https://doi.org/10.2147/IDR.S235009
8. WHO : Attributing illness caused by Shiga toxin-producing *Escherichia coli* (STEC) to specific foods, pp. 1-5, 2011
9. Ram, S., Vajpayee, P., & Shanker, R. (2007). Prevalence of multi-antimicrobial-agent resistant, Shiga toxin and enterotoxin producing *Escherichia coli* in surface waters of river Ganga. Environmental science & technology, 41(21), 7383–7388. https://doi.org/10.1021/es0712266
10. Titilawo, Y., Obi, L., & Okoh, A. (2015). Antimicrobial resistance determinants of *Escherichia coli* isolates recovered from some rivers in Osun State, South-Western Nigeria: Implications for public health. The Science of the total environment, 523, 82–94. https://doi.org/10.1016/j.scitotenv.2015.03.095
11. Osińska, A., Korzeniewska, E., Harnisz, M., & Niestępski, S. (2017). The prevalence and characterization of antibiotic-resistant and virulent *Escherichia coli* strains in the municipal wastewater system and their environmental fate. The Science of the total environment, 577, 367–375. https://doi.org/10.1016/j.scitotenv.2016.10.203
12. Mshana, S. E., Imirzalioglu, C., Hossain, H., Hain, T., Domann, E., & Chakraborty, T. (2009). Conjugative IncFI plasmids carrying CTX-M-15 among *Escherichia coli* ESBL producing isolates at a university hospital in Germany. BMC infectious diseases, 9, 97. https://doi.org/10.1186/1471-2334-9-97
13. Mruk, I., & Kobayashi, I. (2014). To be or not to be: regulation of restriction-modification systems and other toxin-antitoxin systems. Nucleic acids research, 42(1), 70–86. https://doi.org/10.1093/nar/gkt711
14. Suzuki, Y., Niina, K., Matsuwaki, T., Nukazawa, K., & Iguchi, A. (2018). Bacterial flora analysis of coliforms in sewage, river water, and ground water using MALDI-TOF mass spectrometry. Journal of environmental science and health. Part A,

Toxic/hazardous substances & environmental engineering, 53(2), 160–173. https://doi.org/10.1080/10934529.2017.1383128

15. Xie, H., Ogura, Y., & Suzuki, Y. (2022). Persistence of Antibiotic-Resistant *Escherichia coli* Strains Belonging to the B2 Phylogroup in Municipal Wastewater under Aerobic Conditions. Antibiotics (Basel, Switzerland), 11(2), 202. https://doi.org/10.3390/antibiotics11020202
16. Clinical and Laboratory Standards Institute (CLSI) (2017). Performance standards for antimicrobial susceptibility testing. M100-27th ed. CLSI supplement M100.Wayne, PA: Clinical and Laboratory Standards Institute, 1-282, ISBN 1-56238-804-5. https://clsi.org/standards/.
17. Shibata, N., Kurokawa, H., Doi, Y., Yagi, T., Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Ishikawa, S., Kato, H., Ozawa, Y., Shibayama, K., Kai, K., Konda, T., & Arakawa, Y. (2006). PCR classification of CTX-M-type beta-lactamase genes identified in clinically isolated gram-negative bacilli in Japan. Antimicrobial agents and chemotherapy, 50(2), 791–795. https://doi.org/10.1128/AAC.50.2.791-795.2006
18. Uyttendaele, M., van Boxstael, S., & Debevere, J. (1999). PCR assay for detection of the *E. coli* O157:H7 eae-gene and effect of the sample preparation method on PCR detection of heat-killed *E. coli* O157:H7 in ground beef. International journal of food microbiology, 52(1-2), 85–95. https://doi.org/10.1016/s0168-1605(99)00132-4
19. Cebula, T. A., Payne, W. L., & Feng, P. (1995). Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. Journal of clinical microbiology, 33(1), 248–250. https://doi.org/10.1128/jcm.33.1.248-250.1995
20. Oswald, E., Schmidt, H., Morabito, S., Karch, H., Marchès, O., & Caprioli, A. (2000). Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. Infection and immunity, 68(1), 64–71. https://doi.org/10.1128/IAI.68.1.64-71.2000
21. The enterohemorrhagic *E. coli* test and diagnosis manual from the National Institute

of Infectious Diseases. https://www.niid.go.jp/niid/en/iasr-vol33-e/865-iasr/7282-447te.html

22. Logan, L. K., Medernach, R. L., Domitrovic, T. N., Rispens, J. R., Hujer, A. M., Qureshi, N. K., Marshall, S. H., Nguyen, D. C., Rudin, S. D., Zheng, X., Konda, S., Weinstein, R. A., & Bonomo, R. A. (2019). The clinical and molecular epidemiology of CTX-M-9 group producing *Enterobacteriaceae* infections in children. Infectious diseases and therapy, 8(2), 243–254. https://doi.org/10.1007/s40121-019-0237-2
23. Merida-Vieyra, J., De Colsa, A., Calderon Castañeda, Y., Arzate Barbosa, P., & Aquino Andrade, A. (2016). First report of group CTX-M-9 Extended spectrum beta-lactamases in *Escherichia coli* isolates from pediatric patients in mexico. PloS one, 11(12), e0168608. https://doi.org/10.1371/journal.pone.0168608
24. Bajpai, T., Pandey, M., Varma, M., & Bhatambare, G. S. (2017). Prevalence of TEM, SHV, and CTX-M beta-lactamase genes in the urinary isolates of a tertiary care hospital. Avicenna journal of medicine, 7(1), 12–16. https://doi.org/10.4103/2231-0770.197508
25. Vaidya V. K. (2011). Horizontal transfer of antimicrobial resistance by extended-spectrum β-Lactamase-producing *Enterobacteriaceae*. Journal of laboratory physicians, 3(1), 37–42. https://doi.org/10.4103/0974-2727.78563
26. Mendonça, N., Leitão, J., Manageiro, V., Ferreira, E., & Caniça, M. (2007). Spread of extended-spectrum beta-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. Antimicrobial agents and chemotherapy, 51(6), 1946–1955. https://doi.org/10.1128/AAC.01412-06
27. Knudsen, P. K., Gammelsrud, K. W., Alfsnes, K., Steinbakk, M., Abrahamsen, T. G., Müller, F., & Bohlin, J. (2018). Transfer of a bla CTX-M-1-carrying plasmid between different *Escherichia coli* strains within the human gut explored by whole genome sequencing analyses. Scientific reports, 8(1), 280. https://doi.org/10.1038/s41598-017-18659-2
28. Mshana, S. E., Imirzalioglu, C., Hossain, H., Hain, T., Domann, E., & Chakraborty, T. (2009). Conjugative IncFI plasmids carrying CTX-M-15 among *Escherichia coli*

ESBL producing isolates at a university hospital in Germany. BMC infectious diseases, 9, 97. https://doi.org/10.1186/1471-2334-9-97

29. Meng, M., Li, Y., & Yao, H. (2022). Plasmid-mediated transfer of antibiotic resistance genes in soil. Antibiotics (Basel, Switzerland), 11(4), 525. https://doi.org/10.3390/antibiotics11040525

30. Frost, L., Leplae, R., Summers, A. et al. (2005).Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol 3, 722–732 https://doi.org/10.1038/nrmicro1235

Chapter 5. Conclusion

In this thesis, a three-part experiment was conducted to investigate the increase in ARB under aerobic conditions, the incomplete removal of ESBL-resistant bacteria during treatment leading to an outflow of these bacteria into the receiving river, and the resulting spread of resistance from ESBL-producing *E. coli* in the effluent water to STEC bacteria in the river. Transmission of resistance has occurred through conjugation between different bacteria. In summary, the following conclusions can be drawn based on this thesis.

5.1 Aerobic treatment processes in WWTP could increase resistance

The resistance and phylogroup changes under aerobic conditions were investigated for *E. coli*. The main findings are:

- After 14 days under aerobic conditions, the concentration of TOC exhibited a gradual decline, providing evidence of organic matter decomposition in the wastewater during the 14-day aerobic treatment.
- *E. coli* removal efficiencies were observed to be 99.6% and 98.7% in samples collected from plants A and B, respectively. The decrease in *E. coli* content in the samples indicates that the biological treatment of wastewater was successful under aerobic conditions.
- The survival rate of strains belonging to phylogroup B2 was much higher than that of strains belonging to the other major phylogroups (such as phylogroup A, B1, D and F) in municipal wastewater samples.
- The prevalence of AR *E. coli* isolates increases under aerobic conditions, and the survival of phylogroup B2 isolates carrying the β-lactamase gene is relatively high.

5.2 ESBL-producing bacteria would persist and flow into the river

The prevalence of resistance during the wastewater treatment process in a WWTP were investigated for ESBL-producing *E. coli* and coliforms. The main findings are:

- The numbers of *E. coli* in the influent water, biologically-treated water, and effluent water were $1.2 \pm 0.6 \times 10^7$ CFU/100 ml, $5.3 \pm 0.1 \times 10^4$ CFU/100 ml, and 5.7 ± 0.5 CFU/100 ml, respectively. The removal of *E. coli* in the water was 6 logs (99.9999%). The numbers of ABPC-*E. coli* in the influent water, biologically-treated water, and effluent water were $1.7 \pm 0.5 \times 10^6$ CFU/100 ml, $1.0 \pm 0.2 \times 10^4$ CFU/100 ml, and 1.2 ± 0.4 CFU/100 ml, respectively.
- The numbers of coliforms in the influent water, biologically-treated water, and effluent water were $2.0 \pm 0.1 \times 10^8$ CFU/100 ml, $1.8 \pm 0.10 \times 10^6$ CFU/100 ml, and $2.4 \pm 0.01 \times 10^2$ CFU/100 ml, respectively. The removal of coliforms in the water was 5 logs (99.999%). The numbers of ABPC-coliforms in the influent water, biologically-treated water, and effluent water were $.0 \pm 0.4 \times 10^7$ CFU/100 ml, $2.5 \pm 0.1 \times 10^4$ CFU/100 ml, and 42.4 ± 0.1 CFU/100 ml, respectively.
- The changes in prevalence of ESBL- producing *E. coli* compared to the total counts in the wastewater treatment process showed that the average prevalence was 4.5% in the influent, 1.9% in biologically-treated water, and 5.6% in the effluent. And the average prevalence of ESBL-producing coliforms was $25.6 \pm 3.3\%$ in the influent, $4.0 \pm 0.8\%$ in the biologically-treated water, and $11.4 \pm 1.1\%$ in the effluent.
- The opportunistic pathogens such as *Klebsiella* and *Enterobacter* carrying ESBL-producing genes were detected in the effluent.
- Although the chlorination process reduced the number of *E. coli* and coliforms, some ESBL-producing *E. coli* and coliforms were still released into the environment through the effluent.

5.3 Transmission of AR to rivers after treated water

ESBL-producing *E. coli* could transfer their resistance genes to STEC in rivers and the rates of conjugation were determined. The main findings are:

- The rate of transmission of AR on the filter was measured using a filter mating method.
- The rate of transmission on the filter was found to range from 1.10×10^{-3} to 9.77×10^{-7}. The transmission of AR through plasmids can occur at a relatively low frequency.
- ESBL-producing gene CTX-M-9 can be transmitted from ESBL-producing *E. coli* to STEC strains. It is important to note that even when the same combination of strains was tested, slight differences in transmission rates were observed between the donor and recipient strains.

Conclusion

In this thesis, a three-part experiment was conducted to investigate the increase in ARB under aerobic conditions, the incomplete removal of ESBL-resistant bacteria during treatment leading to an outflow of these bacteria into the receiving river, and the resulting spread of resistance from ESBL-producing *E. coli* in the effluent water to STEC bacteria in the river. Resistance is enhanced in wastewater plants as the bacterial count decreases, especially as ESBL-producing bacteria remain in the effluent water after chlorine disinfection then are released into the receiving rivers. When the ARB and ARG associate with the bacteria via conjugation in the river, which increase the AR of the bacteria in the river. The results of the study confirm that WWTPs serve as reservoirs of ARB and ARGs and pose a potential risk to human. Further research is needed to identify the specific mechanisms underlying the spread of ARB and ARGs in the environment and to develop targeted interventions to prevent the transmission of AR and safeguard public health.

Acknowledgements

My PhD thesis has been a fulfilling and unforgettable experience, thanks to the invaluable support and encouragement I received from various individuals during my candidature. I am immensely grateful for the unwavering guidance, mentorship, and inspiration provided by my supervisor, Professor Yoshihiro Suzuki. I express my deepest gratitude and admiration for the time, vision, and patience that he dedicated to my research over the course of my studies. Throughout the three years of my doctoral journey, Professor Suzuki's unwavering support, boundless expertise, and constant encouragement have been instrumental in shaping my research trajectory and personal growth. His passion and enthusiasm for his work have inspired me greatly and will undoubtedly continue to influence my future academic pursuits. I have not only learned immensely from his profound knowledge but also benefited from his character and personality, which have been an embodiment of the ideal mentor. I am truly grateful for the opportunity to work with him and for the lessons learned during my time under his guidance.

I would like to express my gratitude to the Japan Society for Academic Advancement for generously supporting my scholarship during my academic pursuits. The financial aid provided by the organization has been instrumental in enabling me to pursue my academic goals and has had a significant impact on my research. Furthermore, I am extremely grateful for the support provided by the Japan Society for the Promotion of Science (JSPS) KAKENHI, which has made it possible for me to conduct this research.

I would like to express my appreciation and gratitude to the predecessors and juniors in the research laboratory for their invaluable contribution to my PhD research. Their contributions, discussions, and feedback have been instrumental in refining my research ideas and improving the quality of my work. Their support, guidance, and expertise have been essential in shaping my academic research.

I would like to take this opportunity to express my sincere gratitude to my friends who have been an integral part of my academic journey and have accompanied me throughout

my PhD. I would also like to express my sincere appreciation and gratitude to my boyfriend. His presence in my life has been a constant source of strength, inspiration and motivation. His understanding and support during the long hours of research, writing and revising has been invaluable. He has helped me through the many ups and downs of my academic journey.

I would like to express my utmost gratitude and appreciation to my parents for their unwavering love, support, and encouragement throughout my life. Their belief in me and their constant support have been instrumental in shaping my personal growth and academic achievements. I owe my successes to their sacrifices and dedication to my upbringing, for instilling in me a strong work ethic, values, and principles that have guided me throughout my life. Their love and unwavering support have been my source of strength, and I am deeply grateful for all that they have done for me. I cannot thank them enough for the countless sacrifices they have made and for being my pillar of strength, always there to offer a listening ear and a shoulder to lean on. I owe everything I am today to their tireless efforts, unwavering support, and love. I will always cherish and appreciate the sacrifices they have made.