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TECHNISCHE
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MASTER THESIS

Topic: Antibiotic resistance genes and pathogens in
wastewater treatment plant effluents: regrowth of ARG/ARB
after inactivation processes

Author: Hung, Pham Tien - Master

Supervisor: Prof. Susanne Lackner

Contact person: Dr.rer.nat. Laura Orschler

Vietnamese-German University - Water technology,
management and reuse

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Abstract

This study evaluated the effectiveness of advanced treatment processes (photo-Fenton, photocatalyst, membrane bioreactor and constructed wetland) and disinfection processes (UV, chlorination and ozonation) on the removal of ARGs/ARB as well as its capital cost (capex) and operational cost (opex). The findings demonstrated that a number of physico-chemical variables, including SS, pH, DOC, and others, had an impact on the efficacy of conventional disinfection processes such as chlorination, UV irradiation, and ozonation in addition to dosage dependence. It has been found that significant doses of disinfectant are necessary to inactivate ARGs rather than their hosts, despite the fact that doing so causes severe microbial community rearrangements. In addition to disinfection methods, light-supported techniques including photo-Fenton and photocatalyst demonstrated more effectiveness than disinfection techniques, and no regrowth was detected owing to the affecting residue. The performance of the system is also greatly influenced by chemical concentration and light intensity. In terms of constructed wetland (CW) and membrane bioreactor (MBR), they show good removal of ARGs/ARB via its mechanism of separation, adsorption and biodegradation; however the accumulation of ARGs/ARB on the biosolids, surfaces of membrane and substrate are critically concerned. According to the regrowth potential of ARGs/ARB, photo-Fenton and photocatalyst showed the most optimal prevention, compared to disinfection processes; however, the lack of information of the regrowth relates to MBRs and CWs.



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Abbreviations

AMR	Antimicrobial resistance
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistant genes
BOD	Biochemical oxygen demand
CAPEX	Capital cost
CFUs	Colony-forming units
COD	Chemical oxygen demand
CPDs	Cyclobutane pyrimidine dimers
CAS	Conventional activated sludge
CW	Constructed wetland
HGT	Horizontal gene transfer
LB	Luria Bertani
MBBR	Moving bed bioreactor
MBR	Membrane bioreactor
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
OPEX	Operational cost
OTU	Operational taxonomic unit
q-PCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
TOC	Total organic carbon
VBNC	Viable but not cultivable
VGT	Vertical gene transfer
WWTPs	Wastewater treatment plants

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1. Introduction

Antimicrobial resistance is a significant danger to human, animal, and environmental health resulting from the use of antibiotics by humans and agriculture, veterinary medicine as well as waste emissions, particularly pharmaceutical wastewater (AMR). A microorganism's ability to adapt and thrive in the presence of antimicrobial medications at quantities that would otherwise be deadly or growth-inhibiting is referred to as antimicrobial resistance (AMR). Due to the proliferation of mobile genetic elements (MGEs) and resistant bacteria, as well as the development of AMR through improper or excessive use of antibiotics and inadequate empiric antimicrobial therapy, AMR has become a global health concern. In addition to increased trade, travel, and migration, these factors have led to the spread of MGEs and AMR ([Pattis et al., 2022](#)).

WWTPs are regarded as major reservoirs of resistance genes because urban and agricultural wastewater, polluted by potentially large amounts of antibiotics, antibiotic resistant bacteria (ARB) and their ARGs as a result of veterinary and medical use, are collected for treatment ([Gao et al., 2022](#)), as shown in Figure 1.1. Biological processes in WWTPs create potentially favorable environment for development and spread of resistance genes, as bacteria are constantly exposed to antibiotics at sub-lethal concentrations, promoting the proliferation of ARGs and resulting in more and novel ARB ([I. Michael et al., 2013](#)). Besides, heavy metals derived from industrial wastewater sources, known as the stressor, together with the high nutrient load, stable pH and temperature, and the close proximity of cells in sludge induce horizontal gene transfer facilitation of genetic elements encoding for resistance, including antibiotics ([Manai et al., 2018](#)). Besides, oxygen level and spatial distribution of bacteria could also promote the proliferation of ARGs/ARBs ([Nguyen et al., 2021](#)).

Basically, there are two types of wastewater treatment methods for eliminating ARB/ARGs: (i) using advanced oxidation techniques the same as photo-Fenton and photocatalyst to attack cell membranes and destroy the integrity of the entire cell; (ii) capturing or intercepting ARGs/ARB from wastewater using constructed wetlands and membrane separation processes ([J. Wang et al., 2022](#)).

Pathogens in wastewater can be inactivated by ozone, a potent oxidising agent, both directly and indirectly through hydroxyl radicals (OH^*). By quickly reacting with cell components like phospholipids, proteins, peptidoglycan, and liposacharides and unsaturated bonds, O_3/OH^* initially destroyed the structural integrity of the cell envelope. This altered the permeability of the cell wall and membrane, which then caused the leakage of cellular components. Following that, additional O_3 and OH^* enter the inside of the cell, deactivating the components of the cell, including ARGs ([J. Wang et al., 2022](#)).

To prevent the spread of the pathogens, chlorine has been used extensively. In a manner similar to ozone, chlorination can alter the permeability of cell membranes and wall membranes, which can cause the leakage of cellular components like ATP, DNA, and proteins. It does this by reacting with components, such as the N-terminal amino acids of peptidoglycan, which are initially located on the cell membranes. In addition, when chlorine combines with water, OCL⁻ and HOCL are created, which easily pass through the cell membrane and continue to damage nucleic acids and other interior cellular components ([J. Wang et al., 2022](#)). In addition to chlorine, chloramine (NH₂Cl, NHCl₂ and NCl₃) is a replacement for chlorine due to the reduction in formation of disinfection-by products during treatment process ([Ezeuko et al., 2021](#)). However, comparison between chlorine and chloramine in case of removing ARGs/ARB is discussed in below.

Light-dependent disinfection systems, as opposed to chlorination, often utilize the physical element of light as the system's primary initiating element. Since UV irradiation is recognized as a highly effective, practical, and affordable approach, it continues to be the major light-based disinfection technology utilized in WWTPs worldwide ([Manoharan et al., 2022](#)). By easily passing through the transparent cell membrane and cytoplasm, UV light inactivates bacteria by interacting with bases in nucleic acids like pyrimidine and purine. Based on different wavelength ranges, UV is separated into three parts: UVA (320–400 nm), UVB (280–320 nm), and UVC (200 – 280 nm). The primary method by which UV harms bacteria varies depending on the UV wavelength; the level of DNA harm may change depending on the absorption wavelength. More specifically, the photosensitivity of UVA may produce different reactive oxygen species, which cause oxidative alterations in lipids and proteins, harming bacteria as a result. Contrary to UVA, UVB causes light-produced DNA molecules called cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidinone to have an antibiotic effect. Finally, due to its shorter wavelength, UVC may increase the creation of CPDs in bacterial DNA, reducing the physiological functions of bacteria ([Cai et al., 2021](#)). However, despite the fact that UV reduces the concentration of ARGs/ARBs, UV cannot entirely eradicate antibiotic resistance, meaning that there is still a chance that ARGs transfer to the final effluent ([M.-T. Guo et al., 2015](#)). Therefore, it is crucial to investigate in detail how UV-induced disinfection affects the elimination of ARGs/ARB.

Advanced oxidation processes (AOPs) are characterised by the generation and utilisation of OH^{*}, the second-strongest oxidant after fluorine, for the oxidation and degradation of chemical compounds into low harmful or non-toxic small molecules, under particular reaction conditions. Successful applications of Fenton/photo-Fenton, photocatalyst, and other technologies include the removal of bacteria, viruses, and new compounds like ARGs/ARBs in addition to treating conventional chemical pollutants like BOD, COD, and TOC ([Pei et al., 2019](#)).

According to scientific literature, a membrane bioreactor (MBR) technology outperforms a conventional activated sludge (CAS) process in terms of ARG performance. According to reports, ARGs in MBR often remove 1.5–7.0 logs, and those in CAS typically remove 0.8–4.5 logs. Adsorption on sludge particles, membrane rejection (membrane pore diameters are lower than ARGs/ARB sizes), fouling layer, and biodegradation are the main processes of ARGs/ARB in MBR (R. Wang et al., 2021). Regarding CWs, this technique has been widely used to treat wastewater, either alone or in conjunction with more traditional treatment methods. In low income nations, particularly for treating wastewater for agricultural reuse, CWs are particularly promising for decentralised treatment due to their minimal mechanical complexity and energy demands. Additionally, it has been discovered that CWs effectively remove contaminants from both industrial and municipal wastewater (Hazra et al., 2022). Therefore, a holistic assessment combining the operational aspects of improvement in water quality parameters, removal of emerging contaminants, including ARB/ARGs are discussed in Chapter 3.2.

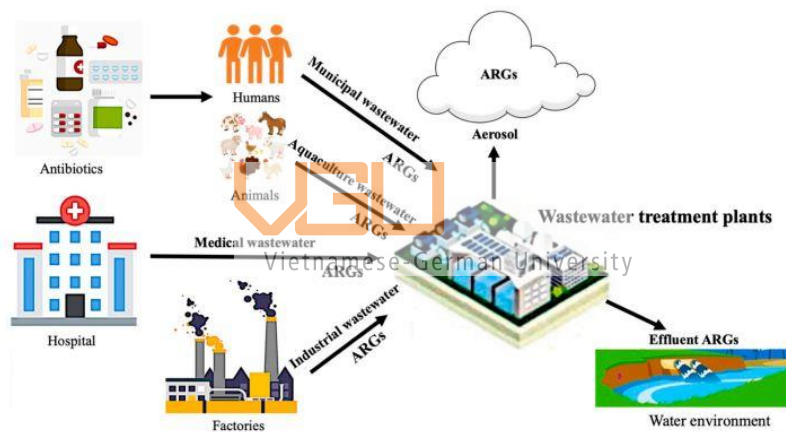


Figure 1. 1: Different sources of ARGs/ARB enter the WWTPs (Gao et al., 2022)

ARGs can be acquired by a bacterial host cell via two distinct mechanisms: (1) horizontal gene transfer (HGT) which is mediated by MGEs, including plasmid, transposons, integrons and bacteriophages via three sub-ways: transformation, transduction and conjugation; (2) vertical gene transfer – transmission of modified genetic information located on bacterial chromosome to subsequent generations of daughter cells, as depicted in Figure 1.2 (Pazda et al., 2019). Furthermore, mutation plays a critical role in the emergence and diversification of resistance mechanism. Multitargeted resistance alters antibiotics action through various mechanisms such as (1) modifying the antimicrobial targets, (2) by decreasing antibiotic uptake, and (3) by activating efflux mechanism to extrude certain molecules, as shown in Figure 1.3 (Hazra et al., 2022).

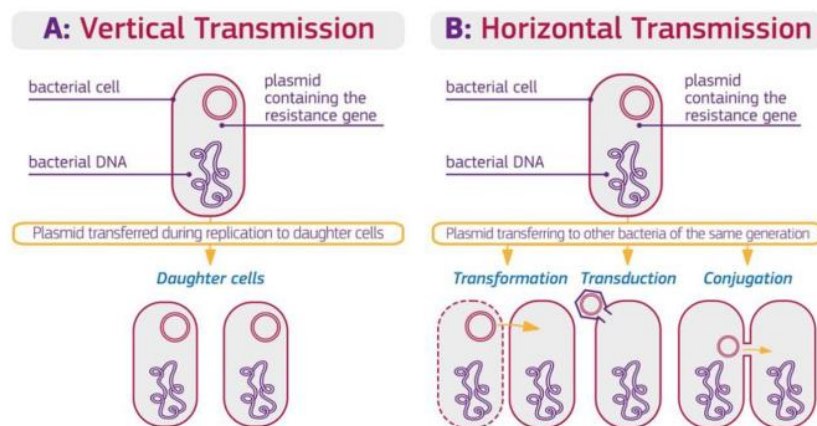


Figure 1. 2: Horizontal gene transfer and vertical gene transfer pathways (Jovanovic et al., 2021)

According to monitoring and detection of ARGs/ARBs during wastewater treatment processes, molecular approaches for quick detection and characterization of ARB or their genes include polymerase chain reaction (PCR), real-time polymerase chain reaction (q-PCR), and DNA microarray (Bouki et al., 2013), sequencing technologies (e.g. metagenomic sequencing) (Pazda et al., 2019). Through the use of specific primers and the development of fluorescence, which emission is proportional to the PCR amplicon produced, due to the use of a fluorescent dye or a probe, molecular approaches, such as qPCR, are designed to monitor in real-time the amplification of a specific gene fragment (Manaiia et al., 2018). Primers are short single-stranded DNA fragments that are particularly designed to bind to one section of the genome and enable for the accurate amplification of the diagnostic target region. Probes are frequently employed in real-time PCR. Probes are fluorescently labelled DNA oligonucleotides and non-specifically fluorescent DNA dyes that are two techniques for the real-time imaging of amplified DNA fragment utilized in pathogen identification (Kralik et al., 2017). The purpose of a probe is to attach to one of the primers during a PCR reaction and to produce a fluorescence signal. The probe is marked with a fluorescent reporter molecule at its 5' end (Arya et al., 2005). However, qPCR still has several limitations that must be considered when data analysis and interpretation are being done. Since the primers are designed based on reference sequences that are already characterized and stored in databases, one key constraint is the inability of designing primer for new or unknown genes. Primers are tough to create, whether they are general or customized. It is difficult to create a universal primer that functions the same way across all taxa. In contrast, a primer set that is excessively specific when ARGs are the targets prevents the detection of gene variations. On the contrary, since primers only require homology with a small fragment of DNA, annealing possibly occur with non-target genes with regions of high identity with primers. However, these false positive reactions can be easily identified based on melting temperature of

the amplicon ([Manaia et al., 2018](#)). Additionally, the manner in which nucleic acids are extracted and the calibre of the isolated DNA are essential for the following PCR gene identification. The identification of particular bacterial species and functional genes is challenging because wastewater samples include complex matrices, high quantities of organic and inorganic debris, inhibitors including humic compounds, and detergents. Reduced detection sensitivity in environmental samples potentially results in an undercount of initial targets or false negative findings ([Pazda et al., 2019](#)).

One of the main advantages of cultivation is the possibility of determining phenotypic traits, many of which are crucial for understanding the ecology of a given bacterial group. In case of antibiotic resistance, this knowledge is the basis to assess the propagation or gene transfer potential of specific ARB conditions. In this aspect, relevant phenotypic traits that can be examined in bacterial isolates including metabolism, required physico-chemical conditions for growth, such as pH, temperature and salinity, biofilm formation capacity, sporulation, motility, tolerance against stressful conditions ([McLain et al., 2016](#)). The most popular method for identifying bacteria with multiple drug resistance is culture-based methods, which are further described as a tool allowing the quantification of viable cells and the assessment of antibiotic resistant profiles, such as minimum inhibitory concentration (MIC) and antibiotic resistance spectrum of isolates ([D. Li et al., 2009](#)). Culture-based treatments lost favour with the introduction of molecular technologies and their increased efficacy in recent decades. Direct DNA analysis were frequently preferred since, in addition to having a limited ability to survey microbial communities, these techniques may be time-consuming, expensive, and need expert operators to perform post-isolation assays properly. Working with ARB isolates necessitates appropriate protective clothing and procedures because to the possibility of biohazard contamination of exposed operators and the surroundings, which is another crucial factor. This can be a limitation in some laboratories. However, the value of cultivation is increasingly becoming clear, particularly because it is now backed by data from databases that primarily refer to DNA analyses, in which gene functions are mainly inferred based on in silico analyses and cultivation, is once more regarded as a crucial need to advance the field of microbiology knowledge. In the future, the optimum strategy for examining the environment resistome could be a combination of culture-based and molecular methodologies ([Manaia et al., 2018](#)).

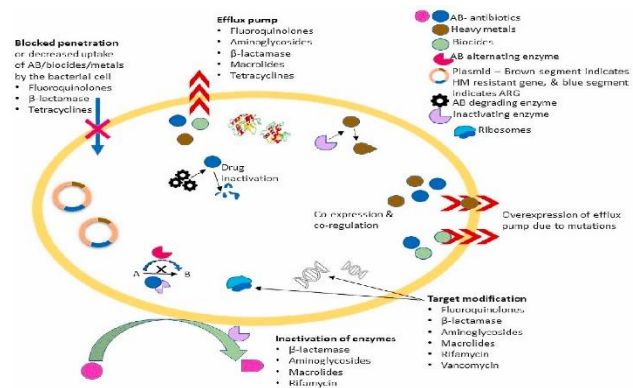


Figure 1. 3: Resistance mechanisms of pathogens to antibiotics (Hazra et al., 2022)

Additionally, certain ARGs from the most popular class of antibiotics, including beta-lactams, quinolones, sulfonamides, and tetracyclines, and colistin are of particular relevance in this study since considerable levels of these chemical compounds have been found in sewage samples (Michael et al., 2013). According to the report of Versporten et al. (2018), Based on a survey of 335 hospitals in 53 countries, the top three antibiotics given globally are quinolones and beta-lactams (including 8 lower-middle-income and 17 upper middle-income countries) (Versporten et al., 2018). Beta-lactam as parent compounds, which account for 50-70% of total antibiotics used by human, are found in minute concentration in wastewater and thus in the environment (Jovanovic et al., 2021). According to research, ampicillin, cefalotin, and cefoxitin all hydrolyze at room temperature (25°C) and pH 7.0 to produce molecules with less antibacterial action. These compounds have half-lives of 5.3, 9.3, and 27 days, respectively. When compared to the parent molecules, the breakdown products of b-lactams are four orders of magnitude more abundant (Segura et al., 2009). The bacterial resistance mechanism to beta-lactams includes modified target and changed permeability of the bacterial outer membrane for the drug through reduction in porin channel density. The third potential route of resistance is the inactivation of antibiotics by beta-lactamase, which is most prevalent in Gram-negative bacteria. Genes indicate the mechanism of antibiotic breakdown by rupturing the beta-lactam ring (amp, bla, oxa). Different MGEs contain specific resistance genes, including ampC on the chromosome and ampR, bla_{TEM}, bla_{CIT}, bla_{CTX-M}, bla_{OXA}, and bla_{SHV} on the plasmid (X.-Z. Li et al., 2007). In addition to its clinical use, quinolones are often used in veterinary medicine to treat a range of illnesses and as preventative purposes (Pazda et al., 2019). The majority of quinolones are eliminated in unmodified form by the urine (45–62%) and faeces (15–25%), which are then released into hospital or municipal sewage, leading to their routine detection in WWTPs across the world (Kaplan et al., 2013). Sulfonamides are now employed in veterinary medicine to treat bacterial and protozoal illnesses as well as pharmacological feed additives. Sulfonamides are mostly used in conjunction with trimethoprim or alone in the treatment of uncomplicated respiratory, urinary tract, and chlamydia

infections in human medicine ([Pazda et al., 2019](#)). According to the available data, sulfonamides constituted 6% (5.1 tons per year) of total antibiotic consumption in Switzerland ([Göbel et al., 2004](#)) and 12% in China ([Xu et al., 2007](#)). Both natural and semi-synthetic antibiotics belong to the class of tetracyclines. They are now only seldom utilized in human medicine, although they are widely employed in the care of animals and aquaculture ([Pazda et al., 2019](#)). Tetracycline is used in veterinary medicine annually in amounts of more than 2500 tonnes in Europe and 1800 tonnes in the US, according to published data. After taking medicine, both people and animals excrete and release more than 70% of tetracyclines in active form into the environment through urine and faeces. They have exhibited great endurance in the aquatic environment due to their extremely hydrophilic nature and low volatility ([Daghrir et al., 2013](#)). Tetracyclines, sulfanomides, and quinolones are primarily adsorbed onto sludge particles when combined with antibiotics-induced selection pressure, a long hydraulic retention time, and sludge recirculation during biological treatment processes, which is attributed to the development of their ARGs/ARB in wastewater treatment plants ([Jovanovic et al., 2021](#)). Colistin, also known as polymixin E, was first used in human medicine in the 1950s ([Ovejero et al., 2017](#)). However, due to its significant nephrotoxicity, it was subsequently repurposed for use mainly in agriculture. Surprisingly, despite the fact that the usage of polymixins was still prohibited in 2013, polymixins ranked as the fifth-most popular class of antimicrobials in European countries ([Jovanovic et al., 2021](#))

This review focuses primarily on the efficacy of advanced treatment processes individually or in combination for ARGs/ARBs removal and evaluation of the potential regrowth of ARGs/ARBs after treatment, as killing of bacteria carrying resistance genes is believed to control the spread of AMR in aquatic environments ([Gao et al., 2022](#)). In addition, these advanced treatment processes are classified and evaluated based on factors such as removal efficiency, installation- and operating cost, energy and spatial footprint, providing operators and decision makers within WWTPs with a holistic picture of current technologies to choose appropriate treatments for limiting the transmission of AMR in aquatic environments.

2. Review method

The American chemical society, Google scholar, and Elsevier are three of the most prominent search and data collection tools. Collecting and analyzing as much data as possible regarding the fate and occurrence of ARM in WWTPs and the receiving environment. Additionally, all documents relevant to advanced treatment unit procedures are gathered and synthesized in order to classify and evaluate the potential application of these treatment processes in WWTPs based on a variety of variables. Using keywords such as "antibiotic resistance genes in WWTPs," "antibiotic resistance bacteria in WWTPs," "antimicrobial resistance in WWTPs," "temporal and spatial distribution of AMR," and "advanced methods for removing ARGs/ARBs," relevant information was discovered.



3. Results and discussions

3.1. Literature review (chapter 3-1)

3.1.1. Spread of antimicrobial resistance in WWTPs – A holistic picture

The results showed that the majority of studies have focused on ARGs and ARBs in WWTPs around the globe. Different ARGs such as sulfonamides, B-lactam, macrolide, and tetracycline, and so on, and its hosts (ARB) were summarized in Table 3.1. In addition, MGEs, such as integron class 1 (int1), are also readily detected since MGEs cause multi-drug resistance in bacteria via horizontal gene transfer and are marked as an indicator of the abundance of ARGs. Studies showed, that during the treatment process a significant amount of ARB and ARGs are removed from the water phase and partitioned into the sludge phase, high concentrations of ARGs in sludge and biosolids—up to 10^9 copies/g—have been found in influent, effluent, and activated sludge of wastewater treatment plants in numerous countries (Nguyen et al., 2021). ARGs are still present in the treated effluent, and as biosolids and effluent are released back into the environment, ARGs and ARBs from wastewater potentially come into touch with humans, domestic animals, wild animals, and other critters as well as environmental microbes. WWTP effluents contribute significantly to the number of detected ARGs, transposon, and integrons in the receiving environment and downstream sediments (Nguyen et al., 2021). To detect ARGs/ARBs in WWTPs, the majority of studies used culturable and molecular techniques to evaluate total DNA or bacterial isolates in influent, effluent, and activated sludge samples.

Table 3. 1: Detection of ARGs in different countries

Country	ARGs/ARB	Methods	Source			Reference
			Inf.	Eff.	AS	
Euro						
Sweden	<ul style="list-style-type: none"> - Mcr-1, mcr-3, mcr-4, mcr-5 (5) - Sul4 (1) - Gar (6) 	(a)	A	A	N/a	(Hutinel et al., 2022)
Poland	<ul style="list-style-type: none"> - Bla_{TEM} (2) - tetA (4) - sul1 (1) 	(a)	A	A	N/a	(Osińska et al., 2020)

	- Intl1 and intl2 (7)					
Portugal	- Bla _{TEM} (2) - Intl1 (7) - vanA (8)	(a)	A	A	N/a	(Narciso-da-Rocha et al., 2014)
Germany	- Sul1 (1) - Bla _{TEM} , bla _{CTX-M32} , bla _{OXA48} , bla _{CTX-M15} and bla _{CMY-2} (2) - tetM (4) - mecA (9) - mcr-1 (5) - vanA (8)	(a)	N/a	A	N/a	(Alexander et al., 2020 ; Hembach et al., 2017)
Romania	- sul1 (1) - tetW and tetO (4) - intl1 (7)	(a)	A	A	N/a	(Lupan et al., 2017)
Spain	- sul1 (1) - Bla _{TEM} (2) - ermB (9) - tetW (4)	(a)	A	A	N/a	(Rodriguez-Mozaz et al., 2015)
Italy	- Bla _{TEM} , bla _{CTX-M} and ermB (2) - Sul2 (1) - qnrS (3) - tetA and tetM (4) - intl1 (7)	(a)	A	A	N/a	(Cacace et al., 2019 ; Di Cesare et al., 2016)

	- mcr1 (5)					
United Kingdom, France and Netherlands	- Bla _{CTX-M-15} , Bla _{CTX-M-32} and bla _{KPC-3} (2) - Sul1 (1) - tetM (4) - mcr-1 (5) - int1 (7)	(a)	N/a	A	N/a	(Cacace et al., 2019)
Asia						
Korea	- Sul1 (1) - aadA1, aaAd13, aadA2 (6)		N/a	N/a	A	(Kang et al., 2022)
China	- sul1 and sul2 (1) - tetW, tetQ and tetX (4) - int1 (7)	(a)	N/a	N/a	A	(H. Zhang et al., 2019)
Sri Lanka	- sul1 (1) - ampC (2) - aac-(6')- 1b-cr, qnrS and qnrB (3)	(a)	A	A	N/a	(Kumar et al., 2020)
Africa						
Tusinia	- Bla _{TEM} (2) - Sul1 (1) - QnrA and qnrS (3) - Int1 (7) - Emr (9)	(a)	A	A	N/a	(Rafraf et al., 2016)

America						
Brazil	- Sul1 (1) - tetA (4) - Bla _{TEM} (2) - ErmB (9) - Int1 (7)	(a)	A	A	N/a	(Leroy-Freitas et al., 2022)
Colombia	- Mcr-5 and mcr-3 (5) - Int1 (7) - Bla _{CTX-M} , bla _{TEM-196} , bla _{GES-23} , bla _{OXA-58} , bla _{KPC} (2)	(b)	A	A	A	(Rodríguez et al., 2021)
USA	- Bla _{TEM} (2) - Sul1 (1)	(a)	A	A	A	(Quach-Cu et al., 2018)
Canada	- Sul1 and sul2 (1) - Int1 (7) - Bla _{CTX-M} and bla _{TEM} , and mecA (2) - ermB (9) - qnrS (3) - tetO (4)	(a)	A	A	N/a	(Neudorf et al., 2017)

(1): Sulfonamide; (2): beta-lactam; (3): quinolone; (4) tetracycline; (5) polymixins; (6) Aminoglycoside; (7):MGEs; (8): glycopeptides, (9) macrolides.

(a): qPCR; (b): Metagenomic sequencing; (c): culture-based methods; (d): DNA microarray.

Table 3. 2: Detection of ARB in different countries

Country	ARB	Methods	Source			Reference
			Inf.	Effl.	AS	
Euro						
Sweden	EBSL-resistance <i>E.coli</i>	(c)	A	A	N/a	(Kwak et al., 2015)
Czech Republic	EBSL-resistant <i>E.coli</i>	N/a	A	A	N/a	(Kutilova et al., 2021)
Germany	B-lactam -resistant <i>E.coli</i> , <i>Klebsiella</i> and <i>E.cloacae</i>	(a), (c)	A	A	N/a	(Savin et al., 2020)
Asia						
India	Multidrug resistance <i>E.coli</i>	(c)	N/a	A	N/a	(Akiba et al., 2015)
Hong Kong - China	Multidrug-resistant <i>Proteobacteria</i> , <i>P. aeruginosa</i> and <i>K. pneumoniae</i>	(b)	N/a	N/a	A	(Yin et al., 2019)
South Korean	Multidrug-resistant <i>Acinetobacter</i> and <i>P.aeruginosa</i>	(c)	A	A	N/a	(Gupta et al., 2018)
America						
USA	Vancomycin-resistant <i>enterococci</i>	(c)	A	A	A	(Goldstein et al., 2014)
Canada	Multidrug-resistant <i>E.coli</i>	(d)	A	A	N/a	(Biswal et al., 2014)

Tetracyclines (tetW, tetG, tetM, etc.), sulfonamides (sul1, sul2, and sul3), B-lactams (blaCTX, blaTEM, etc.), and quinolones (qnrS, qnrB, qnrA, etc.) are frequently found in WWTPs around the world, as the Table 3.1 summarises. ARGs such blaTEM and blaCTX (B-lactam antibiotic resistant genes), sul1 (sulfonamides) were frequently found in all nations and, colistin-resistant genes (mcr1-5), was especially found in Europe. Reports on the frequent detection of mcr-1 in municipal wastewater are thus alarming ([Hembach et al., 2017](#)) as shown in the study of Kneis et al. (2019), where raw municipal wastewater was sampled and evaluated at nine locations all over Germany from 2016 to 2017 ([Kneis et al., 2019](#)). The focus is on one particular gene, the incidence of colistin resistance genes overall is significantly underestimated. More specifically, mcr 1 had an average relative abundance of 1.4×10^{-5} copies per 16S rRNA gene copies, which was less than 10–100 times that of mcr3 and mcr-7. In addition, Ovejero et al. (2017) performed tests to determine if Enterobacteriaceae that harbour the mcr-1 gene were present in water samples taken from nearby rivers and two wastewater treatment plants in Barcelona, Spain ([Ovejero et al., 2017](#)). Mcr-1 was found using PCR, and bacteria were found using MALDI-TOFMS and a broth dilution technique. In addition, incompatibility group PCR, antibiotic susceptibility tests, and transformation studies were used to characterise the plasmid. The findings demonstrated that thirty MDR isolates carrying the mcr-1 gene, including 29 *E. coli* (ST632 and ST479) and one *K. pneumoniae*, were isolated in sewage from two distinct WWTPs, whereas the gene was not present in river water. In addition to MDR isolates, mcr-1 was always connected to an IncI2 plasmid that exclusively offered colistin resistance. This study reveals a significant prevalence of mcr-1 in the sewage of Barcelona, mostly because of the spread of two *E. coli* pulsotypes that are circulating in the community. Mcr-1 was habituated by two dominant *E. coli* clones that were identified in both WWTPs. The studies showed, that due to the high abundance of mcr-1 in the environment, that the disease is highly prevalent in the local population ([Ovejero et al., 2017](#)).

ARB includes multidrug-resistant *E. coli*, *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*. *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Enterococcus spp.*, *Staphylococcus aureus*, and *Enterobacter spp.* are responsible for the majority of life-threatening bacterial infections in critically sick and immunocompromised people globally in healthcare facilities. ([Savin et al., 2020](#)). As seen in Table 3.2, a lot of studies focused on harmful bacteria and antibiotic-resistant microorganisms found in WWTPs ([Biswal et al., 2014](#); [Goldstein et al., 2014](#)). For instance, *E. coli* that is resistant to vancomycin and other drugs in America and Canada, respectively, or *E. coli* that is ESBL resistant in Sweden and the Czech Republic. Diverse ARB were present in these WWTPs, and wastewater treatment had an impact on the makeup and structure of the microbial community, particularly the Proteobacteria class. The risk associated with a particular ARB depends on multiple factors in addition to its classification as reservoir,

carrier, vector or pathogen. These factors include the frequency of exposure to a human body, modes of transmission and portal of entry, the infectious dose, the capacity to acquire and disseminate ARGs to the host microorganisms, and the types and diversity of ARGs it harbours. A pathogen with a low infectious dose, residing in an environmental compartment with high exposure to humans, conferring resistance to last-resort antibiotics or multiple antibiotics, will be classified at the top level of risk.

3.1.2. Effect of different factors to AMR spectrum during WWTPs

By evaluating the abundance of ARGs during WWTPs in different European countries such as France, Norway, Portugal, Germany, Netherlands, Cyprus, Turkey, Austria and United Kingdom, Cacace et al. reported that geographical latitude and plant size are not factors affecting the abundance of ARGs but applied technologies, the number of biological steps in this case, illustrate a significant correlation with abundance of ARGs during WWTPs (Cacace et al., 2019). Furthermore, Cypriot WWTP effluents showed the lowest absolute abundance of ARGs among the investigated plants because only Cypriot WWTP applies membrane bioreactors (MBR) to treat the wastewater, and it is emphasized that the application of wastewater technology affects the development of AMR during WWTPs. In another case, Tong et al. conducted investigations on six full-scale municipal WWTPs with three commonly different treatment processes in Hebei province, China divided into three main groups: (1) Plant A – Cyclic activated sludge system (CASS) and sandfilter, Plant B – Cyclic activated sludge system without sandfilter; (2) Plant C and D – Oxidation ditch and secondary clarifier; and Plant E and F – Anaerobic, anoxic, aerobic reactors, disinfection and tertiary treatment processes (Tong et al., 2019). As a result, the biological treatment processes of six WWTPs showed effective removal of the total ARGs and MGEs, with 12.44-24.63 logs and 3.25-7.22 logs, respectively. Relating to third group, there were 0.42 and 0.15 log reduction of total ARGs and MGEs in the aerobic tank (MBBR process) of plant E, compared to 0.74 and 0.75 logs reduction of total ARGs and MGEs in the aerobic tank without biochips, demonstrating that the less efficiency of the MBBR of plant E on removing ARGs and MGEs, compared to activated sludge system of plant F. Furthermore, there was 2.08 and 7.91 logs reduction of total ARGs during disinfection process with chlorine, and 6.82 and 1.72 of total ARGs during tertiary treatment for plant E and plant F, respectively, illustrating that ARB known as ARG-carrying hosts were effectively destroyed during disinfection and tertiary treatment processes. Therefore, it can be concluded, that disinfection and tertiary treatment are necessary for further application in resistance reduction.

In addition to application of different technologies, climate induces the development of AMR in the water environment. Rodríguez et al. (2021) reported that the climate also showed an increase in the relative abundance of ARGs due to an increase in antibiotic consumption of population in Colombia (Rodríguez et al., 2021). The same results are reported by Wang et al. (2020), the seasonal variations, especially winter season in this case, show the same tendency for the development of AMR (J. Wang et al., 2020). The extensive use of antibiotics has contributed to the development of antibiotic resistance genes and bacteria, reducing the therapeutic potential against human and animal pathogens (I. Michael et al., 2013). This arises from the ability of antibiotics to exert pressure on exposed microorganism thus inducing resistance to itself, or stimulate the transfer of MGEs (e.g. plasmid and conjugative transposon) responsible for the dissemination of resistance determinants (Nguyen et al., 2021). In addition to applied technologies and seasonal variations, wastewater characteristics and sub-lethal concentration of antibiotics possibly show relative correlation between AMR spectrum and WWTPs, especially correlation between AMR spectrum and the treated wastewater in the effluent of WWTPs.

Firstly, wastewater characteristics including different sources such as hospital and industrial, and municipal contributions (Rodríguez et al., 2021; Tong et al., 2019; H. Zhang et al., 2019) is to drive significant WWTP bacterial community among different WWTPs, revealing a significant correlation with ARGs abundance and distribution. Especially, hospital wastewater is well-known as huge distribution of ARGs to WWTPs, but its wastewater only contribute (0.2 – 2%) of the total amount of wastewater to an urban area (Cacace et al., 2019). Fan et al. (2018) collected activated sludge samples of 16 full-scale WWTPs treating different sources of wastewater in different locations in China to find a clear correlation between ARGs, bacterial genera and environmental factors (Fan et al., 2018). As a results, the gene counts of ARGs varied in different WWTPs, however, it was still comparable in most of the WWTPs, suggesting influences of wastewater types on abundance of ARGs. Cacace et.al also reported the same results when evaluating absolute abundance of ARGs in relation to WWTP characteristics of different European countries by separating collected data into two main groups: (1) number of hospital beds in the catchment, number of biological steps, and COD effluent concentration; (2) number of hospital beds in the catchment, geographical latitude and plant size (Cacace et al., 2019). As a result, there is only the factor of number of biological steps illustrating a significant correlation with the absolute abundance of ARGs. Furthermore, wastewater types also cause shifts in bacterial community. The concept of operational taxonomic unit (OTU) which constructs mathematically defined taxa, is widely accepted and applied to describe bacterial communities using amplicon sequencing of 16S rRNA (Lladó Fernández et al., 2019). Depending results of OTUs was reported by Zhang et al. (2019) within 18 wastewater treatment plants in China,

Proteobacteria has the largest dissimilarity contribution, comprising around 47% of the OTUs across all samples, followed by *Bacteroidetes* (30%), *Firmicutes* (7%), *Actinobacteria* (2.2%) and *Chlorobi* (1.2%) ([H. Zhang et al., 2019](#)), as same tendency was also shown in this report ([Fan et al., 2018](#)). *Proteobacteria* and *Bacteroidetes* show higher relative abundance in most of WWTPs treating industrial and mixed wastewater than municipals, while *Actinobacteria* and *Firmicutes* are commonly detected in WWTPs treating municipal wastewater, emphasizing the influences of wastewater characteristics on the dominant bacterial community in WWTPs ([Fan et al., 2018](#)). Moreover, a previous study also demonstrated that *Proteobacteria*, *Actinobacteria* and *Firmicutes* were important hosts of ARGs ([Huerta et al., 2013](#)). There is a need to identify those microorganisms that potentially carrying specific ARGs or integrons and the selective force in different sources of wastewater, intending to design treatment strategies that possibly reduce the problem of antibiotic resistance bacteria in WWTPs.

Secondly, sub-lethal concentration of antibiotics that are several hundred - fold below the minimal inhibitory concentration (MIC) select for resistant bacteria. Sub-lethal concentration of antibiotics can produce reactive oxygen species (ROS) which consequently induce mutagenesis ([Amarasiri et al., 2020](#)). In detail, sub-MIC selection favors the accumulation of multiple small-step mutations, it enriches for mutator bacteria. Bacteria that have high mutation rates are enriched, as they accumulate mutations at an increased rate and thus experience more rapid adaption to the growth-inhibitory environment. This is functionally analogous to the well-known correlation between selection in changing environments and the enrichment of mutators ([Andersson et al., 2014](#))

3.2 Evaluation of advanced treatment processes to reduce ARBs/ARGs (Chapter 3.2)

In this chapter, a detailed overview of the application progress of different traditional, new and hybrid technologies in removing ARB and ARGs, mainly focusing on bacterial inactivation and break-down ARGs of disinfection technologies (chlorination, UV irradiation and ozonation), advanced oxidation processes (Fenton/solar fenton and photocatalysis), biological technologies (membrane bioreactor and constructed wetland), and combination of traditional and latest technologies which find out solutions for optimal reduction of ARB/ARGs. Furthermore, this review also focus on the regrowth potential of ARB/ARGs within those technologies, for example, different pathways of horizontal gene transfers, especially conjugation, promote ARG transfer among bacterial communities. Antibiotic-sensitive bacteria can obtain ARGs through HGT to form new ABR, known as the result of the regrowth.

3.2.1 Disinfection processes

3.2.1.1. Chlorination

Zheng et al. (2017) used NaClO as free chlorine compounds with different doses (2, 4, 8, 16 and 32 mg/l) to treat sulfamethoxazole - and tetracycline - heterotrophic bacteria with 30 min contact time. To determine ARB after disinfection contact, the heterotrophic plate count approach was applied (Zheng et al., 2017). ARB was measured as soon as chlorine contact occurred. The samples were serially diluted (in duplicate), plated, and incubated at 35°C for 72 hours to determine the colony-forming units (CFU) per milliliter (CFU/ml) of the samples. As a result, no presence of antibiotic resistance bacteria were at chlorine dose of 32 mg/l. According to Shi et al. (2022), to make a stock solution of free chlorine, NaOCl was diluted, and the concentration of free chlorine was assessed using the traditional iodometric technique (Shi et al., 2022). To get the mixture's first chlorine additions of 5, 10, 20, 50, 100, and 200 mg/l, respectively, the stock solution was added. For one hour, the reaction mixture was shaken at 120 revolutions per minute (rpm) while remaining at room temperature (25°C). The samples following chlorine contact plated on Luria-Bertani (LB) agar medium were incubated in the dark at 37°C for 48 h, and then CFUs were counted to assess the regrowth of ARB after treatment and subsequent storage. The ratio of the abundance of the targeted ARB in treated samples after 48 hours of storage (C_r) to the abundance of the targeted ARB at the conclusion of treatment was calculated, and values of C_r / C_i that were greater than 1 were regarded as evidence of the targeted ARB's post-chlorination regrowth. The findings revealed that for SMZ-ARB at a chlorine dose of 100 mg/l and GTM-ARB at a chlorine dose of 50 mg/l, respectively, the C_r to C_i ratio was 25.57 and 7.21, and the chlorine concentration of 200 mg/l showed no regrowth of SMZ-ARB and GTM-ARB. Huang et

al. (2011) also use NaOCl to treat multi-drug resistant bacteria under different NaOCl doses (0.5, 1.0, 2.0, 5.0 and 10.0 mg/l) with 10 min contact time ([J.-J. Huang et al., 2011](#)). The treated samples were serially diluted into 10 ml of nutrient agar and were transferred to the plates. The plates were then incubated for 24 hours at 37°C for counting ARB. The degree of inactivation was quantified using the log ratio of inactivated specific bacteria to specific bacteria before chlorination to assess the effects of chlorination on ARB. As a result, at CT value of 50 mg.min/l, highest log reductions were approached for multidrug resistant bacteria, especially 4.5 and 5.5 log reductions for ampicillin - and penicillin - resistant strains, respectively, compared with 3 log reductions of tetracycline-, chloramphenicol- and rifampicin - resistant strains. Huang et al. (2013) also used NaOCl with different dosage ranging from 0 - 2 mg/l with 10 min contact time to inactivate tetracycline - resistant E.coli ([J.-J. Huang et al., 2013](#)). The degree of inactivation was measured using the log ratio of CFU before disinfection to CFU after disinfection in order to assess the effects of chlorination on tetracycline-resistant E. coli. The plates were incubated for 24 hours at 37°C. The results showed that 5 log reduction of ARB approached at CT value of 10 mg.min/L. As results are mentioned, that high reduction of ARB is approached at high CT value. However, to maintain high effectiveness of ARB reduction, at fixed CT value high dose of chlorine and short time contact are recommended. To demonstrate the mentioned statement, Huang et al. (2011) fixed CT value at 50 mg Cl₂/l for three operation modes of chlorination compared between 2.0 mg/l with a contact time of 25 min, 5.0 mg/l for 10 min and 25.0 mg/l for 2 min ([J.-J. Huang et al., 2011](#)). Figure 3.1 illustrated that inactivation of penicillin-, ampicillin-, tetracycline and chloramphenicol- and rifampicin-resistant strains were more effective at two operational modes of 25 mg/l for 2 min and 5 mg/l for 10 min, compared to 2 mg/l for 25 min. Additionally, the performance of chlorination is also dependent on different ARB, especially tetracycline - resistant bacteria which were mentioned in the literature review that was more tolerant to chlorine, compared to the others ([J.-J. Huang et al., 2011](#); [J.-J. Huang et al., 2013](#); [Zheng et al., 2017](#)). As example, when increasing the chlorine concentration from 2 mg/l to 8 mg/l, the number of sulfamethoxazole-resistant bacteria decreased from 4.3x10³ CFU/ml to 2.3x10³ CFU/ml, while the number of tetracycline-resistant strains increased from 1.32x10³ CFU/ml to 6.53x10² CFU/ml. Huang et al. (2011) also reported that at three mode operations of 25 mg/l for 2 min, 5 mg/l for 10 min and 2 mg/l for 25 min, tetracycline-resistant bacteria showed less inactivation compared with inactivation of penicillin- and ampicillin-resistant strains ([J.-J. Huang et al., 2011](#)). Huang et al. (2013) also reported that the susceptibility of tetracycline-resistant bacteria decreased when increasing the chlorine concentration from 1.5 mg/l to 2.0 mg/l, and detection of tetracycline-resistant strains was observed at the chlorine concentration of 8 mg/l, however, in low values of 30 - 300 colonies ([J.-J. Huang et al., 2013](#)). Allard et al. (1992) demonstrated that the

hydrophobicity of the pBR322 tet(A) transmembrane segments in the cell membrane (Allard et al., 1992). The hydrophobicity of bacterial cells rises as tetracycline-resistant *E. coli* with high tetracycline resistance contain more tet(A) efflux protein, which can shield the bacteria from chlorine oxidation and chlorination and reduce the likelihood of inactivation. In other words, *E. coli* with higher tetracycline resistance levels are more tolerant of chlorine. Tetracycline may help remove chlorine from cells because it resembles other proton-dependent transport proteins associated with multi-drug resistance. Additionally, the tetracycline shares similarity with other proton-dependent transport proteins connected to multi-drug resistance, which could aid in removing chlorine from cells. In the end, the results demonstrated that even strains resistant to tetracycline were more chlorine tolerant, and that inadequate inactivation of ARB was detected when exposed to lower chlorine concentrations with longer contact durations. Even at high doses of chlorine, effective reduction is possible.

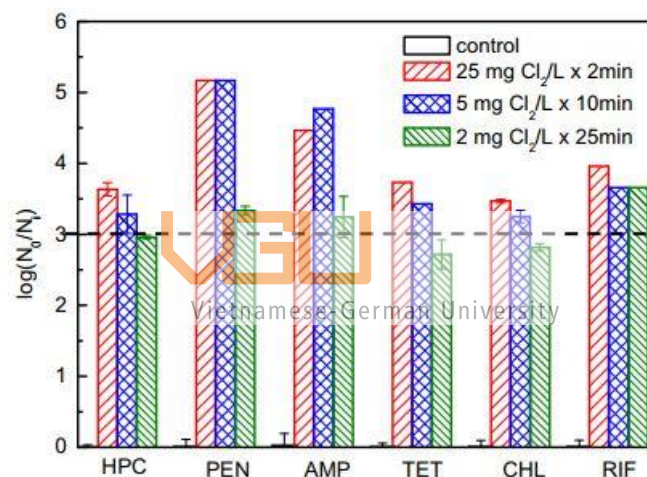


Figure 3. 1: Inactivation of total heterotrophic and antibiotic resistant bacteria by chlorination in different modes (J.-J. Huang et al., 2011)

Compared to inactivating ARB, cases of inactivating ARG call for larger chlorine dosages to approach successful inactivation. As a result of chlorine's initial damage to the cell wall, its potential destruction of the cell membrane due to its effect on peptidolycan components, and its continued entry into the cytoplasm where it acts on ARGs and other cytoplasmic targets. This indicates that intracellular ARGs are acted upon when the chlorine enters the cell membrane, causing ARGs to become inactive (Cai et al., 2021). Zhang et al. (2015) reported that log reductions of ARGs increased slowly from the chlorine dosage of 5 - 20 mg/l, and increased significantly when chlorine concentration reached 30 mg/l, that 1.2 and 1.3 - 1.49 log reductions of sul1 and other genes (tetX, tetG and int1) (Y. Zhang et al., 2015). Zhuang et al. (2015) used NaOCl as free chlorine with different concentrations (5, 20, 40, 80, 120, and 160 mg/l) with 120 min contact time to evaluate the inactivation of sul1, tetG and int1 (Zhuang et al., 2015). Sul1,

TetG, Int11, and 16S rDNA were measured using qPCR to assess ARGs. A melt curve stage was used to confirm specificity after the temperature procedure, which included 10 min at 95°C, 40 cycles of 15s at 95°C, and 1 min at the annealing temperature. Targeted gene qPCR efficiency varied from 90 to 100%, with standard curve R² values above 0.99. The degree of the log ratio of the gene copy number of the particular gene in the original wastewater samples to the gene copy number of the specific genes survived after the disinfection process at a specified dosages was used to characterise the effect of chlorination on ARGs. The results showed that the maximum inactivation of ARGs by chlorine at 160 mg/l was 3.16 log for sul1 and 3.24 log for tetG genes. Yuan et al. (2015) also conducted experiments to inactivate erythromycin- and tetracycline resistant genes with different NaOCl doses (0, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/l) for the contact time of 30 min (Yuan et al., 2015). The results showed that 0.42 and 0.1 log reductions of erythromycin- and tetracycline resistant genes was achieved.

Even while ARB are completely rendered inactive following chlorination, undamaged DNA traces in the ensuing cell debris may still be able to pass on resistance to bacterial populations downstream via HGT. It is thought that competent non-resistant recipient bacteria can accept extracellular ARGs-carrying plasmid or genomic DNA from a donor bacteria (donor cell) into the latter's genome, and the transformed recipient bacteria can express the extracellular ARGs (recipient cell) (He et al., 2019). In addition, ARB recovery after disinfection creates a membrane permeability and induces the synthesis of conjugative pilus on the surface of donor cells, that resulting in facilitation of conjugative transfer (M.-T. Guo et al., 2015).

According to chlorination-induced regrowth potential, sub-lethal dosage of chlorination (M.-T. Guo et al., 2015; Y. Zhang et al., 2017) play a role of promoting ARGs gene transfer between donor and recipient cells resulting in regrowth potential of ARGs/ARB within in wastewater after chlorination. Guo et al. (2015) conducted experiments on multi-drug resistant *E.coli* K12 as donor cells which harbour the plasmid RP4, and *E.coli* NK5449 as recipient donors with different chlorine doses of 0.5, 1, 2, 4, 8 and 16 mg Cl₂/l with 10 min contact time (M.-T. Guo et al., 2015). The number of transconjugants cells per recipient cells was used to indicate the frequency of conjugative transfer. At the CT value of 40 mg Cl.min/l, it showed that ineffective inactivation of donor and recipient cells with 0.09 - 0.3 for donor strains and 0.34 - 0.55 for the recipient strains thereby inducing an increase of conjugative frequency to 2.5 - 5.0 x 10⁻³, compared to the initial value of 1.0 x 10⁻³ at the optimal mating time of 6 hour. However, when increasing the CT values of chlorination to 80 mg.min/l, conjugative frequency approach the value of zero, and it remained at CT values of 120 and 160 mg.min/L latter. Lin et al. (2016) reported, that when increasing chlorine concentration to 0.5 mg/l, the value of conjugative frequency was below detection limit

(Lin et al., 2016). Zhang et al. (2017) also reported, that conjugative frequency increased significantly by from 3.4 to 6.4 fold, compared to the control value of 10^{-5} at the chlorine concentration of 0.1 to 1 mg/l (Y. Zhang et al., 2017).

Because chlorine has been shown to have a significant reactional capacity with membrane lipids and other membrane constituents, chlorination at low dosages resulted in the development of pilus or pores on the surface of conjugative cells, which likely increased cell permeability and ARG transfer routes. Additionally, fewer donor and recipient bacteria meant that conjugative transfer was minimised. But the effectiveness of chlorination also depends on certain ARGs and the microbial ecology.

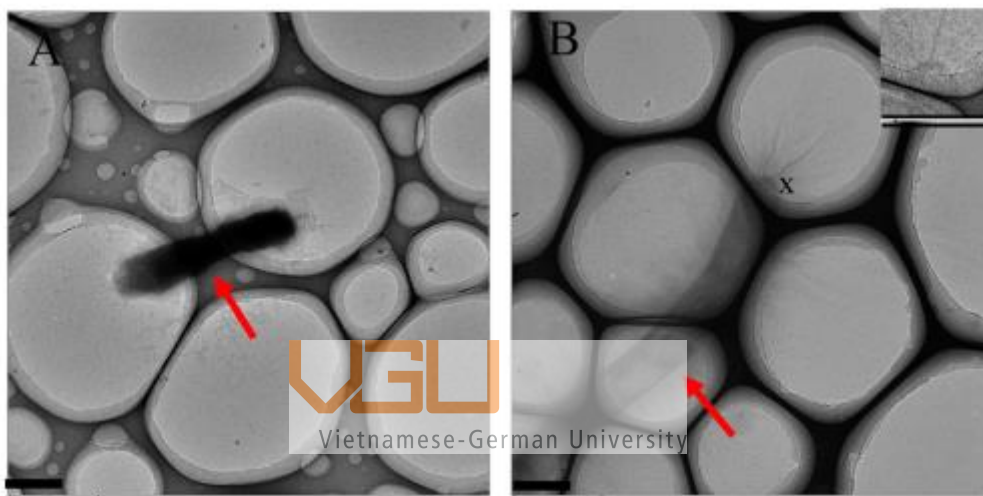


Figure 3. 2: The matting cell during conjugative transfer (left) before chlorination and the matting cell during conjugative transfer after chlorination with low CT-value of 20 mg Cl_2 .min/l (right) (M.-T. Guo et al., 2015)

In addition to impacts of sub-lethath concentration of chlorine, nutrients (N,P) and substract (C-source) were considered essential for the conjugative transfer as either the ARGs transfer by donor or the recipient required energy (M.-T. Guo et al., 2015). Especially, the presence of high concentration of ammonia nitrogen ($\text{NH}_3\text{-N}$) causes a decrease in ARGs/ARB removal (M.-T. Guo et al., 2015; Y. Zhang et al., 2015).

According to Zhang et al. (2015), in the chlorination tests, NaOCl was used to create various free chlorine dosages (5, 10, 15, 20, 25 and 30 mg/l) with a 30-minute contact period to eliminate various ARGs (sul1, tetX, tetG and intl1) (Y. Zhang et al., 2015). Additionally, the $\text{NH}_3\text{-N}$ concentration was established at 2.56 mg/l, 5 mg/l, and 15 mg/l to examine the impact of $\text{NH}_3\text{-N}$ concentration on the elimination of ARGs during chlorination. 2.56 mg/l served as the samples' background concentration in this analysis, and 5 mg/l and 15 mg/l were obtained by adding an appropriate volume of $\text{NH}_4\text{-Cl}$ stock solution. After a 30-minute reaction with 30 mg/l of free

chlorine, the samples were collected for the purpose of detecting ARGs. Detection of ARGs was conducted by using qPCR. The amount of ARGs removed was determined as the log ratio of the given gene's copy number in the initial wastewater effluent to the survival of the gene after disinfection at various dosages. The findings demonstrated that when chlorine was added at dosages of 5 mg/l to 20 mg/l and 25 mg/l, respectively, the elimination of ARGs increased gradually and significantly, respectively. The greatest removal for the *sul1* gene was attained at 30 mg/l free chlorine and was at 1.2 logs, whereas other genes obtained 1.30 to 1.49 logs removal. Regarding influence of $\text{NH}_3\text{-N}$ concentration on the removal of ARGs, higher $\text{NH}_3\text{-N}$ concentrations resulted in lower ARGs removal efficiencies by chlorination. The maximum log removal of target genes (*sul1*, *tetX*, *tetG* and *intl1*) was 1.20 – 1.49 logs at $\text{NH}_3\text{-N}$ concentration of 2.56 mg/l, whereas 0.03 – 0.1 log removal at NH_3 concentration of 15 mg/l. According to Guo et al. (2015), ammonia nitrogen concentration of 250 mg/l was used in the study to test the conjugative transfer under effect of ammonia nitrogen (Case I), compared to the situation without ammonia nitrogen (Case 2) (M.-T. Guo et al., 2015). As a result, Case I showed significant amplification of conjugative transfer being 4.2 – 5.4 fold higher, compared to Case II. Zhang et al. (2017) reported that conjugative transfer caused by chloramine concentration of 1 mg/l increased by 7.5 fold while the conjugative transfer caused by free chlorine concentration of 1 mg/l increased by 6.4 fold, that is compared to the control value of 10^{-5} (Y. Zhang et al., 2017). It is well explained that the reactivity of chloramine was much weaker than of free chlorine (HOCL and OCL) (L.-S. Wang et al., 2007). As a result, chlorine-induced disinfection slowly consumes it, ineffectively removing ARGs. Furthermore, decreased oxidation of chloramine may result in oxidative stress, which may trigger a reactive oxygen species (ROS) response and increase cell permeability, which facilitates the transfer of genes or nutrients. More ammonia nitrogen concentration means more chlorine is needed to remove combined chlorine because the ratio of chlorine to ammonia nitrogen must approach 7.6:1 in order to obtain a high level of free chlorine within the total chlorine residue when ammonia nitrogen is present (Y. Zhang et al., 2015).

Table 3. 3: Chlorination-induced removal of ARGs/ARB

ARGs/ARB	Chlorine dosage	Contact time	Remove efficiency (%)	Regrowth potential	Reference
Antibiotic resistance genes					
<i>Sul1</i> , <i>tetX</i> , <i>tetG</i> and <i>intl1</i>	5 – 30 mg/l	30 min	1.20 log reduction of <i>sul1</i> , and 1.30 – 1.4 log	N/a	(Y. Zhang et al., 2015)

			reduction of tetX, tetG and intl1 with 30 mg/l at 30 min contact time		
Sul1 and tetG	5 - 160 mg/l	120 min	3.16 and 3.24 log reductions for sul1 and tetG genes, respectively at 160 mg/l	N/a	(Y. Zhang et al., 2015)
tetA, tetM, tetO, tetQ, tetW, sul1 and sul2	2, 4, 8, 16, 32 mg/l	30 min	Higher 85% removal of tet genes at 8 mg/l Higher 99% removal of sul genes at 16 mg/l	N/a	(Zheng et al., 2017)
EreA, ereB, ermA, ermB, tetA, tetB, tetM and tetO	0 - 300 mg/l	5 - 180 min	0.42 log reductions of ere genes 0.1 log reduction of tet genes	N/a	(Yuan et al., 2015)
Antibiotic resistance bacteria					
SMZ – , GTM - ARB	- 5, 10, 20, 50, 100 and 200 mg/L	60 min	99.7% with dosage of 200 mg/l	No regrowth potential at all dosage	(Shi et al., 2022)

Multidrug – resistant bacteria	25 mg Cl ₂ /l 5 mg Cl ₂ /l 2 mg Cl ₂ /l	2 min 10 min 25 min	No reactivation was observed when the dosage reached 5.0 mg/l for 10 min	Regrowth occurs at 2 mg Cl ₂ /l x 25min (chloramphenicol and tetracycline - resistant strains)	(J.-J. Huang et al., 2011)
tetA – resistant E.coli harbouring pBR322	0-2 mg/l	10 min	5 log reduction of tetA – resistant E.coli at 1.0 mg Cl ₂ /l	>1.0 mg Cl ₂ /l increase average tetracycline resistance of tetracycline-resistant E.coli	(J.-J. Huang et al., 2013)
Tetracycline – resistant, sulfamethoxazole – resistant bacteria	2, 4, 8, 16, 32 mg/l	30 min	No detected bacteria at 32 mg/l	Slight increase in tetracycline – resistant bacteria at 8 mg/l	(Zheng et al., 2017)
Multidrug-resistant E.coli HB101 and E.coli K12 harbouring RP4	0 - 0.5 mg/l	N/a	High doses of 0.3 - 0.5 decreased conjugative transfer	Low doses of 0.02 - 0.2 mg/l	(Lin et al., 2016)
Multidrug resistant-E.coli K12 and NK5449 harbouring RP4 plasmid	0.5, 1, 2, 4, 8, 16 mg/l	10 min	< 10 ⁴ CFU/ml at CT value of higher 80 mg.min/l	Low doses of 0.5 – 4 mg/l and high concentration of NH ₄ -N	(M.-T. Guo et al., 2015)
Multidrug E.coli S17-1 (donor cells) and E.coli K12 and S. typhimurium (recipient cells) harbouring	0.1 - 5 mg Cl ₂ /l as free chlorine	30 min	N/a	Low dose of 0.1 – 1 mg/l	(Y. Zhang et al., 2017)

pUA139 and pBHR1 plasmids, respectively	0.1 - 1 mg Cl ₂ /l as chloramine				
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3.2.1.2. UV irradiation

Without harming a cell's membrane, UV light inactivates microorganisms by creating CPDs and 6-4 photoproducts de their DNA. No major taxonomic alterations in the bacterial populations were seen as a result of this inactivation mechanism. Before and after UV disinfection, the frequency of ARGs per total bacterium, as determined by the 16rRNA gene, remained constant. The absence of a substantial difference is attributed to the measuring technique since the molecular approach is unable to distinguish between living and dead cells, regardless of whether pretreatment is used to distinguish between the two ([Jovanovic et al., 2021](#)).

Guo et al. (2015) conducted UV-induced disinfection on the plasmid RP4 – carrying *E.coli* K12 and *E.coli* NK5449 with different antibiotic resistance (Tetracycline, erythromycin and gentamycin) as a donor and a recipient, respectively to test frequency of conjugation ([M.-T. Guo et al., 2015](#)). The UV irradiance values were fixed at 0.17 mW/cm² throughout the experiment, while the exposure time was changed for various plates to reach UV doses of 1, 2, 4, 8, 10, 20, and 40 mJ/cm². As a result, below 10⁴ CFU/ml of bacterial survival was approached at UV dose of higher than 10 mJ/cm², compared to initially microbial concentration of 10⁷ CFU/ml, that greatly inhibited the frequency of conjugative transfer. Because low bacterial number causes lower chance of cell - cell contact which form a pilus or pore that allows for the passage of plasmids. Furthermore, Huang et al. (2013) evaluated UV₂₅₄ performance on the removal of tetracycline-resistant *E.coli* with different UV light doses from 0 to 80 mJ/cm² ([J.-J. Huang et al., 2013](#)). Evaluation of effects of UV irradiation on tetracycline-resistant *E.coli* was conducted by determining the degree of inactivation. The degree of inactivation was quantified using plate counts, that based on log ratio of CFU before disinfection to CFU after disinfection. The results showed that over 4 log reduction of tetracycline - resistant *E.coli* was achieved at the UV influence of 10 mJ/cm², and increased slightly from 20 mJ/cm² to 80 mJ/cm² of UV influences. The effective performance of UV irradiation on inactivation of ARB depended strongly on specific microbes ([McKinney et al., 2012](#); [Shen et al., 2020](#)). Huang et al. (2016) used the UV doses which were in the range of 0 to 40 mJ/cm² to calculating the inactivation rate of different tetracycline-resistant strains, that was to indicate the tolerance to UV light ([J.-J. Huang et al., 2016](#)). The UV dose at which inactivation ratios reach stability could be considered as the minimum UV dose needed to inactivate a tetracycline strain effectively. The degree of inactivation was measured using plate

counts to assess how UV irradiation affected tetracycline-resistant bacteria. The log ratio of CFU after inactivation to CFU before to UV disinfection served as a measure of inactivation degree. It was shown that the maximum inactivation ratios of the isolates (*Aeromonas* 1-4, *Enterobacter* 1-3, *Escheria* 1-5, *Citobacter*, *Hafnia* and *Klebsiella* 1-2) from the secondary effluent of the municipal WWTP in China ranged from 4.9-log to 6.5-log reduction at the UV influence of 40 mJ/cm². Additionally, it was shown that the minimum of UV dose for inactivating *Escheria*, *Aeromonas*, *Klebsiella*, *Citrobacteria*, and *Hafia* strains were 10 mJ/cm², compared to 20 mJ/cm² required for inactivating *Enterobacter* strains. Shen et al. (2020) evaluated the performance of UV-LED with different levels of influence (0 - 50 mJ/cm²) to inactivate two Gram-positive tetracycline - resistant bacteria strains (*Bacillus cereus* as TRB-3 and *Bacillus pumilus* as TRB-5) at two different wavelengths (268 nm and 275 nm) (Shen et al., 2020). After UV inactivation, the quantity of ARB was counted by broth microdilution. Tetracycline-resistant bacteria solution and nutritional broth were combined, and the mixture was incubated at 37°C for 16 to 20 hours. By comparing the colony count before to disinfection to the colony count after disinfection, the log inactivation ratio of bacteria was used to examine the effectiveness of bacterial inactivation. Regardless of using 268nm or 275 nm, the UV doses to cause complete inactivation, that is lower than limit of detection, of TRB-3 were lower than that of TRB-5. In details, it was shown that a fluence of 15.36 by both 268 and 275 nm UVC-LED is able to inactivate TRB-3 complete, at 5.4 log reduction, while TRB-5 could be completely inactivated (5.7 log reduction) by 23.04 and 30.72 mJ/cm² at 268 and 275 nm, respectively. It is clear that a strain of *B. pumilus* which was found to be more resistant to UVC-LED disinfection than TRB-3. This is because *B.pumilus* possess much higher resistance to UV irradiation than other species of *Bacillus* and thereby enables survival under standard disinfection practices (Gioia 2007; Kempf 2005; Newcombe 2005; Setlow, 2006). According to McKinney and Pruden (2012), UV fluence rate of approximately 0.2 mW/cm² with fluence times of 10 to 5000 second was used for inactivating two antibiotic resistant-Gram-positive strains (*Staphylococcus aureus* and *Enterococcus*) and two antibiotic resistant - Gram-negative strains (*E.coli* and *P. aeruginosa*) (McKinney et al., 2012). Each individual data point was analysed in triplicates for each UV disinfection experiment, which was carried out in experimental triplicates. Following serial dilution, the UV-exposed material was plated on LB agar with two antibiotic resistant-Gram-negative bacteria and brain heart infusion with two antibiotic resistant-Gram-positive strains to count CFU. For 24 to 48 hours, plates were incubated in the dark at 37°C. By comparing the CFUs before UV exposure to the CFUs after disinfection, the effectiveness of UV irradiation was described. The results showed that 4-5 log reductions of ARBs at low UV doses of 10 - 20 mJ/cm². It was found that the two Gram-positive bacteria were more resistant to UV disinfection than two Gram-negative bacteria.

As a result, the thicker peptidoglycan layer characteristics of Gram positive or organism may offer some protection against UV inactivation. Furthermore, two Gram-positive bacteria also possessed smaller total genome size (2,872,769 and 2,826,716 bps for *Staphylococcus aureus* and *Enterococcus*, respectively) which could also have reduced their susceptibility to UV, compared to two Gram-negative strains (5,068,389 and 6,264,404 bps for *E.coli* and *P. aeruginosa*, respectively). Chang et al. (2017) also illustrated the genome size of multidrug resistant *P. Aeruginosa* was 6500 kbp that needs the UV dose of 2 mJ/cm² to achieve 1 log reduction, compared to vancomycin resistant *E.faecium* whose genome size was 3000 kbp requiring 8 mJ/cm² for 1 log reduction, that demonstrated large genome sizes of bacteria easily damaged by UV disinfection ([Chang et al., 2017](#)).

The literature illustrates that higher UV doses required to inactivate ARGs, compared with inactivation of ABR. Zheng et al. (2017) tested different influences (10, 20, 40, 80 and 160 mJ/cm²) at UV wavelength of 253.7 nm on removing different ARGs (tetA, tetM, tetO, tetQ, tetW, sul1 and sul2) ([Zheng et al., 2017](#)). The results showed that 52.0% - 73.5% at 40 mJ/cm² or less, and 79.7% - 92.0% at 160 mJ/cm² for tet gene removals, and 78.1% and 71.1% for sul1 and sul2 removals (at 160 mJ/cm²), respectively. According to McKinney et al. (2012), the removals of 4 target ARGs were evaluated at different UV doses from 50 mJ/cm² to 400 mJ/cm² ([McKinney et al., 2012](#)). The effects of UV irradiation on the removals of target ARGs were evaluated through the surviving fraction of the specific genes before and after UV exposure, that was determined from qPCR measurement. The results showed that reductions of mec(A), van(A), tet(A), amp(C) were 4-5 log which were achieved at high UV doses of 200 to 400 mJ/cm² ([McKinney et al., 2012](#)). In terms of removing tetL, Shen et al. (2020) showed that the highest reductions of tetL expression were 0.93 and 0.95 fold-decrease observed in the 268 nm with influences of 38.4 and 46.08 mJ/cm², for *Bacillus cereus* and *Bacillus pumilus*, respectively ([Shen et al., 2020](#)). McKinney et al. (2012) illustrated various responses of ARGs (ampC, tetA, vanA and mecA) that the order of least susceptible to most susceptible to UV performance was ampC > tetA > vanA > mecA because mecA carried the most potential T = T (thymine dimers) sites, exactly 263 counts, compared to 40 counts of ampC and was the most readily damaged ([McKinney et al., 2012](#)). It was found that mechanism of UV inactivation is not only depend on pyrimidine dimer formation known as the direct damage of DNA. ARG inactivation actually decreased with increase T-T (adjacent thymine) sites per genome, but surprisingly increased with C-C sites per genome, however the opposite trend was witnessed within ARG inactivation ([McKinney et al., 2012](#)). It was same reported by Chang et al. (2017) that the bla_{TEM-1} genes contains 1.5X the number of adjacent thymidine bases than tetA results in 1.2X faster reaction of bla_{TEM-1} than tetA, and emphasized that the adjacent thymidine base content of DNA is arguably more important than

DNA size when predicting a genome's reactivity with UVC inactivation ([Chang et al., 2017](#)). Zhang et al. (2017) reported that that log reductions of tetB, sul2 were 1.18 and 0.85, respectively at UVC dose of 80 mJ/cm² ([C. Zhang et al., 2017](#)). However, when increasing UV dose to 400 mJ/cm², the relative abundance of tetB and sul2 increased from 8.24 to 14.73 and from 2.07 to 19.32, respectively within SER2 strain (contains tetB, tetM, tetW and sul2), following the ARGs to 16S rRNA ratio, and within SER6-1 strain (tetA, tetM, tetW, sul1, sul2, bla_{TEM}, bla_{CMY-2}, and bla_{CTX-M}), the relative abundances of tetA and sul2 genes increased from 0.18 to 9.43 and from 1.42 to 51.59, respectively. As a results of ARG tendencies after UV inactivation, the sul2 genes are more difficult to damage, which could be attributed to the differences in potential adjacent thymine and thymine dimers. UV light can primarily be adsorbed by pyrimidine nucleobases compared with purine nucleobases, depending on the higher susceptibility of thymine and uridine to the 254 nm wavelength ([Durbbeej et al., 2003](#)).

Meanwhile, a paradoxical phenomenon is that, the UV-damaged DNA can be recovered by way of two major repair mechanism such as photoreactivation by the enzyme photolyase that requires light of 330 - 480 nm and nucleotide excision repair well-known as the dark repair ([Friedberg 1995; Harm, 1980; Kelner, 1949; Oguma 2001](#)). It was mentioned that CPDs are induced from UV irradiation with maximum absorption around 260 nm. Where CPDs are monomerized by photolyase with light of 330-480 nm, the inactivated bacteria can be recovered. In nature, photolyase is a large family of enzymes and can be widely found in bacteria as an adaption to UV light exposed habit. Regarding photoreactivation after UV disinfection, Shen et al. (2020) prepared samples for photoreactivation on two Gram positive bacteria which were resistant to tetracycline (*Bacillus cereus* - TRB3 and *Bacillus pumilus* - TRB5) after contacting two different UV wavelength (268 and 275 nm) with different influences (7.68, 15.36, 23.04, 30.72, 38.4 and 46.08 mJ/cm²) ([Shen et al., 2020](#)). These samples are diluted and plated at 2, 4, 8, 12 and 24 hour to evaluate the extent of repair. It was shown in Fig 3.3 that there were no detections of photoreactivation of both *Bacillus* strains after disinfection by UV wavelength of 268 nm with high levels of influences (23.04, 30.72, 38.4 and 46.08 mJ/cm²) at the 2, 4, 8 and 12 - hour platings. However, there were greater increases in the percentage of photoreactivation with lower UV influences (7.68 and 15.36 mJ/cm²) for both of *Bacillus* strains, that linearly rises with longer time required for plating. In addition to UV wavelength of 268 nm, UV wavelength of 275 nm only showed significant rises of photoreactivation in TRB-5 at UV influences of 7.68, 15.36, 23.04 and 30.72 mJ/cm² at 4, 8 and 12 - hour platings, and there was no detection of photoreactivation at high levels of UV influences (38.4 and 46.08 mJ/cm²).

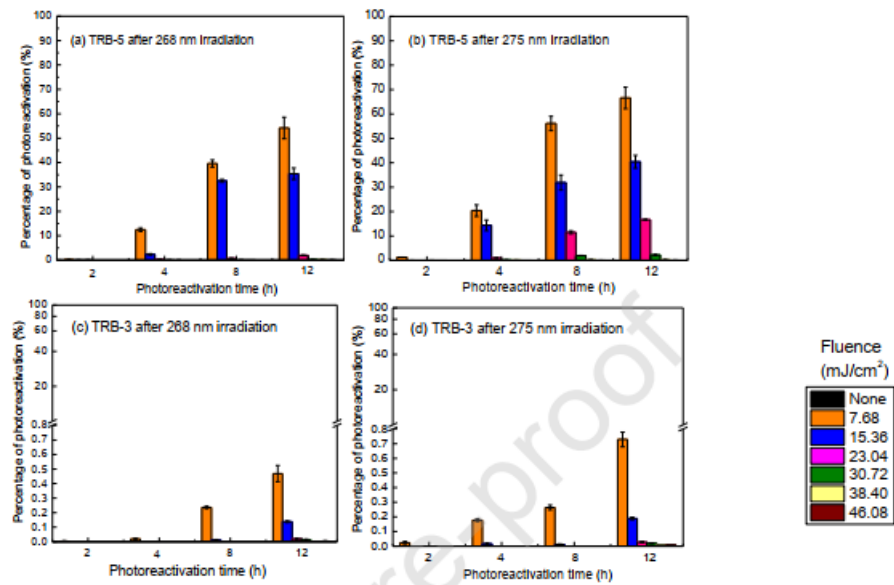


Figure 3. 3: Photoreactivation of two *Bacillus* strains at two different UV wavelength with different influences under 2, 4, 8 and 12 - hour platings ([Shen et al., 2020](#))

In terms of dark repair, Shen et al. (2020) prepared samples for dark repair by taking these in tubes covered with aluminum foil after UVC-LED irradiation and kept in a cardboard box over the same time period (24h) ([Shen et al., 2020](#)). The serial dilution and plating were done at 2, 4, 8, 12 and 24 h, to evaluate the extent of repair. Figure 3.4 showed that regardless of inactivation by 268 nm or 275 nm, higher levels of UV doses, higher than 15.36 mJ/cm², prevented effectively dark repair-caused reactivation. However, when lasting time storage from 8 hour to 12 hour, there were detections of reactivation at all UV doses. In terms of results of Huang et al. (2016), there was detection of an increase in survival of tetracycline-resistant strains in secondary effluent after UV disinfection with high UV doses of 20 mJ/cm² and 40 mJ/cm², and with 24-hour incubation ([J.-J. Huang et al., 2016](#)).

The tendency of regrowth was noticed after a longer duration of incubation based on the regrowth potential of ARB/ARGs within dark repair and photoreactivation, as well as those results that were described before. There is typically a delay before regrowth is initially noticed due to the time it takes for reactivation following DNA repair. These findings revealed that the regrowth period needed for reactivation spans from 12 to 24 hours. According to the results above, the prevalence of ARGs, particularly tetracycline resistance genes, has a tendency to rise under high UV levels (> 10 mJ/cm²). According to the argument, under the prolonged pressure of UV, the resistance profile of the surviving strains could change as a result of mutations, lesion of ARGs, or changes to the bacterial. It is argued that under the long-term pressure of UV, the resistance profile of the surviving strains could change as a result of mutations, lesion of ARGs, or changes in the bacterial metabolism to antibiotics ([C. Zhang et al., 2017](#)).

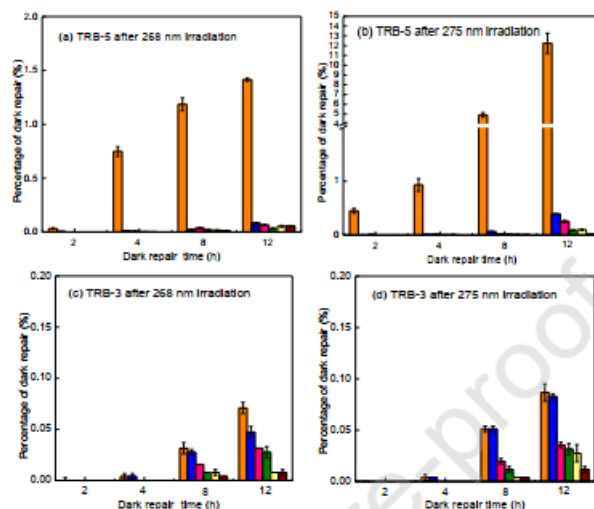


Figure 3. 4: Dark repair of two *Bacillus* strains after UV - induced inactivation at different times (2, 4, 8, 12h) (Shen et al., 2020)

Table 3. 4: UV-induced removal of ARGs/ARB

ARGs/ARB	Operational conditions	Contact time	Remove efficiency (%)	Regrowth potential	Reference
Vietnamese-German University					
Antibiotic resistance genes					
tetA, tetM, tetO, tetQ, tetW, sul1 and sul2	10, 20, 40, 80 and 160 mJ/cm ² at 254 nm	N/a	79.7 – 92% removal of tet genes at 160 mJ/cm ² 78.1% and 71.1% reduction of sul1 and sul2, respectively at 160 mJ/cm ²	N/a	(Zheng et al., 2017)
tetL	7.68, 15.36, 23.04, 30.72, 38.4 and 46.08 mJ/cm ² at 268 nm and 275 nm	N/a	0.93 and 0.95 reductions of tetL at 268 nm with 38.4 nm and 46.08, respectively	Photoreactivation and dark repair	(Shen et al., 2020)

	(0.38 mW/cm ²)				
Mec(A), van(A), tet(A), amp(C)	2 - 1000 mJ/cm ² (0.2 mW/cm ²)	10 - 5000s	3 - 4 log reduction at 200 - 400 mJ/cm ²	Thymine adjacent and dimers	(McKinney et al., 2012)
tetB and sul2	0, 20, 40, 60 and 80 mJ/cm ² (0.1 mW/cm ²)	40 - 4000s	1.18 and 0.85 log reductions for tetB and sul2, respectively at UV dose of 80 mJ/cm ²	Thymine adjacent and dimers	(C. Zhang et al., 2017)
Antibiotic resistance bacteria					
tetA – resistant <i>E.coli</i>	2, 4, 5, 10, 20 mJ/cm ²	N/a	Exceeded 4 log reduction at 10 mJ/cm ²	High doses of UV	(J.-J. Huang et al., 2013)
Tetracycline – resistant bacteria (TRB)	0, 2, 5, 10, 20 mJ/cm ²	N/a	3 log removal at 10 mJ/cm ²	Dark repair and high doses of UV	(J.-J. Huang et al., 2016)
Tetracycline – resistant <i>Bacillus pumilus</i> and <i>Bacillus cereus</i>	0 - 50 mJ/cm ² at two different wavelengths 268nm and 275 nm (UVA – LED)	N/a	5.7 and 5.4 log reduction of <i>Bacillus pumilus</i> and <i>Bacillus cereus</i> by 15 mJ/cm ² and 23.04 mJ/cm ² at 268 nm respectively	Photoreactivation and dark repair	(Shen et al., 2020)

Tetracycline-, erythromycin-, gentamycin-resistant <i>E.coli</i>	1, 2, 4, 8, 10, 20, and 40 mJ/cm ²	N/a	<10 ⁴ CFU/ml at > 10 mJ/cm ²	Bacterial concentration > 10 ⁴ CFU/ml increase conjugative transfer	(M.-T. Guo et al., 2015)
Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA), vancomycin-resistant <i>Enterococcus faecium</i> (VRE), Tetracycline-resistant <i>E.coli</i> SMS-3-5, and ampC-resistant <i>P. aeruginosa</i> 01	2 - 1000 mJ/cm ² (0.2 mW/cm ²)	10 - 5000s	4 - 5 log reduction at 10 - 20 mJ/cm ²	Thymine adjacent and dimers, and genome sizes	(McKinney et al., 2012)

As a result of research surrounding UV disinfection, it was stated that ARG damage needs a far greater influence than what required for bacterial inactivation indicates a more complex cellular response UV light rendering ARG destruction than ARB inactivation ([McKinney et al., 2012](#)). Lethal UV influences and wavelengths, microbial diversity, and ARG structures are other key topics covered in this section to further assess the effectiveness of the inactivation and potential regrowth of AMR within wastewater after UV disinfection, which ultimately optimises the operational conditions of UV disinfection.

3.2.1.3. Ozone (O₃)

Ozone is a potent oxidant that has antimicrobial effects on a number of microorganisms. Bacterial ozonation's primary mode of inactivation involves O₃ attacking and cleaving the cell wall. As a result, proteins' carbon and nitrogen bonds are broken and nucleic acids undergo oxidative denaturation, which results in depolymerization. To amino acids and unsaturated C-C bonds found in proteins, peptidoglycans, and lipids in cell walls and membranes, ozone is known as a highly reactive gas. O₃ may therefore continue to assault intracellular ARGs after the cell wall

and cell membrane have already been damaged ([Dodd, 2012](#)). The target microorganism's physical properties, contact time, and O₃ doses all have an impact on the outcome of ozonation. Different bacteria exhibit different ozonation sensitivities, and these particularly correspond to the genome's guanine-cytosine composition of the target organism. However, the reactivity of ozone to double-stranded DNA is not as strong as anticipated because of the hydrogen bonds between DNA double-strands. This raises concerns regarding how effectively ozonation removes ARGs ([Cai et al., 2021](#)). Additionally, the specific O₃ dose expressed, which is the applied O₃ concentration normalised to dissolved organic carbon, has an impact on the effectiveness of ozonation to reduce bacterial load ([Iakovides et al., 2021](#)).

Regarding ARB removal, the performance of ozonation to reduce ABR within wastewater that possibly depends on different opportunistic bacteria. Alexander et al. (2016) investigated the impact of ozone treatment on ARB by adjusting ozone concentration to 0.9g O₃/gDOC ([Alexander et al., 2016](#)). Samples were taken once every two weeks and were processed immediately for DNA extraction. The reduction of ARB prior to and after ozone treatment was determined by using q-PCR through the use of genetic markers. The results showed that 98.9% reduction of MDR *Enterococci*, respectively, compared to lower removal percentage of *P.aeruginosa* at ozone dose of 0.9 gO₃/gDOC. PCR-DGGE analyses showed that a high G-C content in the amplicon of 16S rDNA fragment were more abundant in the wastewater population of the ozone outflow due to their stronger migration within the gel, while bacteria with low content of G-C within genomic structure seemed to be more efficiently reduced by the ozone. Furthermore, the degeneration of DNA by ozone was reported to be T- and G- dependent, because these two nucleotide bases are more susceptible to ozone. Additionally, *P. aeruginosa* is more tolerant to ozone than *enterococci* because the mucoid character of *P. aeruginosa* with increased production of extracellular polymeric substances (EPS) might be responsible for the reduced impact of ozone. In addition to microbial diversity, flocs or suspended solids also cause negative impacts on ozonation process ([Czekalski et al., 2016](#); [Pak et al., 2016](#)). Park et al. (2016) evaluated how efficiently O₃ removed, and transconjugants (pB10), and explored optimal ozone dose (3, 5, 7 and 10 mg/l) ([Pak et al., 2016](#)). A culture-based method was used to calculate the decline in the number of viable ARB colonies after ozonation. ARB-containing disinfected samples were serially diluted with PBS to yield 30 to 300 colonies on each LB agar plate. The colonies on the plates were counted after 16 hours of incubation at 37°C. Additionally, qPCR was used to assess how much the amount of pB10 plasmid decreased following disinfection. After being cleaned, the culture was centrifuged for five minutes at room temperature, and DNA was extracted from the pellet. Using a highly specific primer, q-PCR was used to validate the extraction of the pB10 plasmid. The survival log ratio of ARB, pB10 concentration, and pB10 plasmid transfer after

interaction with ozone at experimentally provided amounts were used to determine the inactivation efficiency of pB10 and ARB. When the ozone dose was 7 mg/l, the removal efficiency of MDR *E.coli* DH5 α reached 4-log at CT value of 122.73 mg.min/L, compared to higher CT value of 127.15 that was required for reducing 4 log of pB10. However, when the ozone doses decreased to 3 or 5 mg/l, log reductions of MDR *E.coli* DH5 α were 2, and the decrease of pB10 plasmid transfer and the removal of pB10 plasmid were not sufficient in practice. It was explained that there is possibility that even though the ARB cell surface was damaged by ozonation, the plasmid on ARG were not inactivated because the mechanism of ozone disinfection was caused by cell wall disruption rather than penetration into cell wall (Cai et al., 2021). Under disturbance of flocs, viable and MDR bacteria were detected, albeit at low levels, even at the highest specific ozone dose of 0.85 gO₃/gDOC, while reduction of sul1 was 1.4 log at ozone dose of 0.5 - 0.6 gO₃/gDOC in case of the presence of flocs, compared to below limit of detection (LOD) at ozone dose of higher 0.4 gO₃/gDOC in case of the absence of flocs. Park et al. (2016) also reported that results for removal of ARB, pB10 plasmid, and pB10 plasmid transfer showed a decreasing in the ozone disinfection efficiency as the increased flocs concentration (Pak et al., 2016). Interestingly, the observed different in qPCR and plate count data suggested that flocs greater than 10 μ m can shield targeted bacteria and their ARG from inactivation (Czekalski et al., 2016). Additionally, flocs or suspended solids are firstly decomposed into high-molecular-weight compounds and then further into low-molecular-weight compounds by ozonation process. Therefore, floc or suspended solid-induced consumption of ozone leads to ineffective performance of ozone oxidation (Pak et al., 2016)

According to ARGs removal, intracellular ARGs (iARGs) might be less susceptible to ozonation because iARGs are attacked after cell wall and cell membrane are damaged by ozone oxidation (Dodd, 2012). Iakovides et al. (2019) used differently specific O₃ doses (0.125, 0.25, 0.5 and 0.75 gO₃/gDOC) to remove various ARGs (int11, aaDA1, dfrA1, qacED1 and sul1) with different hydraulic retention time (10, 20, 40 and 60 min) (Iakovides et al., 2019). Quantitative polymerase chain reaction (q-PCR) using SYBR Green chemistry was employed to measure the abundance (per ml of sample) of the target ARGs. The results illustrated that 4 - 6 log reduction of ARGs approached optimally at 40 min contact time and O₃ dose of 0.75 gO₃/gDOC. Alexander et al. (2016) also reported that high reductions of vanA, bla_{VIM}, ampC and ermB were 49.9%, 18.7%, 69.8% and 99.3% at ozone concentration of 0.9 \pm 1 gO₃/gDOC, respectively (Alexander et al., 2016). Czekalski et al. (2016) evaluated the optimal removal of sul1 genes under different O₃ doses of 0.45 - 0.55 gO₃/gDOC, and there was no detection of sul1 when the O₃ doses were recorded at the values of higher 0.4 gO₃/gDOC (Czekalski et al., 2016). A more recent study used various O₃ doses (0, 2, 4, 6, 8 and 10 mg/l) with 10 min contact applied to inactivate tet genes (tetA, tetM,

tetO, tetW and tetQ) and sul genes (sul1 and sul2) resulting in 49.2 % and 34.2% of tet and sul genes, respectively at 2 mgO₃/l. However, at higher doses of 4, 6, 8 and 10 mg/l showed lower levels of ARG reductions ([Zheng et al., 2017](#)). The ability eliminate ARGs was not dramatically improved by increasing the ozone dose. This is attributed to ozone's strong oxidizing ability which results in its reaction with a variety of cell material and reduces the opportunity to DNA or ARGs. Additionally, at a high concentration of ozone, the phenomenon of self-decomposition of ozone could lead to lowly effective performance of ozone ([Foroughi et al., 2021](#))

It should be noted that oxidizing agents (O₃ and OH^{*}) produced during ozonation process possibly causes induction of "Viable but not cultivable" (VBNC) state in bacteria which might have ability to transfer ARGs among bacterial community ([Iakovides et al., 2019](#); [Pak et al., 2016](#)). Additionally, ozone time, the applied ozone concentration, the physical characteristics of the examined ARB and the wastewater composition, are the major parameters affecting the bacterial regrowth after ozone disinfection ([Iakovides et al., 2019](#)). Furthermore, oxidative stress caused by sub-lethal of antibiotics possibly not only activate anti-oxidative and oxidative damage-associated responses, typically recA system which is known as an induction of horizontal gene transfer ([Alexander et al., 2016](#)). The regrowth potential of trimethoprim (TMP) - and sulfamethoxazole (SMX) - resistant *E.coli* were tested in two experiments: (1) low ozone dose and high contact time (0.25gO₃ and 40 min), and (2) higher ozone dose and shorter contact time (0.75 and 20 min), and antibiotic-resistance *E.coli* of two experiments were incubated in different time periods (24,48, or 72h) in the dark at both 24±1°C and 44±1°C. As results shown in Figure 3.5, high dose of ozone and short contact time induced the regrowth of antibiotic-resistance *E.coli* at all of incubated times, while there were no detections of the regrowth of antibiotic resistance bacteria in the mode of low ozone dose and high contact time ([Iakovides et al., 2019](#)). Sousa et al. (2017) conducted ozonation experiments to evaluate the efficiency of ozone treatment on the removal of different ARB from conventionally treated effluents ([Sousa et al., 2017](#)). These tests were conducted with ozone flowing at a constant flowrate of 150 cm³/min and an input concentration of 50 g/Nm³ for various contact times (15, 30 and 60 min). The amount of ARB in wastewater samples was analysed in order to gauge the effectiveness of ozonation and the possibility for ARB regrowth. Following a series of dilutions, treated samples were filtered using cellulose nitrate membranes. For the purpose of counting ARB, the filtered samples were cultured on culture medium at 37°C for 24 hours. The effectiveness of the treatment was determined by the log ratio of untreated to treated ARB. In order to determine the ARB's ability to develop following ozonation, treated samples were kept at room temperature and light in cotton-pumped Erlenmeyer flasks with a liquid to air ratio of 1:2. According to the findings, the log decrease of MDR *E. coli* A2FCC14 was 4.4 at a 15-minute contact duration. MDR *E. coli* A2FCC14 were

inactivated to levels below the limit of detection (0.5 CFU/100 ml) during a prolonged ozone exposure (60 min). Furthermore, no reactivation was observed after 60 min ozonation, while for the shorter time tested, 15 min, regrowth potential of multidrug resistant *E.coli* were detected. It might be attributed to wastewater matrix, for example, concentration and type of dissolved organic material, or the presence of flocs or particles, reduce the stability of ozone or can shield microbes from its effects, thereby decreasing the ozone (Czekalski et al., 2016; Sousa et al., 2017).

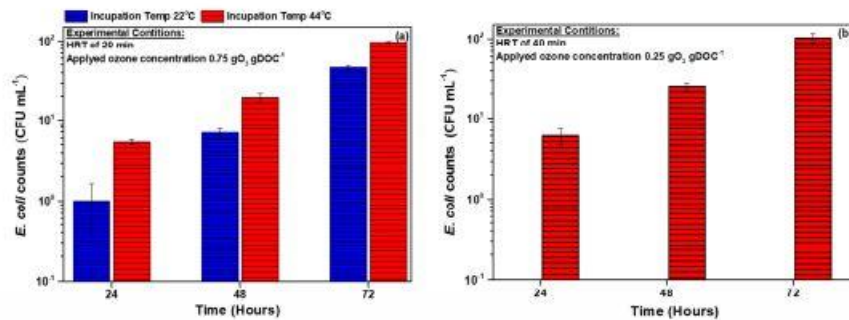


Figure 3. 5: Regrowth potential of examined total and antibiotic-resistance *E.coli* at two mode operations: (a) high ozone dose and low contact time ; (b) low ozone dose and high contact time in different time incubation (24, 48 and 72h) and different temperature (22°C and 44°C) (Iakovides et al., 2019)

Table 3. 5: Ozone-induced removal of ARGs/ARB

ARGs/ARB	Operational conditions	Contact time	Remove efficiency	Regrowth potential	Reference
Antibiotic resistance genes					
tetA, tetM, tetO, tetQ, tetW, sul1 and sul2	0, 2, 4, 6, 8, 10 mg/l	10 min	49.2% removal of tet genes at 2 mg/l 34.5% removal of sul genes at 2 mg/l	N/a	(Zheng et al., 2017)
Int1, aaDA1, dfrA1, qacED1 and sul1	0.125, 0.25, 0.5 and 0.75 gO ₃ /gDOC	10, 20, 40 and 60 min	4 - 6 log reduction at 0.75 gO ₃ /gDOC and 40 min contact time	N/a	(Iakovides et al., 2019)

Sul1	0.45 - 0.55 gO ₃ /gDOC	N/a	< LOD at higher 0.4 gO ₃ /gDOC	N/a	(Czekalski et al., 2016)
vanA, ampC and bla _{VIM} , and ermB	0.9 gO ₃ /gDOC	18 min	49.9% and 18.7, 69.8% and 99.3% of vanA, bla _{VIM} , ampC and ermB respectively	N/a	(Alexander et al., 2016)
Antibiotic resistance bacteria					
Multidrug resistant <i>E.coli</i> A2FCC14 (bla _{TEM} and intl1)	Ozone flowrate (150 cm ³ /min STP) and ozone inlet concentration (50g/Nm ³)	15, 30 and 60 min	<LOD at 60 min	Short contact time (15 - 30 min)	(Sousa et al., 2017)
Trimethoprim (TMP) - and sulfamethoxazole (SMX) - resistant <i>E.coli</i>	0.125, 0.25, 0.5 and 0.75 gO ₃ /gDOC	10, 20, 40 and 60 min	No detection at ozone doses of 0.125 - 0.75 gO ₃ /gDOC by 60 min	Short contact time (20 min)	(Iakovides et al., 2019)
Multidrug resistant <i>E.coli</i> DH5α harbouring pB10 plasmid and <i>P. aeruginosa</i> PAK exoT	3, 5, 7 and 10 mg/l	1, 3, 5, and 10 min	5-6 log reductions at 7- 10 mg/l for 10 min contact time	N/a	(Pak et al., 2016)
Multidrug- resistant bacteria	0.45 - 0.55 gO ₃ /gDOC	N/a	1.4 - 1.6 log reduction of multidrug	N/a	(Czekalski et al., 2016)

			resistant bacteria		
Multidrug resistant bacteria (<i>Enterococci-23SrDNA</i> , <i>Staphylococci-mecA</i> , <i>Enterobacteriaceae-ampC</i> and <i>P. aeruginosa-ecfX</i>)	0.9 gO ₃ /gDOC	18min	98.9% reduction of MDR Enterococci	Bacterial diversity and genomic structure	(Alexander et al., 2016)

The target microorganism's physical features, the length of contact, and the amount of O₃ used all have an impact on the outcome of ozonation disinfection. In greater detail, longer O₃ treatment times are needed for ARG/ARB inactivation to be more effective, and the O₃ dose needed for ARG elimination is larger than the O₃ dose needed for ARB inactivation. Additionally, different bacteria exhibit varying ozonation sensitivities, and these particularly relate to the genome's guanine-cytosine composition of the target organism. ARGs with less guanine-cytosine may be more vulnerable to O₃ damage. However, the amount of double-stranded DNA is not as big as anticipated because of the hydrogen bonds between DNA. This raises concerns regarding how effectively ozonation eliminates ARGs ([Cai et al., 2021](#)).

3.2.2. Advanced oxidation processes

3.2.2.1. Photo-induced Fenton

Due to their great efficacy, ease of use, and relatively low cost, Fenton and photo-Fenton are promising tools for removing microorganisms. When Fe²⁺/Fe³⁺ react with H₂O₂ in an acidic medium during the Fenton oxidation process, OH* can be generated and employed to damage cells. The permeability of the cell membrane may be improved by the presence of H₂O₂. Due to the entry of H₂O₂, iron ions and the resulting Fenton and Haber-Weiss reaction damage and even kill cells ([J. Wang et al., 2022](#)). The Fenton reaction process, however, involves radical reactions and is highly complicated. Fenton's reagent can be coupled with UV visible light, which has a wavelength range of 300 to 600 nm, to boost efficiency ([Gmurek et al., 2022](#)). As a result, photo-Fenton is discussed in this section because to its benefits, including (i) acceleration of the Fenton process under light influence by promoting radical generation, (ii) very effective oxidation and

mineralization of organic pollutants, and (iii) low cost of application. Figure 3.6 illustrates the two mechanisms by which bacteria are inactivated during the photo-Fenton process: (1) light oxidation, and (2) reactive oxygen species (ROS) mostly derived from H_2O_2 and OH^* . In more detail, ROS cause DNA lesions directly whereas UVC and UVB radiation and ROS also induce membrane damage. CAT and ROS enzymes are activated by UVA and visible light, which indirectly causes DNA damage. Intracellular photo-Fenton reaction is caused by the diffusion of Fe^{2+} and H_2O_2 into bacterial cells, which encourages an additional internal photoreaction, especially with naturally occurring iron (Gmurek et al., 2022). H_2O_2 also alters the steady-state ROS levels inside of cells. Additionally, bacteria are rendered inactive by intracellular ROS generation and a rise in water temperature (Moreira et al., 2018). According to Giannakis et al. (2018), H_2O_2 residuals could be neutralized by adding excess catalase, and 1U ($\mu\text{mol}/\text{min}$) of enzyme could decomposes $1\mu\text{mol}$ of H_2O_2 at pH 7 (Giannakis, Watts, et al., 2018). However, one major constraint of this process is the formation of oxidation products deriving from the dissolved organic matter, which may induce toxic effects (S. G. Michael et al., 2019)

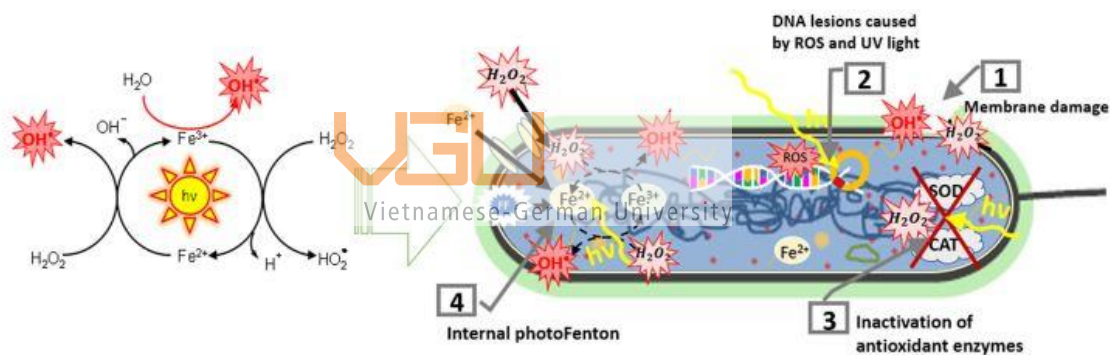


Figure 3. 6: Mechanism of photo-Fenton to inactivate ARB/ARGs (Polo-López et al., 2018)

As for reaction time, it was found that the removal efficiency of the targeted ARB decreased with the increase in the treatment time. Giannakis et al. (2018) reported that 4-log reduction of Streptomycin-resistant *E.coli* within photo-Fenton process (Giannakis, Watts, et al., 2018). In this experiment, Fe^{2+} to H_2O_2 ratio was 1/20 combined with stimulation of UVB (310 nm) with different contact time (15-120 min), and 90 min was the optimal condition for inactivating Streptomycin-resistant *E.coli*. Fiorentino et al. (2019) also reported that cefatoxime-resistant bacteria of two examined urban wastewater treatment plants in Spain (El Bobar and El Toyo) were below limit of detection when using 20 mg/l of Fe^{2+} and 50 mg/l of H_2O_2 with effect of UVA (solar irradiation) (Fiorentino et al., 2019). The inactivation of cefatoxime-resistant bacteria increased linearly with increased contact time, and completely inactivate cefatoxime-resistant bacteria in 30-40 min. Interestingly, it was found that although water turbidity increased because of iron precipitation, a sufficient amount of ROS was produced to effectively inactivate

cefatoxime-resistant bacteria. According to Giannakis et al. (2018), complete inactivation of multidrug-resistant E.coli, S.aureus and K.pneumoniae also were recorded at 90 min of photo-Fenton with $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ ratio of 1/20 (Giannakis, Le, et al., 2018). It is well explained that OH^* deriving from photo-Fenton process has the high reactivity and low selectivity, and easily react with dissolved organic matters (DOMs) (Yoon et al., 2017), therefore, longer treatment time is necessary for subsequent reaction of OH^* within ARGs/ARB inactivation. One more study reported that 6.17 log reduction of multidrug-resistant E.coli DH5 α was achieved by visible LED-induced UVA with 30 min reaction time (Ahmed et al., 2020).

In addition to treatment time, pH values could affect the production of HO^* , and in turn influence the efficiency of the performance. It was reported that ARBs, resistance to SMX and CLA, could be more efficient abated at pH 4.0, compared to that at neutral pH (Karaolia et al., 2014). On the other hand, iron-induced flocs was performed due to that iron complexes or precipitation at pH > 4.0 under form of $\text{Fe}(\text{OH})_3$, leading to abatement of the dissolved iron species in solution, which in turn affected the removal efficiency of ARGs. By contrast, regarding to different journals (Fiorentino et al., 2019; Giannakis, Le, et al., 2018; Giannakis, Watts, et al., 2018) and (Ahmed et al., 2020), although pH is an important operational parameter, the application of the photo-Fenton can be used also in neutral pH value.

Regarding ARG removal, the effective reduction of ARGs increases linearly with an increase in reaction time (Giannakis, Le, et al., 2018; S. G. Michael et al., 2019). Because photo-Fenton process was proven to be an intracellular process (Giannakis, Le, et al., 2018), so longer treatment time is necessarily required for oxidative reagents which could react with cytoplasmic components, that result in degrading ARGs. Micheal et al. (2019) used $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ ratio of 1/10 combined with solar irradiation under acidic medium to evaluate the performance of photo-Fenton on degrading targeted ARGs (Sul1, qnrS, bla_{TEM}, bla_{OXA}, bla_{CTX-M} and tetM) (S. G. Michael et al., 2019). The results showed that except bla_{TEM}, targeted ARGs were completely degraded after 90 min treatment time, and no detection of targeted ARGs, except bla_{TEM}, was observed after 180 min storage. Bla_{TEM} genes was found to be the most prevalent among the tested ARGs, detected in all samples examined, suggesting its lower susceptibility towards photo-Fenton. Furthermore, this may be attributed to the oxidative damage of bacteria during photo-Fenton process, causing them to release the specific gene, as well as a lower inter- and intra-species selective pressure. Fiorentino et al. (2019) also reported the same results, when B-lactam resistant genes (bla_{TEM} and bla_{CTX-M}) were not affected by photo-Fenton process (Fiorentino et al., 2019). However, in the case of Giannakis et al. (2018), B-lactam-resistant genes (bla_{CTX-M1} and bla_{CTX-M9}) was no further detected in wastewater after 15-min photo Fenton (Giannakis, Le, et al., 2018). It might be

attributed to light intensity which was used in different experiment. In experiments of Giannakis ([Giannakis, Le, et al., 2018](#)), light intensity was 750 W/m^2 , compared to approximately 25-fold higher than light intensity values which were used in Fiorentino ([Fiorentino et al., 2019](#)) and Michael ([S. G. Michael et al., 2019](#)). In addition to high light intensity, high H_2O_2 concentration of 170.7 mg/l also caused $6.75 - 8.56 \text{ log}$ reduction of tetA and bla_{TEM} ([Ahmed et al., 2020](#)) after 20 min reaction time, and the detection limit of targeted ARGs was achieved at 340.2 mg/l of H_2O_2 after 10 min treatment time. Interestingly, it was found that bla_{TEM} genes were faster degraded than tet genes at lower H_2O_2 concentration of 340.2 mg/l because the adjacent T-T bases of bla_{TEM} is 1.5 higher than of tet genes. The degradation rate of targeted ARGs is also influenced by amplicon length. The rate constant of long amplicon genes were higher than the short amplicon. For example, the inactivation rate of bla_{TEM} long amplicon is 0.98 min^{-1} , which is a higher of 0.88 min^{-1} of bla_{TEM} short amplicon, that indicated the easier degradation rate of tetA gene (1200 bp), compared to degradation rate of bla_{TEM} (861 bp) ([Ahmed et al., 2020](#)). This finding may explained that hard degradation of bla_{TEM}, compared to other ARGs in mentioned journals.

According some diggings surrounding the regrowth potential after photo-Fenton inactivation of ARB/ARGs, no regrowth was observed in case of long treatment time ($60 - 90 \text{ min}$) with high light intensity ([Fiorentino et al., 2019](#); [Giannakis, Le, et al., 2018](#); [Giannakis, Watts, et al., 2018](#)). However, when decreasing treatment time below 90 min , regrowth of targeted ARB was detected, and it increased linearly with decreased treatment time from 90 min to 15 min ([Giannakis, Le, et al., 2018](#); [Giannakis, Watts, et al., 2018](#)). As a result, longer exposure times cause the irreversible DNA damages ultimately preventing the bacteria multiplication. However, in case of Ahmed et al. (2020), complete inactivation of multidrug-resistant E.coli DH5 α was achieved at 30 min of exposure time under visible LED-induced UVA with high H_2O_2 concentration of , then no ARB regrowth occurred after 48-h dark storage, demonstrating that the process is very effective to result in permanent inactivation of ARB ([Ahmed et al., 2020](#)). Results of Ashmed et al. (2020) might be attributed to high concentration of H_2O_2 (340.2 mg/l) ([Ahmed et al., 2020](#)), compared to 20 mg/l ([Giannakis, Watts, et al., 2018](#)), 10 mg/l ([Giannakis, Le, et al., 2018](#)), and 50 mg/l ([Fiorentino et al., 2019](#)). Interestingly, Ashmed ([Ahmed et al., 2020](#)) illustrated that high H_2O_2 concentration instead of high light intensity, complete ARB reduction was completely achieved at shorter time than other mentioned journals. It is well explained that DNA damage is mostly indirect via ROS, less by UVA or visible light itself. During photo-Fenton process, ROS is produced at sufficient level to break down the defense system of the microorganism for both achieving complete inactivation and avoiding bacterial recovery or regrowth. Due to residual disinfecting effect which is remarkable advantage of photo-Fenton, and H_2O_2 has a questionable

privilege and prevalence in disinfection, therefore its extra generation during photocatalytic process causes an additional residual effect. Moreover, further ROS generation from H₂O₂ inhibits bacterial reproduction, acting as an “after illumination residual disinfectant. Additionally, when photo-Fenton is performed, the light intensity as well as H₂O₂ concentration are a key factor ([Gmurek et al., 2022](#))

Table 3. 6: Photo-Fenton-induced removal of ARGs/ARB

ARGs/ARB	Operational conditions	Contact time	Remove efficiency (%)	Regrowth potential	Reference
Antibiotic resistance genes					
Sul1, qnrS, bla _{TEM} , bla _{OXA} , bla _{CTX-M} and tetM	Fe ²⁺ : 5 mg/l H ₂ O ₂ : 50 - 100 mg/l pH: 2.8 - 2.9 UVA: <400 nm Light intensity: 30 W/m ²	0 - 300 min	Abundance of all ARGs < LOD except bla _{TEM}	Bla _{TEM} increase after 120 min reaction time	(S. G. Michael et al., 2019)
Bla _{CTX-M-1} and bla _{CTX-M-9}	Fe ²⁺ : 1 mg/l H ₂ O ₂ : 10 mg/l pH: 6.1 - 6.2 UVA Light intensity: 750 W/m ²	0 - 90 min	Complete reduction of bla _{CTX-M-1} after 15 min treatment	No regrowth	(Giannakis, Le, et al., 2018)
Sul1, qnr, bla _{TEM} and bla _{CTX-M} , and intl1	Fe ²⁺ : 20 mg/l H ₂ O ₂ : 50 mg/l pH: 7.0 Light intensity: 21±7.9 W/m ²	0 - 180 min	Relative abundance of sul1 < LOD	Bla _{TEM} , Bla _{CTX-M} and intl1 were not affected	(Fiorentino et al., 2019)

	and 17.4±6.6 W/m ²				
bla _{CTX-M} , bla _{TEM} , vanA, sul1 and qnrS	Fe ²⁺ : 10 mg/l H ₂ O ₂ : 20 mg/l pH: 5.5 UVA: 300 - 400 nm Light intensity: 40 W/m ²	0 - 4 h	3 log reduction for bla _{CTX-M} 1 log reductions for qnrS, bla _{TEM} and sul1 vanA < LOD	No regrowth	(Moreira et al., 2018)
Antibiotic resistance bacteria					
Streptomycin- resistant E.coli	Fe/H ₂ O ₂ : 1/20 UVB: 310 nm pH: 7.0	15 - 120 min	4 log reduction at 90 min	No regrowth	(Giannakis, Watts, et al., 2018)
Cefotaxime- resistant E.coli, Enterococcus and total coliform	Fe ²⁺ : 20 mg/l H ₂ O ₂ : 50 mg/l pH: 7.0 UVA	0 - 80 min	Cefotaxime- resistant bacteria < LOD at 30 - 40 min contact time	No regrowth	(Fiorentino et al., 2019)
Multidrug- resistant S.aureus, E.coli and K.pneumoniae	Fe ²⁺ : 1 mg/l H ₂ O ₂ : 10 mg/l pH: 6.1 - 6.2 UVA Light intensity: 750 W/m ²	15 - 120 min	< LOD after 90 min reaction, except K.pneumoniae	Short treatment time (15 - 60 min)	(Giannakis, Le, et al., 2018)
Bla _{TEM-1} and tetA resistant E.coli DH5α harbouring	Fe ²⁺ /H ₂ O ₂ : 1/20 UVA: 425 - 525 nm pH: 6.85 - 6.96	0 - 60 min	6.17 log reduction at 30 min reaction	No ARB regrowth after 48-h storage	(Ahmed et al., 2020)

plasmid pWH1266					
Bla _{CTX-M} , bla _{TEM} , vanA, sul1 and qnrS	Fe ²⁺ : 10 mg/l H ₂ O ₂ : 20 mg/l pH: 5.5 UVA: 300 - 400 nm Light intensity: 40 W/m ²	0 - 4 h	3 log reduction for bla _{CTX-M} 1 log reductions for qnrS, bla _{TEM} and sul1 vanA < LOD	No ARB regrowth after 3-day storage	(Moreira et al., 2018)

Peer-reviewed articles state that after using the photo-Fenton procedure, ARGs and ARB exhibit no regrowth during long-term storage, demonstrating the effectiveness of the therapy in avoiding the emergence of AMR. In addition, two crucial factors—light intensity and H₂O₂ concentration directly affect how well photo-Fenton works. The performance will be more efficient and the length of the therapy will be reduced the more both of these factors are present.

3.2.2.2. Photocatalysis process

ARBs and ARGs in wastewater can be effectively treated by the photocatalysis technique. ([Gmurek et al., 2022](#); [J. Wang et al., 2022](#)). The ROS oxidation and the adsorption of the photocatalytic particles for ARG reduction may be responsible for the mechanism involving the ARGs/ARB by photocatalysis process. When cells come into touch with photocatalysts, damage to the cellular surface may really occur. This damage may then change the permeability of the membrane, allowing for the leaking of cellular components. As additional photocatalytic particles entered the cell, the cell damage increased. These continuous oxidative reactions caused damage to lipid, anti-oxidative enzymes (CAT and SOD), protein, and DNA. On the other hand, under irradiation with energy larger than or equal to the band gap, photocatalysts might be activated to form e⁻/h⁺ (electron-hole pairs), which could diffuse to the particle surface and lead to a strong oxidation. The subsequent reactions of e⁻/h⁺ with hydroxyl groups and O₂ can result in the production of ROS (OH* and O₂*-), respectively. Additionally, UVA and visible light cause indirect DNA damage by producing ROS, whereas UVC and UVB damage DNA directly.

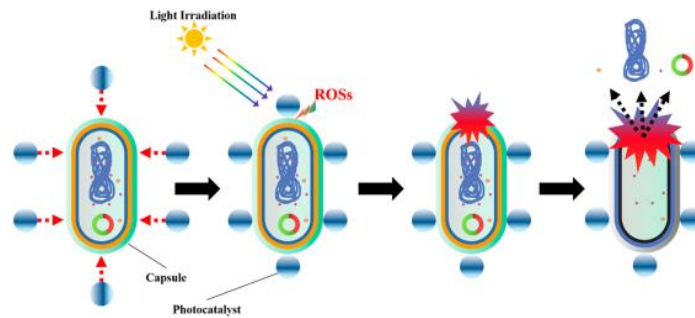


Figure 3. 7: Mechanism of photocatalyst on inactivation ARGs/ARB (Cai et al., 2021)

Table 3. 7: Photocatalyst-induced removal of ARGs/ARB

ARGs/ARB	Operational conditions	Contact time	Remove efficiency (%)	Regrowth potential	Reference
Antibiotic resistance genes					
ampC and mecA	TiO ₂ - thin film UVC (254 nm): 0 - 120 mJ/cm ²	N/a	5.8 log mecA and 4.7 log ampC at 120 mJ/cm ²	N/a	(C. Guo et al., 2017)
ampC, sul1 and ermB	TiO ₂ , TiO ₂ -rGO-PH and TiO ₂ -rGO-HD: 100 mg/l UVA Light intensity: 62 W/m ²	0 - 60 min	Sul1 (2.37 - 2.92 log) ampC (nearly 2.4 log with only TiO ₂) ermB (0.2 - 1.4 log)	N/a	(Karaolia et al., 2018)
Int11, qnrS, bla _{CTX-M} , bla _{TEM} and sul1	TiO ₂ -GO: 4 wt.% of GO UVA	0 - 5 hours	Low influences on all ARGs	Only bla _{CTX-M} were below LOD	(Moreira et al., 2018)

	Light intensity: 40 W/m ²				
Antibiotic resistance bacteria					
Multidrug-resistant <i>E.coli</i>	UVA: 0 - 24 kJ/L TiO ₂ :H ₂ O ₂ : 10:100 and 50:100 mg/l TiO ₂ suspension: 100 mg/l	0 - 6h	Complete inactivation in 150 min with 100 mg TiO ₂ /l and 20 kJ/L	No regrowth	(Fiorentino et al., 2015)
Methicillin-resistant <i>S.aureus</i> , multidrug-resistant <i>A. baumannii</i> and vancomycin-resistant <i>E. faecalis</i>	UVA: 400 and 800 μW/cm ² TiO ₂ suspension: 0.0625 and 0.125 mg/l	5 - 80 min	Completely inactivation in 20 - 30 min treatment time with 0.125 mg/l and 800 μW/cm ²	N/a	(Tsai et al., 2010)
Multidrug resistant <i>E.coli</i>	UVA/LED (325nm): 60, 70 and 80 W/m ² TiO ₂ - thin film	30 min periodic illumination + 240 min dark time 60 min periodic illumination + 240 min dark time	Below limit of detection after 90 min treatment	No regrowth after 4- hour incubation	(Xiong et al., 2013)

		90 min periodic illumination + 240 dark time			
mecA- resistant <i>S.aureus</i> and ampC- resistant <i>P.aeruginosa</i>	TiO ₂ - thin film UVC (254 nm): 0 - 12 mJ/cm ²	N/a	5.5-5.8 log reductions at 12 mJ/cm ²	N/a	(C. Guo et al., 2017)
MDR <i>E.coli</i>	TiO ₂ , TiO ₂ - rGO-PH and TiO ₂ -rGO-HD: 100 mg/l UVA Light intensity: 62 W/m ²	0 - 180 min	<LOD after 180-min reaction	Regrowth occurs at only TiO ₂ with 30 and 60- min reaction times	(Karaolia et al., 2018)
MDR <i>enterococci</i> and <i>faecal</i> <i>coliform</i>	TiO ₂ -GO: 4 wt.% of GO UVA Light intensity: 40 W/m ²	0 - 5 hours	<LOD after 5- hour reaction	No regrowth	(Moreira et al., 2018)

Application of TiO₂ as a photosensitizer is most commonly used in wastewater treatment due to its high efficiency, affordability, no toxic by-products as well as high stability ([Iervolino et al., 2020](#)). Therefore, application of TiO₂ in removing ARGs/ARB within wastewater are considered. Fiorentino et al. (2015) reported that multidrug-resistant *E.coli* was completely removed after 150 min inactivation time with 100 mg/l, and UVA light intensity of 20 kJ/L ([Fiorentino et al., 2015](#)). Furthermore, the inactivation rate of multidrug-resistant *E.coli* increased linearly with an increase in light intensity. Tsai et al. (2010) also evaluated different light intensities (400 and 800 μW/cm²)

and TiO₂ powder concentrations (0.0625 and 0.125 mg/l) on inactivation of three typical ARB (methicillin-resistant *S.aureus*, multidrug-resistant *A.baumannii*, and vancomycin-resistant *E.faecalis*) with treatment time ranging from 5 to 80 min (Tsai et al., 2010). The results showed that optimal reductions of three typical ARB (90 - 99%) were achieved by 800 μW/cm² and 0.125 mg/l with reaction time ranges of 20-30 min, as shown in Table 3.7. It is emphasized that high light intensity and TiO₂ concentration affect positively the performance of TiO₂-induced photolysis process on ARB inactivation. Because higher photon flow and more catalyst applied could not only shorten the delay region, but also accelerate inactivation rate in the following log reactivation area (Xiong et al., 2013). Besides, use of TiO₂ powder is known to achieve the high efficiency in inactivation of ARB and ARGs because it has large surface area, and furthermore relates its penetration of catalyst particles inside cells causing continuous oxidative reactions (Gmurek et al., 2022). The TiO₂ powder, however, has restricted its wide application in practical use because they are difficult to be separated and recovered in watery solution (Gmurek et al., 2022; C. Guo et al., 2017). Therefore, TiO₂ thin film draws attention to researchers as an alternative solution for TiO₂ suspension for solving the separation and recovery problems (C. Guo et al., 2017; Xiong et al., 2013). Xiong et al. (2013) used the TiO₂ thin film with thickness of around 15 μm and the total coated TiO₂ weight of 73.0 mg, compared to 100 g of initially used TiO₂ powder (Xiong et al., 2013). With different light intensities ranging from 6 to 8 mW/cm², a UVA/LED system was set up. All inactivation brought on by the three distinct light intensities was undetectable throughout the first 20 minutes of reaction time. This delay may be caused by the fact that bacteria require a certain amount of damage before they start to manifest negative consequences. However, after 100 minutes of reaction time, 8 mW/cm² was required to obtain the maximum reduction of MDR *E. coli*, at 4.5 log, as opposed to 4 log and around 1 log by 7 and 6 mW/cm², respectively. Intriguingly, the experiment revealed the presence of a saturation stage when the inactivation rate increment from 7 to 8 mW/cm² was less than one from 6 to 7 mW/cm², as seen in Fig. It may be argued that since the coated TiO₂ surface is constant, subsequent increases in light intensity would not considerably accelerate the inactivation rate after the reaction system is saturated with photons. Guo et al. (2017) also evaluated the performance of TiO₂ thin film on inactivation of different ARB (mecA-resistant *S.aereus* and ampC-resistant *P.aeruginosa*) (C. Guo et al., 2017). According to the findings, MRSA and *P. aeruginosa* log reductions of 4.5–5.0 and 5.5–5.7, respectively, were attained under 6 and 12 mJ/cm², respectively. However, under 120 mJ/cm² UV₂₅₄, TiO₂ thin film was able to reduce mecA and ampC by 5.8 log and 4.7 log, respectively. Comparing the inactivation of ARB and ARGs, it was found that ARB was more affected by various treatments than ARGs, indicating that ARGs needed more light intensity to be removed than ARB. because the oxidant first attacked the

membrane, enzymatic, and transport systems of microorganisms before attacking the inner organisms, such as RNA and DNA. Although the bacteria had a defence mechanism to prevent ROS oxidation, too much ROS might cause oxidative stress and attack membrane lipids, which would eventually result in DNA or membrane damage (M. Li et al., 2011). Additionally, light intensity has a role in the efficiency of the TiO₂-induced photolysis process on the inactivation of ARGs and ARBs in addition to the influence of TiO₂ content. According to Table 3.7, increased light intensity resulted in higher ARG/ABR inactivation rates. because a faster inactivation rate was produced by increased light intensity. A large flow of photons was available at high intensities. However, the additional photon may directly affect ARB and speed up inactivation. In addition, photons that penetrate water increased at high light intensities. The penetration of photons into water may cause the creation of ROS, which then attacks bacteria and greatly increases the pace at which bacteria are destroyed (Xiong et al., 2013).

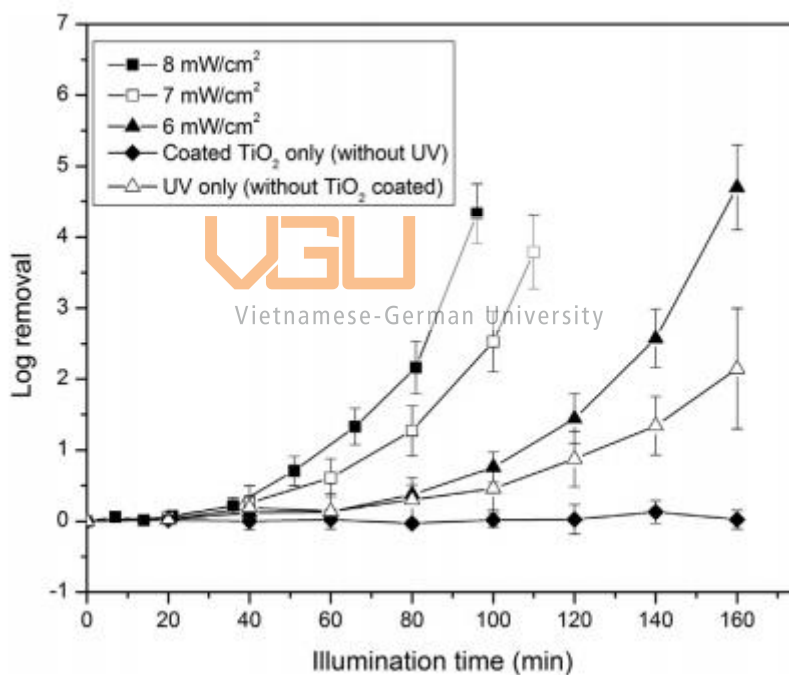


Figure 3. 8: Inactivation of MDR E.coli by TiO₂ thin-induced photocatalysis process with different light intensities (Xiong et al., 2013)

On the other hand, photolysis process could be combined with H₂O₂ to accerlerate inactivation rate (Fiorentino et al., 2015). Fiorentino et al. (2015) showed that complete inactivation of MDR E.coli was achieved after 150 min reaction when only TiO₂/sunlight was applied (Fiorentino et al., 2015). However, when adding H₂O₂ to TiO₂/sunlight process with two ratios of TiO₂:H₂O₂ (10:100 and 50:100 mg/l), the reaction time decreased from 150 min to 60 min, and light flow rate also decreased from 20 kJ/L to 3 and 4 kJ/L, respectively. Furthermore, TiO₂:H₂O₂ ratio of 50:100 mg/l showed the faster inactivation rate then one of 10:100 mg/l .Guo et al. (2017) also

reported the same trend when combining H_2O_2 with TiO_2/UVC (C. Guo et al., 2017). Additionally, when increasing H_2O_2 concentration from 0 to 100 mmol/l, inactivation rates of methicillin-resistant *S.aureus* and ampC-resistant *P.aeruginosa* also increased. Additionally, light intensities required for only TiO_2/UVC and $\text{H}_2\text{O}_2/\text{TiO}_2/\text{UVC}$ have the same trend as the previous results. The addition of H_2O_2 in presence of TiO_2 promotes the formation of HO^*_2 , and low values of OH^* . It might be explained by the change in the extent of decomposition of H_2O_2 , comparing effective area/volume ratios. On the other hand, it is possible to speculate whether a loss of effective surface area due to an agglomeration of particles occurs, resulting in a decreased formation of OH^* . Besides, agglomeration is possibly due to interactions among HO^*_2 to surface of TiO_2 . For this reason, the presence of HO^*_2 is stabilized at high concentrations, with a best performance of inactivation (Fiorentino et al., 2015)

In addition to oxidative reactions, interfacial adsorption could contribute to reduction of ARB/ARGs within photocatalysis process, especially when combining TiO_2 with graphene oxide (GO) (Karaolia et al., 2018; Moreira et al., 2018; Zhou et al., 2020). Because GO was rich in sp^2 backbones and functional oxygen groups, which efficiently captured and enriched the ARGs via π - π interactions and hydrogen bonds (Zhou et al., 2020). Furthermore, GO also has other properties such as high (1) specific area, thus increasing adsorption of microcontaminants onto TiO_2 -graphene composites; (2) high transparency due to its one-atom thickness; (3) extended light absorption range towards the visible region of the electromagnetic spectrum (Karaolia et al., 2018). Karaolia et al. (2018) evaluated the inactivation rate of MDR *E.coli* during 180-min reaction time with only TiO_2 and two modified TiO_2 -GO (TiO_2 -rGO-PH and TiO_2 -GO-HD by two ex-situ synthesis methods, namely hydrothermal treatment (HD) and photocatalytic treatment (PH) (Karaolia et al., 2018). The results showed that there were differences between ERY-resistant -, CLA-resistant- and SMX-resistant *E.coli* after 30-min reaction time, however, these ARB were not detected when increasing to 180-min reaction for all types of photocatalysts. In addition to ARB inactivation, ARGs (sul1, ampC and ermB) which were measured in all samples ranging from 3.52 - 3.95 log through *enterococci* known as a host. After 60-min reaction, sul1 is hardly removed by TiO_2 , TiO_2 -GO-PH and TiO_2 -GO-HD photocatalysts remaining 2.37 - 2.92 log within all samples, thus indicating that bacterial carrier of the mentioned ARGs was relatively stable under photocatalytic treatment. AmpC abundance had no changes after treatment with TiO_2 , but there was no detection of ampC in samples treated with two synthesized TiO_2 -GO catalysts. EmrB was reduced 4-fold, compared to the initial log of 1.2, after treatment with TiO_2 , however, there were no changes with two modified TiO_2 -GO catalysts (Karaolia et al., 2018). It was well explained that the reduction of ARGs can be attributed to intracellular DNA damage of the host produced by photo-induced TiO_2 -GO and TiO_2 suspension. Additionally, DNA damage

by photocatalytic treatment occurs at specific sites on the DNA double strands causing its photo-cleavage and causing base modification, producing mutaton in the next generation ([Pigeot-Rémy et al., 2011](#)). Moreira et al. (2018) also reported that MDR enterococci and faecal coliform were completely inactivated by TiO₂-GO with 4 wt.% of GO after 5-hour treatment ([Moreira et al., 2018](#)). However, TiO₂-GO photocatalyst caused low influences on targeted ARGs. It was emphasized that ARG removal harder removed than ARB inactivation with the same time reaction. Furthermore, it might be attributed to the ARG reductions which possibly caused by cell inactivation and not to ARG loss. These findings suggested that hosts might changed into the state of VBNC, being able to reproduce during long time storage.

Regarding regrowth potential of ARB/ARGs within photocatalyst process, most cases shown in Table 3.7 showed no regrowth of ARB after being treated with TiO₂ particles and its combination with GO. Xiong et al. (2013) evaluated the photoreactivation and dark repair of MDR *E.coli* which were incubated for 4 hours ([Xiong et al., 2013](#)). In details, for photoreactivation, bacteria samples were stirred in the Petri dishes and were exposed to a 20W fluorescent light with wavelength of 400 - 700 nm for 4 hour and samples were taken at hourly interval for bacteria concentration determination. For dark repair, bacteria samples were also stirred for 4 hour, and samples were taken at hourly intervals for the bacteria concentration determination. The results showed that neither photoreactivation nor dark repair was found after 4-hour incubation. Additionally, bacteria concentration decreased to under limit of detection. It could explained by the residual disinfection affect. Fiorentino et al. (2015) also reported that the regrowth of MDR *E.coli* occurred after 24-hour storage ([Fiorentino et al., 2015](#)). Although the cultivability of the ARBs was completely inactivated, the regrowth and repair of the ARB need to be concerned during photocatalysis process, due to recovery ability of bacteria under sub-lethal concentration of reactive oxgen species (ROS) stress. The regrowth of the ARBs was observed, revealing the necessity to achieve ARBs complete deactivation and prevention of further recovery. Furthermore, the regrowth potential probably depends on the different ARB ([Karaolia et al., 2018](#); [Moreira et al., 2018](#)), especially being reported in two cases of applying TiO₂-GO. Moreira et al. (2018) considered that the bacterial community is much more diverse and complex than that assessed (two targeted ARB above) based on the cultivation methods used, and that some bacteria may be injured and hence, unable to grow, so culture-independent method (qPCR) was carried out to give additional insights on the effectiveness of TiO₂-induced photocatalyst process on inactivation of ARB/ARGs ([Moreira et al., 2018](#)). The results showed that two class classes of Proteobacteria (*Beta- and Gammaproteobacteria*) or that at least some bacteria belonging to these classes have higher capacity to regrow after 3-day storage. It might be attributed to the alteration

in composition and structure of the target ARB resulting in bacterial defense against ROS (Karaolia et al., 2018).

Eventually, the effectiveness of photocatalyst process depends mainly on TiO₂ concentration as well as light intensity. More TiO₂ concentration and light intensity are used, more effectiveness is achieved. Compared to inactivation of ARB, high ARG reduction seem be hardly achieved because except TiO₂ concentration and UVA light intensity, bacterial community and VBNC state which are known as two factors causing the regrowth potential within TiO₂ photocatalyst process, that being further researched to achieve the optimal performance of reducing ARGs/ARB.

3.3.3 Biological processes

3.3.3.1. Constructed wetlands

Constructed wetland is an effective and sustainable wastewater treatment solution, especially with regard to the removal of ARGs. The three main methods of eliminating ARGs are biodegradation, substrate adsorption, and plant absorption. Plants indirectly help to eliminate ARGs by supplying oxygen to microbial populations. On the other hand, substrate provides a surface for biofilm to form on, improving microbial clearance effectiveness and reducing ARG abundance. How ARGs turn out depends on the type of CWs, which include two main configurations such as (1) surface flow CWs and (2) subsurface flow CWs (horizontal subsurface flow - CWs and vertical subsurface flow - CWs), substrate type, plant species, and HRT (T.-t. Zhu et al., 2021).

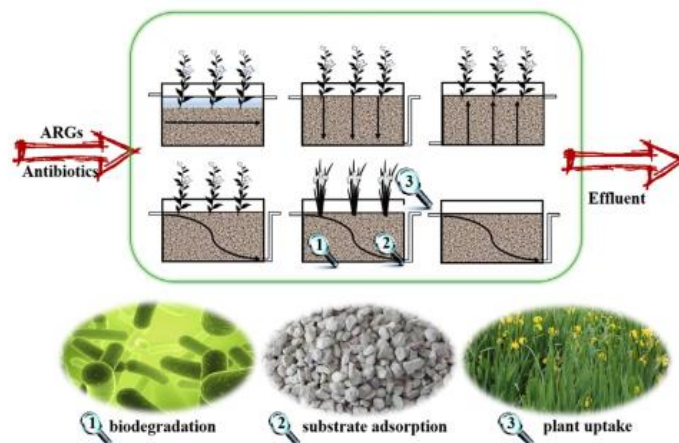


Figure 3. 9: A scheme of different constructed wetland configurations and its mechanism of removing ARGs/ARB (Chen, Ying, et al., 2016)

Table 3. 8: Constructed wetland-induced removal of ARGs/ARB

ARGs/ARB	Operational conditions	HLR and HRT	Remove efficiency	Regrowth potential	Reference
Antibiotic resistance genes					
ermB, sul1, tetM and intl1	HSSF CWs: CW1: 40-cm tuff and Phragmites australis CW2: 40-cm tuff and P.australis and Typha angustifolia CW3: gravel and P.australis CW4: gravel, P.australis and Typha	180 L/h and 24 h	CW1: 79.6 - 86.6% CW2: 89.8 - 94.7% CW3: 74.3 - 76.9 CW4: 79.7 - 85.3%	N/a	(Abou-Kandil et al., 2021)
Sul1, sul2, sul3, tetG, tetM, tetX, tetO, ermB, ermC, qnrS, qnrD, qnrB, cmlA, fexA, fexB, floR, intl1 and intl2	12 HSSF-CWs: CW1, 2, 3: 65-cm layer of oyster shell and Cyperus alternifolius L. respectively CW4, 5, 6: 65-cm layer of zeolite and C.alternifolius L. CW 7, 8 and 9: 65-cm layer of medical stone and C.alternifolius L.	10, 20, 30 cm/d	50.0 - 85.8% of all targeted ARGs	N/a	(Chen, Wei, et al., 2016)

	CW 10, 11 and 12				
Sul1, sul2, ermB, qnrS and bla _{TEM} , bla _{KPC} , bla _{NDM} , and bla _{OXA-48} , and vanA	2 VSSF: CW1: 0.1-m layer of sand and 0.7-m layer of fine gravel, and P.australis CW2: 0.1-m layer of sand and 0.7-m layer of fine gravel, and P.australis	133 mm/d	sul1, sul2, ermB, qnrS and bla _{TEM} : 21 - 93% vanA, tetM and tetW, bla _{KPC} , bla _{NDM} , bla _{OXA-48} : <LOD	N/a	(Ávila et al., 2021)
tetW, tetA, tetX and intl1	VSSF: CW1 and CW2: 450-mm brick particles and P.australis CW3 and CW4: 450-mm oyster shell and P.australis	5.1 cm/d	33.2 - 99.1% of tet genes and intl1	N/a	(X. Huang et al., 2017)
tetG, tetM, tetO, tetX, and sul1, sul2, sul3, and ermB, ermC, and cmlA and floR	6 CWs: CW1: surface flow - Thalia dealbata Fraser - 40 cm of zeolite CW2: VSSF - T. dealbata Fraser - 65 cm of zeolite - down-flow direction CW3: VSSF - T. dealbata Fraser - 65	20 cm/d	63.9 - 84% of all targeted ARGs	N/a	(Chen, Ying, et al., 2016)



cm of zeolite - up-flow direction				
CW4: HSSF - T. dealbata Fraser - 65 cm of zeolite				
CW5: HSSF - Iris tectorum Maxim - 65 cm of zeolite				
CW 6: HSSF - 65 cm of zeolite without plantation				

Four horizontal subsurface CWs were constructed and labelled as CW1, CW2, CW3 and CW4. Among 4 CWs, CW1 and CW2 were equipped with 40-cm tuff and *P. australis*, and *P. australis*-Typha, respectively, while CW3 and CW3 were equipped with 40-cm gravel and *P. australis*, and *P. australis*-Typha in order, that evaluating the removal of 4 targeted ARGs (*ermB*, *sul1*, *tetM* and *intl1*). The results showed that the total ARG removal of tuff-filled *P. australis*, tuff-filled *P. australis*-Typha, and their gravel-filled CWs counterparts ranged between 79.6 and 86.6%, 89.8%-94.7%, 74.3-76.9%, and 79.7% and 85.3%, respectively as shown in Table 3.8. As result, the two-layer of plantation and tuff outperformed the monolayer of plantation and gravel. It was attributed to high rate of adsorption of the targeted ARGs on the high-surface area of the tuff, and biodegradation and uptake of the serial plantation *P. australis*-Typha ([Ávila et al., 2021](#)). Chen et al. (2016) conducted 12 HSSF-CWs for optimization of wetland substrates and hydraulic loading ([Chen, Ying, et al., 2016](#)). Twelve mesocosm-scale CWs were filled with three replicas of each of four substrates (oyster shell, zeolite, medicinal stone, and ceramic). Each CW contained a 65-cm-thick substrate layer and a 60-cm-thick water layer. The CWs were built to run at three different hydraulic loading rates (10, 20 and 30 cm/d), but were intended to be horizontal subsurface flow planted with *C. alternifolius* L. The results showed that the removal rates ranged between 50.0% and 85.8%, greatly reducing the total concentrations of different ARGs in the effluents. In general, when HLR increased, the removal for all ARGs dropped. The CW using zeolite as the substrate and an HLR of 10 cm/d had the highest removal rate for all ARGs.

Regarding VSSF-CWs, literature illustrated that the high removals of ARGs/ARB also were achieved in addition to HSSF-CWs, as shown in the Table 3.8. Ávila et al. (2021) conducted 2 VSSF-CWs filled with 0.1-m sand and 0.7-m fine gravel in order with *P. australis* plants (Ávila et al., 2021). The saturation condition of the two parallel CWs, one of which was partially saturated with 0.35 out of 0.8 m filter materials and the other which was unsaturated, was the only difference between them for evaluating the possibility of removing 11 different ARGs as shown in the Table 3.8. The results showed that the removal of *sul1*, *sul2*, *emrB*, *qnrS* *bla*_{TEM} ranged from 21 - 93%, and *intl1* was lowest removed in both of two CWs, while the rest of targeted ARGs was below the limit of detection. Huang et al. (2017) used brick and oyster as filtration materials instead of using gravel or sand for four VSSF-CWs (X. Huang et al., 2017). Two CWs were specifically filled with 450 mm of brick particles, and the rest were filled with oyster shell. All CWs were filled to a height of 100 mm with a red soil and humus soil combination at the top, and *P. australis* were planted there. To evaluate the ARG removal, two of their CWs ran with an up-to-down flow, whereas the others ran with a down-to-up flow. According to the findings, the average computed removal efficiency for the *intl1* and *tet* genes among the four CWs was higher than 62%, and 99.1% was the highest removal efficiency for the ARG. The clearance rates of the *tet* genes and *intl1* were also comparable across the four treatments. It has been noted that the relative abundance of *tet* genes was higher in the down-to-top VSSF effluent than it was in the top-to-down flow direction, increasing the likelihood that comparatively more ARB in relation to the total amount of bacteria would be released into the environment. However, the average removal efficiencies of down-to-top flow were significantly higher than those of top-to-down flow when the removal percentage of 16sRNA genes was taken into account. This is likely because the lower oxygen supply in down-to-top flow inhibited the growth of some bacteria in wastewater (X. Huang et al., 2017). Therefore, a higher removal of 16S RNA may contribute to the removal of ARGs.

In comparisons between subsurface and surface CWs, Chen et al. (2016) reported that VSSFs had good removal efficiencies for all types of ARGs in wastewater, the effectiveness of entire ARG elimination inside up-flow, as can be seen in Figure 3.10, VSSF CWs outperformed HSSF-CWs and SF-CWs, whereas SF-CWs displayed the lowest percent of ARG removal (Chen, Wei, et al., 2016). It might be argued that sub-surface flow CWs are significantly more effective at filtering out ARG hosts than surface flow CWs, leading to the more effective eradication of ARGs (X. Huang et al., 2017). In addition to different CW types, applied substrates also contribute its affects to the removal percentage of ARG/ARB within CWs (Chen, Wei, et al., 2016; X. Huang et al., 2017). Since intestinal microbes frequently carry ARGs, Huang's findings (X. Huang et al., 2017) that bacteria, and particularly intestinal microorganisms, may be easily filtered by substrates and

that this removal of ARB by the substrate may be the source of the eradication of ARGs in CWs. The removal of ARGs from wastewater in CWs is also influenced by the presence of various substrate types. By employing brick and oyster shell, respectively, ARGs had differing removal efficiencies of tet genes, being 78.5–97.2% and 63.8–96.8%, respectively (X. Huang et al., 2017). Furthermore, compared to an oyster shell substrate, a brick substrate had a higher efficacy of int1 and 16s rRNA removal (X. Huang et al., 2015). It can be linked to the brick particles' high percentage of crystalline iron oxide, higher porosity, average micropore size, and larger average micropore size. Another study (Chen, Wei, et al., 2016) evaluated applications of different substrate types (oyster shell, zeolite, medical store and ceramic) for removing ARG within wastewater. The findings demonstrated that zeolite is the best substrate type out of the four because it exhibited porous morphology, which suggested that zeolite had a larger specific surface area than the others. Additionally, as shown by SEM and FTIR, the zeolite exhibited micropore structure with a 2 nm diameter and bridging hydroxyls (Si-OH). Zeolite's micropore structure can offer a lot of surface area for chemical sorption and microbiological adhesion.

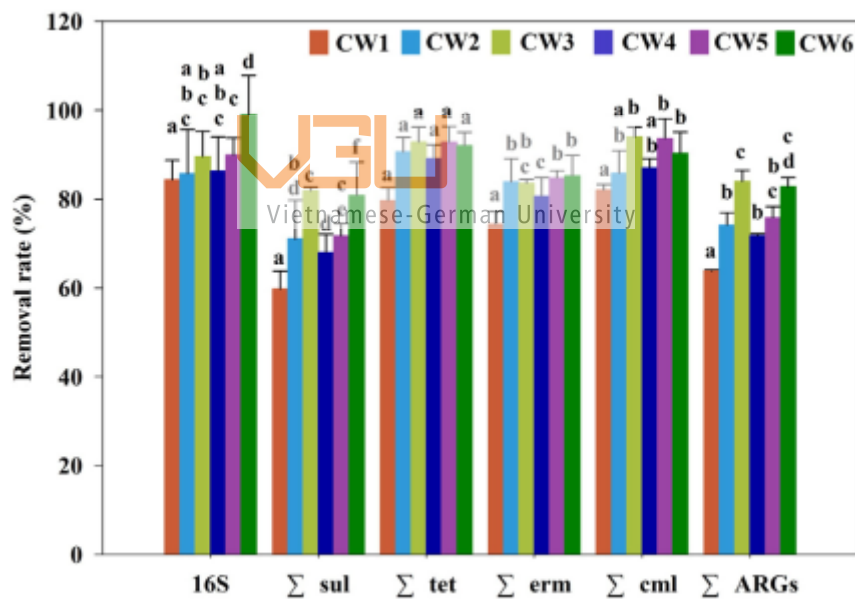


Figure 3. 10: Removal percentage of ARGs within different configurations of CWs (Chen, Ying, et al., 2016)

Besides the efficiency removal of targeted ARGs, substrate is a ideal reservoir for ARGs (Abou-Kandil et al., 2021; Ávila et al., 2021). This findings demonstrated that accumulation of ARGs in the substrate and enrich for ARGs in wastewater effluent. ARGs enrichment in CW sediments and wastewater could result from transmission of ARGs among bacterial population via HGT, leading to release of ARGs from substrate biofilm to the effluent of the WWTPs (X. Huang et al.,

2017). Therefore, further treatments followed the CWs, such as AOP and disinfection processes, for polishing ARGs/ARB to prevent its spread in downstream.

3.3.3.2. Membrane bioreactor (MBR)

As it has been widely used as an alternative to traditional activated sludge processes for dealing with organic pollutants (such as BOD and COD), suspended particles, antibiotics, and bacteria with a suitable efficiency, the membrane bioreactor can no longer be considered as a developing technology. MBR systems typically keep suspended particles and bacteria inside bioreactors by using different sizes of membrane pores, such as micro- or ultrafiltration in combination with aerobic or anaerobic biological treatment units. When compared to other conventional wastewater treatment units, MBR systems have recently demonstrated significantly greater ARB and ARG eradication at both full and lab scales (Pei et al., 2019)

Table 3. 9: Membrane bioreactor-induced removal of ARGs/ARB

ARGs/ARB	Operational conditions	Contact time	Remove efficiency (%)	Regrowth potential	Reference
Antibiotic resistance genes					
Int1, bla _{TEM} , ermB, tetO and tetW	AMBR: MF - hollow membrane - 0.4 µm	N/a	95.9 - 97.9%	N/a	(S. Wang et al., 2020)
Sul1, sul2, tetC, tetX, int1 and ereA	AMBR: MF - sheet membrane - 0.1 µm - 40 kPa	SRT: 60 d HRT: 6h	0.6 - 5.6 log	N/a	(Y. Zhu et al., 2018)
Bla _{ND<-1} , bla _{CTX-M-15} and bla _{OXA-48}	AnMBR: MF - 0.3 µm	HRT: 18.5h	1.9 - 3.9 log	N/a	(Cheng et al., 2017)
qnrA, qnrB, int1, vanA, tetO, tetM, dfrA, sul1, sul2, ermB,	AMBR: MF - sheet membrane - 0.4 µm	SRT: 14-28 d HRT: 6.1 - 7.7	Up to 4.8 log	N/a	(Le et al., 2018)



cfr, bla _{NDM1} , bla _{SHV} , bla _{CTX-M} , bla _{KPC} , aac6					
tetQ, tet36, tetX, tetM, tetW, tet39, tet32, sul1, sul2, ermF, msrE, cfrA, OXA-347, APH(6)-Id, APH(3'')-Ib	UF: 50 nm - 0.2 mpa RO: 0.8 mpa	N/a	99.79% of ARGs	N/a	(Liang et al., 2021)
Antibiotic resistance bacteria					
Cultivable bacteria resistant to 10 antibiotics	AMBR: MF - sheet membrane - 0.4 μm	SRT: 14-28 d HRT: 6.1 - 7.7	<LOD	N/a	(Le et al., 2018)
Amp-, Ery-, Tet-, Kan- and Cip - resistant bacteria	AMBR: MF - hollow membrane - 0.4 μm	N/a	70 - 97.5%	N/a	(S. Wang et al., 2020)

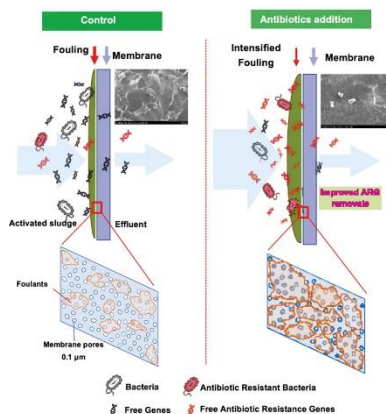


Figure 3. 11: A scheme of mechanism of removing ARGs/ARB by MBR process (Y. Zhu et al., 2018)

As shown in Table 3.9, MBR process showed the effective separation of ARGs/ARB within wastewater. Le et al. (2018) conducted submerged microfiltration membrane flat sheet with the pore size of 0.4 μm treating 16 ARGs as well as cultivable bacteria resistant to 10 antibiotics. The results showed that log reductions of 16 targeted ARGs reached up to 4.8, and there no detections of ARB after being treated by MF flat membrane (Le et al., 2018). Wang et al. (2020) also reported the same effective trend of reducing ARGs and ARB with 95.9 - 97.9% and 70 - 97.5%, respectively (S. Wang et al., 2020). Furthermore, the percentage of ARG removal could be enhanced by applying filtration membrane with smaller size, especially RO and UF (Liang et al., 2021). The swine wastewater after being pre-treated by flocculation and sedimentation steps for reducing membrane fouling and extending membrane application by removing large particulate matter from waste. Then, treated wastewater was passed through a membrane filtration, including UF and two-stage RO for evaluating effective removal of 15 ARGs. The results showed that 92.83% of the total ARGs were retained after UF separation. Furthermore, total ARG removal increased from 92.83% to 99.19% and 99.79% after passing RO1 and RO2 subsequently (Liang et al., 2021).

On the other hand, the effect of fouling membrane could enhance the removal efficiency ARB as well as the targeted ARGs as mentioned in the literature review (Cheng et al., 2017; Y. Zhu et al., 2018). Zhu et al. (2018) also reported the effective performance of MF which was applied in treating 6 different ARGs (sul1, sul2, tetC, tetX, int1 and ereA), with 0.6 - 5.6 log reductions of these targeted ARGs (Y. Zhu et al., 2018). Cheng et al. (2017) also investigated the effect of fouling membranes on plasmid-borne ARGs removed by the anaerobic MBR (Cheng et al., 2017). Results showed that 1.9 - 3.9 log reductions of bla_{NDM-1}, bla_{CTX-M-15} and bla_{OXA-48} were achieved with the support of the fouling membrane, and the removal efficiency increased with the fouling progress. Interestingly, membrane fouling was reported to have positive correlation with ARG removal. Membrane fouling caused by EPS and SMP could enhance ARG reduction within MBR

process because severe membrane fouling can lead to the membrane pore blocking. In detail, appropriate membrane fouling can reduce the effective pore size of the membrane, and thus favor the retention of more microbes or even smaller size DNA fragments, and EPS and SMP contained in the foulants can retain a portion of free ARGs, such as eARGs, which effectively prevents a part of the ARGs leaking from the membrane ([Cheng et al., 2017](#); [Y. Zhu et al., 2018](#))

In terms of regrowth potential of the ARGs/ARB within MBR treatment, it is known that the main mechanism of ARGs/ARB removal in MBR systems as well as filtration membranes is related to the separation and rejection of ARGs/ARB ([Y. Zhu et al., 2018](#)). Detection of ARB and ARGs in the permeability due to two factors: (i) smaller sizes of targeted ARGs/ARB compared to pore sizes of the applied membranes ([Le et al., 2018](#); [Liang et al., 2021](#); [Y. Zhu et al., 2018](#)); (ii) the pressure gradient ([Liang et al., 2021](#)). The pressure-driven membrane filtration may deform ARB and cause the intracellular liquid out of the cell to decrease the cell volume. In detail, high pressure on one side of the membrane squeezes cells, while low pressure on the other side attract them. Therefore, the pressure gradient causes a change in the intracellular fluid volume of the cell by pushing the fluid out the cell. This phenomenon causes cells cross the membrane ([Liang et al., 2021](#)).

The performance of the MBR process is primarily influenced by the size of the pores and the thickness of the fouling layer on the membrane surface. The applied membrane's pores in the MBR range from 0.1 to 0.4 micrometres, however a single bacterium with a diameter of 0.5 to 1.0 micrometres can rarely get through. However, because DNA is a thin, flexible molecule that is about 1 kb long and has 100 helical turns, and because double-stranded DNA has a diameter of around 1 nm, unbound ARGs, which are a type of DNA, could theoretically fit lengthwise through a membrane pore. As a result, it could provide a threat to individuals ([Y. Zhu et al., 2018](#)). Furthermore, ARGs/ARB in the effluent was effectively eliminated, a majority of ARGs and ARB were transferred into sludge phase, which was considered a pool of ARGs ([S. Wang et al., 2020](#)). Therefore, more research is needed on the removal of ARBs and ARGs during the subsequent sludge disposal. Long SRT, high effluent quality, efficient microbial elimination, and improved membrane filtration are all characteristics of MBR in addition to its modest footprint and reactor requirements. In particular, colloidal formation can significantly enhance the clearance of ARG logs. Because MBR applications require a lot of energy, membrane fouling which is thought to be a problem for MBR treatment. Therefore, additional research is required to determine how to maintain the proper level of fouling on the membrane ([Pei et al., 2019](#)).

3.3. Classification and evaluation of available advanced treatment processes (Chapter 3.3)

In addition to effective reduction of ARGs/ARB, footprints, capital and operational costs are necessarily mentioned in this review because it directly affect the operators or decision makers to choose which is the best option for each typical wastewater treatment plant for removing, or at least preventing the spread of AMR development in downstream area. Therefore, in this part, these factors are discussed in details.

3.3.1. OPEX and CAPEX

In comparison to chlorination, UV/chlorination, and UVC irradiation, the Fenton procedure provided the best ARG elimination, according to Zhang's findings from 2015 to 2016 regarding oxidative reaction-induced inactivation of ARGs/ARB. With 0.312 dollars/m³, the Fenton procedure is not economically viable when compared to other treatment methods when chemical requirements are taken into account. Because photo-Fenton is administered under both artificial and natural light, it may be modified to lower the amount of chemicals needed for the standard Fenton process, which reduces both treatment time and operating costs ([Y. Zhang et al., 2015](#)). Furthermore, since H₂O₂/Fe²⁺ must work at acidic conditions to enable the catalyst's dissolution, pH is a crucial operational parameter. Better Fenton process performance often results from keeping the pH value around 3. These operational circumstances may restrict the technology's applicability because they necessitate a further neutralisation step before discharging the treated effluents, which raises operational costs ([Herraiz-Carboné et al., 2021](#)). The threat associated with ARB can be reduced economically by using chemical treatments like ozonation and chlorination. Chemical use, however, can encourage the development of DPBs because it contains organic components that are known to cause human cancer and mutagenicity ([Herraiz-Carboné et al., 2021](#)). Furthermore, as reviewed above, the more concentration of chlorine, or ozone is required for further removal of ARGs because the oxidative reactions happen on the outer membrane of microbes, and then continuously react with biological components inside the cells. This problem also occurs when applying UV irradiation, one double strand DNA break per kbp of DNA corresponded to a UV dose of approximately 2000 mJ/cm² ([Chang et al., 2017](#)). In fact, the real WWTPs could not apply the concentration of disinfectants that were used in most reviewed journal because it probably increases the operational cost.

Table 3. 10: Efficiencies, operational cost and footprints of applied technologies

Classification	Remove efficiency (%)	Cost	Footprint and energy requirements	Reference
Chlorination	1.65 - 2.28 log reductions of selected ARGs	0.006 dollars/m ³	N/a	(Y. Zhang et al., 2015)
Photocatalyst	< LOD after 90 min reaction	Cheaper than TiO ₂ particles	Visible light require less energy than UV-light	(C. Guo et al., 2017 ; Xiong et al., 2013)
UV	0.8 - 1.21 log reduction	0.007 dollars/m ³	N/a	(Y. Zhang et al., 2016)
Photo-Fenton	No regrowth of ARB/ARGs	Less concentration of chemicals	N/a	(Fiorentino et al., 2019 ; Moreira et al., 2018)
Fenton	2.58 - 3.79 log reduction (pH = 3.0) 2.26 - 3.35 log reduction (pH=7)	0.312 dollars/m ³	N/a	(Y. Zhang et al., 2016)
Constructed wetland	63.9 - 84% of all targeted ARGs	Low cost, easy craft and operation	Large area requirement	(Chen, Wei, et al., 2016)
MBR	95.9 - 97.9 of all targeted ARGs	Higher economic cost than disinfection	High energy requirement in cases of the fouling membrane	(S. Wang et al., 2020)

UV light and TiO₂ particles are two elements generating an increase in the operational cost of photo-assisted processes. Especially, when applying TiO₂ particles, The focus of researchers switched to the catalyst in the integrated form and to employing visible light to start the photocatalysis in order to address these two shortcomings ([Gmurek et al., 2022](#)). Guo ([C. Guo et al., 2017](#)) and ([Xiong et al., 2013](#)) also applied TiO₂-thin film as the replacement for TiO₂ particle. After being stored in the dark for a long time, there was no ARB regrowth. Due to the effect of light, which speeds up the reaction rate of the process, photo-Fenton requires less time and concentration of H₂O₂/Fe²⁺. Additionally, lingering effects, as discussed in the photocatalyst and photo-Fenton portions, sustain the efficacy of wastewater disinfection. As with ozonation, UV irradiation, and chlorination, higher light intensity and H₂O₂/Fe²⁺ concentration are added to achieve a more effective elimination of ARGs/ARB.

Constructed wetlands are a well-known alternative to chemical and physical-chemical treatment methods in terms of sustainability. With zeolite material, VSSF-CWs demonstrated the best removal percentage of ARGs/ARB. However, a buildup of ARGs has been found in both the substrate and the solid phase, which could lead to HGT in bacterial communities that are favourable to the spread of ARGs/ARB. According to reports, removal of ARB from membrane bioreactors is effective, while removal of ARG is less successful since ARGs are smaller than the pore diameters of the applied membrane. Additionally, membrane fouling reduces the pore size of the membranes and retains ARGs/ARBs, which helps to increase the removal percentage. However, the more fouling membrane there is, the more energy is needed, which raises the cost of operation. Furthermore, applications of CWs and MBR are observed to lack regeneration potential. It may be attributed that, in contrast to disinfection and AOPs, CWs and MBR were unable to stop the possibility for regrowth of ARB/ARGs. Therefore, it is necessary that disinfection and AOPs should be followed to the MBR, or CWs for polishing and prevent the regrowth of ARGs/ARB in downstream

4. Conclusion

The current study has highlighted the application and efficiency of disinfection and advanced treatment process as well as its merits and demerits in the context of preventing the proliferation of ARGs/ARB. There are three point that is critical emphasized:

In terms of chemical (chlorine, ozonation), physcial (UV irradiation) and physico-chemical (photo-Fenton and photocatalyst), the effective removal of ARG requires more concentration of chemicals as well as time reaction and light intensity. Regarding the prevention of the regrowth potential of ARGs/ARB, photo-Fenton and photocatalyst could maintain no regrowth of ARG/ARB after long time storage in ideal condition, compared to chlorination, UV irradiation and ozonation.

According to CWs and MBR, there is lack of data mentioning about the occurrence of the regrowth potential of ARGs/ARB. Additionally, disinfection and AOPs are necessary for following treatment of CWs and MBR-treated water due to accumulation of ARGs/ARB in the membrane surface as well as substrates possibly contribute to the dissemination of ARGs/ARB in the receiving environment.

In terms of capex and opex , there is lack of data mentioning clearly about the economic picture of these application in removing the ARGs/ARB. It might be attributed that most of researchers conducted these processes as a lab scale. Therefore, pilot-scale and industry-scale process are needed to provide operators a holistic picture of these treatment process , that optimally and suitably choose the treatment options for each typical wastewater and geographical diversity.

5. Biobilgraphy

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